IN VITRO APOPTOSIS INDUCTION AND INHIBITION OF NF-κB SIGNALING PATHWAY BY BISEUGENOL B IN PC3 HUMAN PROSTATIC CANCER CELLS

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ABSTRACT

Prostate cancer is considered as the second most common cancer across the world in men. Apoptosis induction in prostate cancer cells (PC3) revealed an efficient therapeutic strategy for cancer therapy. The conventional cancer treatment approach including chemotherapy and radiotherapy cause severe adverse effects and treatment failure. Given the drawbacks of modern cancer medicines, the search for new synthetic agents from medicinal plants and natural compounds is emerging. Recent phytochemical investigation and clinical studies have shown the potential anti-cancer value of natural compounds. Recent studies have shown selective cytotoxicity of biseugenol B in PC3 cells compared to other cancer cell lines. Therefore, there is a need to evaluate if this natural compound possesses potential anticancer activity. The aim of the study is to evaluate the apoptosis-induction effect of 2, 2'-oxybis (4-allyl-1-methoxybenzene), biseugenol B, a natural compound from *Litsea costalis*, on human prostate cancer cell line (PC3) via activation of extrinsic, intrinsic and inhibition of NF-κB signaling pathways using an *in vitro* model.

The potential therapeutic activity of biseugenol B, isolated from *Litsea costalis* to inhibit human prostate cancer cells PC3 through apoptosis was evaluated *in vitro*. In this study, the cell death mechanism of biseugenol B was investigated. MTT assay was used to evaluate biseugenol B-induced cell viability. The apoptosis effect of biseugenol B in PC3 cells were confirmed by using double-staining propidium and acridine orange and AV-FITC staining. Cell cycle arrest was examined using flow cytometry whilst protein and the expression level of mRNA Bcl-2, Bax, and HSP70 were assessed using Western blotting and RT-PCR. Cell permeability, nuclear condensation, release of cytochrome c and mitochondrial membrane potential (MMP) were observed in PC3 cells treated with biseugenol B using high content screening (HCS). The level of caspase-3/7, -8 and -9 were examined and the activity of NF-κB was evaluated using HCS assay.

Biseugenol B showed a significant cytotoxicity (IC_{50} < 5 µg/mL) towards human prostate cancer cells (PC3) when compared to normal human prostate cells (RWPE-1). Propidium and acridine orange double-staining and AV-FITC staining results showed a significant apoptosis induction effect of biseugenol B in PC3 cells. Early apoptosis cells significantly increased in PC3 cells with exposure to different dose of biseugenol B (P < 0.001). The results indicated that a number of the cells in sub-G0 phase were significantly increased after exposure to different dose of biseugenol B (P < 0.001). The results demonstrate that biseugenol B significantly increased the cell arrest of PC3 in G0/G1 phase after exposure with different dose of biseugenol B. A significant up-regulation of Bax and down-regulation of Bcl-2 and Hsp70 were observed (P < 0.001). Meanwhile caspases-3/7, caspase-8 and caspase-9 levels were noted to be significantly increased (all P-values < 0.001). The translocation of NF-κB from the cytosol to the nucleus was significantly inhibited (P < 0.01) by biseugenol B.

Our findings suggest that biseugenol B could potentially induce apoptosis in prostate cancer cells and thus serve as a promising compound in the prostate cancer treatment.

ABSTRAK

Kanser prostat dikategorikan sebagai kanser kedua paling tinggi di dunia. Induksi apoptosis dalam sel kanser prostat (PC3) didedahkan sebagai strategi terapeutik yang efisyen untuk terapi kanser. Rawatan kanser secara konvensional termasuk kemoterapi dan radiografi mengakibatkan kesan buruk dan kegagalan rawatan. Disebabkan oleh kegagalan fungsi ubat moden, telah wujud kajian berkenaan agen sintetik dari tumbuhan dan sebatian semulajadi. Kajian-kajian penyiasatan fitokimia terkini dan klinikal telah menunjukkan nilai potensi anti kanser dari sebatian-sebatian semulajadi. Kajian-kajian terkini telah menunjukkan ketoksikan terpilih biseugenol B dalam sel PC3 berbanding jujuran sel kanser yang lain. Oleh demikian, wujud kepentingan untuk menilai jika sebatian ini mempunyai potensi aktiviti anti-kanser. Tujuan kajian ini adalah untuk menilai kesan induksi apoptosis 2, 2'-oxybis (4-allyl-1-methoxybenzene), biseugenol B, kompaun asli dari *Litsea costalis*, ke atas sel prostat kanser manusia (PC3) melalui pengaktifan ekstrinsik, intrinsik dan perencatan isyarat jalanan NF-κB menggunakan model *in vitro*.

Potensi aktiviti terapeutik biseugenol B, diasingkan dari *Litsea costalis* untuk menghalang sel kanser prostat PC3 manusia melalui apoptosis telah dinilai melalui kaedah in vitro. Dalam kajian ini, sel mati metabolik biseugenol B telah dikaji. Ujian MTT digunakan untuk menilai keberdayaan biseugenol-B teraruh. Kesan apoptosis biseugenol B dalam sel PC3 telah disahkan melalui pelumuran berganda propidium dan akridin jingga dan pelumuran AV-FITC. Penahanan kitaran sel telah diuji menggunakan aliran sitometri, manakalan protein dan aras lambang mRNA Bcl-2, Bax, and HSP70 telah ditaksir menggunakan penodaan Western dan RT-PCR. Kebolehtelapan sel, kondensasi nuklear, pelepasan sitokrom c dan potensi membran mitokondrial (MMP) telah diperhatikan dalam sel PC3 diperlakukan dengan biseugenol B dengan

menggunakan penyaringan kandungan tinggi (HCS). Aras caspase-3/7, -8 and -9 telah dikaji dan aktiviti NF-κB telah diuji menggunakan ujian HCS.

Biseugenol B menunjukkan sitotoksisiti signifikan (IC_{50} < 5 μg/mL) terhadap sel prostat kanser manusia (PC3) apabila dibandingkan dengan sel kanser manusia yang normal (RWPE-1). Pelumuran berganda propidium dan akridin jingga dan pelumuran AV-FITC menunjukkan signifikan aruhan apoptosis kesan daripada biseugenol B dalam sel PC3. Sel apoptosis awal telah meningkat secara signifikan dalam sel PC3 dengan pendedahan kepada dos biseugenol B (P < 0.001) yang berlainan. Keputusan menunjukkan beberapa sel dalam fasa sub-G0 telah meningkat secara signifikan selepas didedahkan kepada dos biseugenol B (P < 0.001) yang berlainan. Keputusan menunjukkan biseugenol B meningkatkan penahanan sel PC3 secara signifikan dalam fasa G0/G1 selepas didedahkan dengan dos biseugenol B yang berlainan. Regulasi menaik Bax dan regulasi menurun Bcl-2 dan Hsp70 adalah signifikan (P < 0.001). Manakala aras caspase-3/7, caspase-8 dan caspase-9 telah diperhatikan dengan peningkatan yang signifikan (semua nilai (P < 0.001). Penghalangan lokasi rentas NF-κB dari sitosol ke nukleus dari biseugenol B adalah signifikan (P < 0.01).

Dapatan kami, mencadangkan bahawa biseugenol B berpotensi untuk mengaruh apoptosis dalam sel kanser prostat dan menjadikannya kompaun yang berpotensi untuk rawatan kanser prostat.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
/	:	Divide by
˚C	:	Degree Celsius
<	:	Less than
>	:	More than
±	:	Plus, minus
µl	:	microliter
µm	:	micrometer
ANOVA	:	Analysis of variance
AO	:	Acridine orange
Apo3L/DR3	:	Accumulation of photosystem one 3 ligand/death receptor 3
AR	:	Androgen receptor
ATCC	:	American Type Cell Collection
AV	:	Annexin V
Bax	:	BCL2-Associated X Protein
BCIP®/NBT	:	5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium
Bcl-2	:	B-Cell CLL/Lymphoma 2
Bcl-10	:	B-Cell CLL/Lymphoma 2
BPE	:	Bovine Pituitary Extract
CDKs	:	Cyclins and cyclin-dependent kinases
cDNA	:	Complementary DNA
CEMSS	:	Human T4-lymphoblastoid cell line
CO2	:	Carbon dioxide
DCFH-DA	:	2', 7' dichlorofluorescin diacetate
DISC	:	Death-inducing signaling complex

DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
EA	:	Early apoptosis
et al.	:	and other people
FBS	:	Fetal bovine serum
Fas receptor	:	First apoptotic signal receptor
FADD	:	Fas associated via death domain
Fas	:	Fas cell surface death receptor
FITC	:	Fluorescein isothiocyanate
HBSS	:	Hank's Balanced Salt Solution
HPV	:	Human papilloma virus
HSP70	:	Heat shock protein 70 kD
HPV	:	Human papilloma virus
IC_{50}	:	Inhibitory Concentration of 50%
IκB	:	κB inhibitor proteins
IKK	:	IκB Kinase
KB cell lines	:	Cervical adenocarcinoma
Kg	:	Kilogram
K-SFM	:	Keratinocyte-SFM serum-free medium
LA	:	Late apoptosis
MDA-MD-231	:	Human Caucasian breast adenocarcinoma
MCF7	:	Human breast adenocarcinoma cell line
mg	:	milligram
Min	:	Minute/s
ml	:	milliliter
mmol	:	millimole

MMP	:	Mitochondrial membrane potential
MPT	:	Mitochondrial permeability transition
MTT assay	:	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide assay
NaCl	:	Sodium Chloride
NF-κB	:	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
Nf-κB/P65	:	Subunit of NF-kappa-B transcription complex
NF-κB/Rel	:	NF-κB /proto-oncogene, NF-kB subunit
NIK	:	NF-kappa-B-inducing kinase
NF-κB	:	Nuclear factor kappa-light-chain-enhancer of activated B cells
nm	:	nanometer
NP40	:	Nonyl phenoxypolyethoxylethanol
P53	:	Tumor suppressor genes p53
p65 (RelA)	:	RELA proto-oncogene, NF-kB subunit
PBS	:	Phosphate buffer saline
PC3	:	Human prostate cancer cell line
PD	:	Population doubling
PH	:	Potential hydrogen
PI	:	Propidium iodide
PMSF	:	Phenylmethylsulfonyl fluoride
PSA	:	Prostate specific antigen
PTEN	:	Phosphatase and tensin homologue
PVDF	:	Polyvinylidene difluoride
RB	:	Retinoblastoma tumor suppressor gene
RelB	:	RELB proto-oncogene, NF-kB subunit
REL	:	Proto-oncogene, NF-kB subunit

rhEGF	:	Human Epidermal Growth Factor
RIP	:	Ribosome-inactivating protein
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species generation
RPMI	:	Roswell Park Memorial Institute medium
RS	:	Replicative senescence
RT-PCR	:	Reverse transcription-polymerase chain reaction
RWPE-1	:	Human prostate epithelial cell line
SDS-PAGE	:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SN	:	Secondary necrosis
SPSS	:	Statistical Package for Social Sciences
TBST	:	Tris Buffered Saline with Tween
TBS	:	Tris-buffered saline
TNF	:	Tumor necrosis factor
TGF-α	:	Transforming growth factor alpha
TRADD	:	TNFRSF1A associated via death domain
TrisHCL	:	Trisaminomethane hydrochloride
VI	:	Viable cells
WHO	:	World Health Organization
WRL68	:	Hepatic fetal human epithelial cell line

CHAPTER 1: INTRODUCTION

Traditional and herbal medications have been used for years for treatment of different types of diseases, especially for the diseases that do not have deterministic cure like cancers. Phytochemicals are classified into primary and secondary bioactive constituents in which chlorophyll, proteins and sugars are included in primary, while alkaloids, terpenoid and phenolic groups are included in secondary compounds (Krishnaiah, Sarbatly, & Bono, 2007). Alkaloids and terpenoids exhibit different pharmacological properties such as anti-cancer, anti-inflammatory, anti-viral, anti-cholesterol, anti-malarial, anti-bacterial and anesthetic properties (Wadood et al., 2014).

Later studies have indicated that phytochemicals have anti-oxidant, pro-apoptotic and anti-proliferative effects on blood cells (Salim et al., 2013), liver (Muhammad Nadzri et al., 2013), lung (Isa et al., 2013), breast (Ibrahim et al., 2014; Paydar et al., 2014; Arbab et al., 2013), brain (Karmakar, Banik, & Ray, 2007), skin (Katiyar, 2011) and pancreatic cancers (Lev-Ari et al., 2007) in both *in vitro* and *in vivo*. The anti-cancer effects of herbal medicines have been observed in several studies where phytochemicals induced apoptosis by targeting different cellular and molecular pathways in prostate cancer (Yu et al., 2010; Ye, Jiang, Volshonok, Wu, & Zhang, 2007).

Litsea costalis, with the official name *Litsea costalis (Nee) Kosterm*, belongs to a large family of the plants that are known as *Lauraceae* and have been widely used in China and Malaysia for curing several diseases (Hosseinzadeh, et al., 2013b). *Litsea costalis* contains structurally diverse and biologically active secondary compounds such as aporphine alkaloids (Wang, Huang, Lu, Li, & Yang, 2010), anolides (Hosseinzadeh, et al., 2013a), flavonoids and sesquiterpenes (Zhang et al., 2003).

Biseugenol B or 2,2'-oxybis (4-allyl-1-methoxybenzene) is a new compound isolated from *Litsea costalis* bark is an oxyneolignan that is classified under the major group of natural products, lignans and neolignans (Hosseinzadeh, et al., 2013a).

Lignans and neolignans belong to a category of natural plant products which are biochemically related to phenylalanine metabolism, cinnamic acid (Häusler, Ludewig, & Krueger, 2014). Meanwhile, they are characterized by the coupling of two C_6C_3 units (DellaGreca, Zuppolini, & Zarrelli, 2013). The difference between lignans and neolignans is based on the pattern of bonding between the C_6C_3 units. Briefly, if two C_6C_3 units are directly bound together in 8,8'-bond pattern structure, the molecule, thus, will be called lignin. However, if the binding between two C_6C_3 units happened to be other than 8,8'-bond the parent structure, the molecule would be neolignan. In a subgroup of neolignan, the two C_6C_3 units are not directly bonded together but are linked by an oxygen atom. In this case, the molecule is called as oxyneolignan (Li et al., 2011).

Lignans and neolignans have been used for medical purposes for years. Lignans are broadly used in Chinese traditional medicine for protection of the liver and treatment of viral hepatitis (Teponno, Kusari, & Spiteller, 2016). Moreover, physiological activity like tumor-inhibiting has been observed in many lignans and neolignans. This anti-tumor activity leads to using lignans and neolignans to interfere with cell division in animals including humans (Qin et al., 2010). On the other hand, oxyneolignans belong to the major class of phytoestrogens which are also known as “plant estrogens” and have antioxidants function (Sirotkin & Harrath, 2014; Korkina, 2007).

Lignans and neolignans (as well as oxyneolignans as a subgroup of neolignans) play an anti-oxidant role as a defense mechanism against biotic and abiotic factors in plants, also in basic research models of human diseases have shown anti-oxidant and anti-inflammatory activity (Korkina, Kostyuk, De Luca, & Pastore, 2011).

Prostate cancer, or carcinoma of the prostate, affects the gland of the male reproductive system known as prostate (Siegel, Ma, Zou, & Jemal, 2014). It is considered as the second most common type of malignancy and the fifth leading cause of cancer-related death in males worldwide (Sultana et al., 2014). In Malaysia, it accounts for 5.7 percent of cancer cases in men and is the sixth most frequent cancer (Sothilingam, Sundram, Malek, & Sahabuddin, 2010). Although prostate cancer is considered as a treatable disease, however, the management of the treatment can be complicated. It is vital that patients are offered various treatment options and play a willful and active role in the process of the decision-making.

Currently, there are many cancer therapies available such as radiotherapy, chemotherapy, hormonal therapy, immune therapy, surgery, symptomatic and supportive therapy (Urruticoechea et al., 2010). Considering the rising number of non-responding and drug-resistant cases (Armstrong, Garrett-Mayer, de Wit, Tannock, & Eisenberger, 2010), the current treatment strategy of cancer is focused on systemic, nonspecific, high-dose chemotherapy. Therefore, finding the alternative synthetic chemotherapy drugs that are safe and effective is noteworthy.

Natural compounds are good alternatives, because plants are not only significant sources of anti-cancer agents, but also possess lesser side effects (Abreu, McBain, & Simoes, 2012). The low cost of natural products makes them preferable to be investigated as potential synthetic chemotherapy drugs. Overall, by considering the anti-cancer effects of biseugenol B on human prostate cancer cells (PC3), the study of the effects of this compound on PC3 and detecting the mechanism of its anti-cancer effect on PC3 is justifiable. The present study hypothesized the potential anti-cancer properties of biseugenol B on PC3 through the mechanism of apoptosis-induction in human prostate cancer cells.

In normal circumstances, apoptosis has a crucial role in the maintenance of the tissue homeostasis (Wynn, Chawla, & Pollard, 2013). Apoptosis has been broadly studied in cancer therapy. It has been uncovered that one of the most effective ways to remove a tumor cancer is by applying the compound to induce apoptosis in cancer cell. Apoptosis is an irreversible method in which tumor cells destroy themselves. Since most of the anticancer drugs exert their effect by inducing the apoptosis in tumor cells, the best way to assess the anti-cancer effects of the compound is to evaluate the apoptosis induction effect of the compound (Fulda, 2015).

The apoptosis effect of anticancer drugs is detected by various methods. As DNA damage has been caused by the build-up of reactive oxygen species (ROS), the measurement of ROS is one of the most reliable methods in anticancer drug research (Huang et al., 2013; Azad, Chen, & Gibson, 2009). Activation of caspase 9 is another indicator of apoptosis that follows the release of cytochrome *c* into the cytosol that is caused by disruption of the mitochondrial membrane potential (MMP) (Harmand, Duval, Delage, & Simon, 2005). Controlling the ratio of Bcl-2/Bax proteins in the mitochondria is the other way to determine the tumor susceptibility to the apoptosis induction by chemotherapeutic agents (Mohan et al., 2010). Another group of proteins is heat shock proteins (HSP) which not only is considered as promoting tumorigenesis (Richardson et al., 2011), but also play a role in protecting the cell from oxidative stress by refolding the denatured proteins (Calderwood & Ciocca, 2008; Azad, Zoubeidi, Gleave, & Chi, 2015). Malignant tumor cells have shown to increase the expression of HSP70. HSPs can preclude the drug-induced apoptosis. Governing of apoptosis in the cell is conducted not only by HSPs but also nuclear factor-kappa B, a ubiquitous transcription factor (Cheng et al., 2009). Therefore, in this study, these methods will be used to assess the apoptosis inducing effects of a new compound (biseugenol B) on the human prostate cancer cell.

The pathway(s) that has/have been activated by the compound that lead(s) to apoptosis will also be identified.

1.1 Problem statement and significance of the study

The conventional cancer treatments including chemotherapy and radiotherapy have been documented to cause severe adverse effects and treatment failure (Hodgson, 2015). Therefore, an alternative solution for cancer treatment is inevitable. It can be stated that natural compounds have been suitable sources for alternative cancer therapy (Basmadjian et al., 2014). Natural products are the important sources of therapeutics: more than 60% of anticancer agents have been derived from plants, marine organisms and microorganisms (Kinghorn et al., 2009). Medical plants contain therapeutic value constituents that produce physical action on human body (Halliwell & Gutteridge, 2015). The use of plants for curing cancer commenced long ago (Nobili et al., 2009). Recent phytochemical investigation and clinical studies have shown the potential anti-cancer value of medicinal plants (Sharma, Parihar, & Parihar, 2011; Bhanot, Sharma, & Noolvi, 2011). Therefore, there is a need to focus and evaluate the extracts of medicinal plants to see if they possess an anticancer activity (Shoeb, 2006). The new natural compound, biseugenol B, which is extracted from *Listea costalis*, belongs to the large family of natural plant compounds: lignans and neolignans. Compounds of lignans and neolignans family have revealed anti-cancer property such as podophyllotoxin (Lu, Chen, Xiao, Li, & Miller, 2012), honokiol (Liu et al., 2008) and 2-phenoxy-1-phenylethanone (De Souza et al., 2011). Biseugenol B as an oxyneolignan, which is a subgroup of neolignans, is expected to have anti-cancer attributes.

The aim of this research is to analyze the apoptosis-induction effect of the 2, 2'-oxybis (4-allyl-1-methoxybenzene) or known as biseugenol B, a natural compound isolated from

Litsea costalis in human prostate cancer cell (PC3) through activation of extrinsic, intrinsic and inhibition of NF-κB signaling pathways using *in vitro* model.

1.2 Hypotheses

Ha: Natural compound 2,2'-oxybis (4-allyl-1-methoxybenzene), biseugenol B from *Litsea costalis* induces apoptosis through activation of extrinsic, intrinsic and inhibition of NF-κB signaling pathways in human prostate cancer PC3 cells.

Ho: Natural compound 2,2'-oxybis (4-allyl-1-methoxybenzene) or biseugenol B from *Litsea costalis,* does not induce apoptosis through activation of intrinsic or extrinsic pathway or inhibition of NF-κB signaling pathways in human prostate cancer PC3 cells.

1.3 Aim

The aim of this study is to assess the potential apoptosis induction activities of biseugenol B from *Litsea costalis* in the human prostate cancer cell lines.

1.4 Objective

1.4.1 General Objective

To assess the potential apoptosis induction activities of biseugenol B from the bark *Litsea costalis* in the human prostate cancer cell lines.

1.4.2 Specific Objectives

1: To assess the *in vitro* cytotoxicity and selective activities of biseugenol B against human prostate cancer cell line (PC3).

2: To determine the apoptosis mechanism induced by biseugenol B on human prostate cancer cells (PC3) through caspase activation

3: To determine the modulation of apoptotic signaling pathway in PC3 cells induced by biseugenol Bthrough cell cycle status, RT-PCR and western blotting analyses.

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

Cancer is a life threatening and dreadful disease characterized by ungovernable cell division. These cells attack the other tissues and cause the destruction of these tissues (Gennari et al., 2008). Cancer cells can spread to not only adjacent tissues but to distant organs through blood vessels and lymphatic stream. Generally, cancer occurs due to the disturbance of two types of genes: oncogenes, which are responsible for the cancer cell growth and tumor suppressor genes, which prevent cancer from developing (Levine & Puzio-Kuter, 2010; Kent & Mendell, 2006). Even though the exact cause of cancer is still unknown, though there are some factors known as cancer-increasing risk factors such as alcohol, tobacco, infectious agents, environment pollution and life style (Vineis & Wild, 2014). Around 10-15% of cancers exclusively occur due to genetic inheritance (Hildesheim & Wang, 2012; Anand et al., 2008).

Cancer is considered as one of the leading causes of mortality and morbidity in the world. In 2012, 14 million new cases have been diagnosed worldwide and 8.2 million cancer related deaths have been recorded (Group, 2014; Siegel, Ma, Zou & Jemal, 2014). In fact, the number of new cancer cases is increasing and it is expected to increase by more than 70% over the next two decades (Smith, Smith, Hurria, Hortobagyi, & Buchholz, 2009; Torre et al., 2015).

Cancer is diagnosed by several characteristics including self-sufficiency in growth signaling, insensitivity to anti-growth signals, the digression of apoptosis, enabling of unlimited proliferation, angiogenesis induction and finally, metastasis (Wender et al., 2013; Weinberg, 2013). The progression of turning a normal cell to the cell that can form tumor includes multiple stages that are identified as malignant progression (Hanahan & Weinberg, 2011).

Some risk factors for cancer include tobacco (25-30%), obesity (10-20%), poor diet (30-35%), alcohol (4-8%), environmental pollution and ionizing radiation (10-15%) (Siegel, Miller, & Jemal, 2015; Anand et al., 2008). Nearly 20% of cancers in the developed countries occur due to virus infections such as human papillomavirus, hepatitis B and C which probably can enter the human genome and cause mutations (Belpomme et al., 2007). Naturally, cancer develops after massive changes of human genetic profile (Siegel, Ma, Zou, & Jemal, 2014).

There are several options for cancer treatment such as chemotherapy, radiation therapy and surgery (Yan, Rosen, & Arteaga, 2011). One of the most challenging parts in the management of cancer is to overcome the adverse effects that are caused by the anticancer agents such as chemotherapy and radiotherapy (DeSantis et al., 2014). The clinical outcome or survival chances would depend on the type and stage of cancer and the severity of cancer when treatment was initiated (Kurman, Carcangiu & Herrington, 2014).

2.2 Biology of cancer

The evolution of the normal cell to the cancer cell is involved in the mutations that occur in the normal hemostatic mechanism that controls cell proliferation and apoptosis. Cell division is required for human body functions such as reproduction, growth and repair. Furthermore, cell division is controlled by cell cycle checkpoints. Cell cycle makes a balance between the cell division/cell loss (Kastan & Bartek, 2004). The conversion of a normal cell to malignancy requires the series of mutations that result in DNA damage.

2.2.1 Cell cycle

Cell growth and division occur repeatedly in all organisms on earth, ranging from the simplest unicellular to the most complex multicellular mammals. Rudolf Virchow was the first to apply cell cycle theory to compare the cell division in both healthy and diseased tissues to distinguish the diseased cells derived from the healthy cells of normal tissues

(Weinberg, 2013). In the process of cell growth, first, DNA is replicated and then the cell is divided into identical daughter cells. Cell cycle is divided into interphase and cell division. Interphase is divided into three different stages: Gap1 or G1, Synthesis or S and Gap2 or G2. Interphase is the phase in which normal cell duplicates its DNA and gets prepared for mitosis, while mitosis and cytokinesis occur in the cell division state. In human being, the length of cell cycle is approximately 24 hours. The duration of cell cycle phases differs remarkably in various types of the cells (Salomoni & Calegari, 2010). The accuracy of cell cycle requires the perfect cooperation of distinct macromolecular synthesis, assembly and movement. For faultless cell division, the molecule of DNA replicates followed by chromosome condensation and segregation. Cell cycle accuracy is controlled by three "checkpoints": the G1, the G2/M and the metaphase checkpoints. The checkpoint is a control mechanism in eukaryotic cell to ensure proper cell division. Each checkpoint assesses cell condition for the accuracy of the cell function (Medema & Macůrek, 2012).

2.2.1.1 Cell cycle phases

Generally, cell cycle contains four different phases as follows:

G1 phase: The first, longest and most variable in length in different cells. It begins when the previous cell division is completed and is followed by S-phase. In this phase, the cell enlarges and is ready to duplicate the DNA. G1 checkpoint control mechanism ensures that the cell is ready to duplicate the DNA. At this point, the cell can continue up to go either to S-phase in order to complete cell division or go into inactive stage (G0-phase) (Diaz-Moralli, Tarrado-Castellarnau, Miranda, & Cascante, 2013).

S phase: DNA replication is limited to this phase which usually lasts for six hours. In this phase, each chromosome is duplicated and makes two pairs of sister chromosomes (Williams & Stoeber, 2012).

G2 phase: The last and often shortest phase before mitosis, the G2 phase, is a gap between DNA synthesis and mitosis. Cell growth continues until DNA synthesized and chromosome replicates, which started once S-phase completed. G2 checkpoint determines whether the cell is ready to continue into M-phase for division or not. Cells with damaged DNA are prevented from division by G2 check-point. Hence, the genomic stability of the cell is protected and the spread of damaged cells is prevented. G2 phase is determinative in investigations on molecular cause of cancer (Diaz-Moralli, Tarrado-Castellarnau, Miranda & Cascante, 2013; Kim, Åberg, Salvati, & Dawson, 2012).

M phase: In this phase, mitosis (division of the cell genome into two nuclei) and cytokinesis (division of the cell into two daughter cells) occurs. Mitosis and cytokinesis together are defined as M phase. Errors in mitosis result in either major defect in the cells that propel the cell to apoptosis or causes mutations that may result in cancer (Williams & Stoeber, 2012).

2.2.1.2 Checkpoints

The cell cycle is regulated and monitored by checkpoints (Kastan & Bartek, 2004). Cell cycle progression is paused at specific points to verify the necessary process and repair of the DNA damage. Checkpoints generally include a network of regulatory proteins that monitor the cell cycle progression at different levels (You & Bailis, 2010).

There are different checkpoints in the process of the cell cycle to ensure that damaged or incomplete DNA is not proceeded and transferred to daughter cells. The main checkpoints are:

G1/S or restriction point: its responsibility is to check whether the cell possesses the main material for DNA replication (Kumar, Abbas, Fausto, & Aster, 2014).

G2/M: it checks the cell before proceeding to mitosis. This checkpoint determines the right time to replicate the cell (Reinhardt et al., 2010).

Metaphase checkpoint: it is a minor checkpoint during mitosis that ensures that the duplicated chromosomes are aligned at the spindle to separate equally to different poles of the nuclei (Lara-Gonzalez, Westhorpe, & Taylor, 2012). The mutations that prevent the cell to pause and check in or allow the cell to speed through different checkpoints cause many types of cancers. In these cases, cell consecutively goes from S to M and back to S phase without going to any of G1 or G2 phases. It results in the DNA mutations that may have occurred if they were ignored and passed to the daughter cells due to the loss of checkpoints. P53, a well-known tumor suppressor, plays a vital role in the control mechanism of both G1/S and G2/M checkpoints (Williams & Stoeber, 2012).

2.2.1.3 Role of cell cycle in tumor formation

Cell cycle deregulations might result in tumor formation (Tsaniras et al., 2014). The mutations in the cell cycle inhibitors such as RB or p53 may lead to uncontrolled cell division and eventually, cause tumor formation. The length of the cell cycle in tumor cell is approximately similar to or even longer than normal cell (Eisen, 2013; Sherr & McCormick, 2002). However, in tumor cells, the ratio of the cells that proceed to division versus quiescent cells that are arrested in G0 phase is significantly higher than normal cells (Malumbres & Barbacid, 2009). Therefore, there is a net increase in the number of the cells as the higher population of the cells go through cell division, while the number of the cells that are sentenced to apoptosis remains the same. One of the targets in cancer therapy is the mass of the cells that actively undergo cell cycle. The DNA is comparatively unprotected in these cells during the cell division and subsequently is more susceptible to damage by radiation or drugs. This fact is useful in cancer therapy. This way, a significant tumor mass is removed in a process known as debulking, which removes a remarkable number of cancer cells (Vergote et al., 2010). This reduction in the number of cancer cells in tumor mass increases the availability of oxygen, growth factor and nutrition and leads to pushing the remaining cells from G0 to G1 phase. On the other

hand, chemotherapy and radiation following tumor mass removal destroy the cells that have newly entered the cell cycle (Kumar, Abbas, Fausto & Aster, 2014).

2.3 Prostate cancer

Prostate gland, which is located in front of the rectum, between the bladder and the penis, is the male sexual gland (Seisen et al., 2012). Prostate cancer, or carcinoma of the prostate, affects the gland of the male reproductive system known as prostate (Siegel, Ma, Zou, & Jemal, 2014). Prostate cancer is the second most frequent malignancy in (Daniyal et al., 2014) and the fifth leading cause of cancer-related death in men globally (Sultana et al., 2014). Prostate cancer particularly affects older men: 80% of cases are diagnosed after age 65. Although the incidence and mortality rates are highly variable for prostate cancer in different countries, overall, developed countries have shown a higher rate of prostate cancer incidence and mortality (Torre et al., 2015).

Generally, prostate cancer is a slow-growing type of cancer. Prostate cancer could be metastatic and spread to other organs, mainly lymph nodes and bones (Ruddon, 2007). Prostate cancer may show no signs of symptom in early stages, but its appearance at the late stages of life is devastating (Heidenreich et al., 2014b). The symptoms are blood in urine, difficulty in urinating and pain in the pelvic back or even painful urinating (Ruddon, 2007).

Prostate cancer treatment includes hormone therapy, radiation therapy and chemotherapy (Sweeney et al., 2015). Prostate cancer is considered as treatable cancer when it only occurs inside the prostate (Siegel, Ma, Zou & Jemal, 2014). For the metastatic cases in which cancer has already spread to bones or lymph system, pain medications, bisphosphonate and target gene therapy are possibly needed. The survival rate of prostate cancer depends on the person's age and the extensiveness and aggressiveness of cancer (Siegel, Ma, Zou & Jemal, 2014). Most patients do not end up

dying due to prostate cancer. In the United States, the survival rate of five years is almost 99% (Heidenreich et al., 2014a).

According to the Malaysian National Cancer Registry, prostate cancer ranks ninth overall and when subdivided to cancers in men, prostate cancer ranks fourth (7.3% of all cancers). The overall age standardized incidence is 12 per 100,000 population; the highest incidence is among Chinese (15.8 per 100,000) followed by Indians (14.8 per 100,000) and Malays being the lowest (7.7 per 100,000) (Baade, Youlden, Cramb, Dunn, & Gardiner, 2013).

2.4 Prostate cancer cell line, PC3

In 1979, PC3 cells were isolated from bone metastatic prostate cancer obtained from a 62-year old Caucasian male. PC3 is entirely composed of carcinoma cells (Mizutani et al., 2009). This cell line is hormone insensitive and has no androgen receptor (AR) or prostate specific antigen (PSA). PC3 is a highly aneuploidy line which represents 58 chromosomes in karyotype. Transferrin receptor and epidermal growth factor receptor are expressed in PC3 cell line after treating the PC3 cell line with the transferrin which is derived from bone marrow (Barua & Rege, 2009). The transferrin receptor is a cellular surface receptor which interacts with transferrin in order to uptake the cellular iron. This receptor is an attractive target for cancer therapy since it is upregulated on the surface of many cancer cell types (Daniels et al., 2012).

EGF-R (epidermal growth factor receptor) and TGF-α (Transforming growth factor alpha) are highly expressed in PC3 cell line in autonomous growth condition, which explains the hospitability of bone as a metastatic site. Moreover, p53 is improperly expressed with a C deletion in codon 138. It resulted in a nonsense codon at 169 and consequently, phosphatase and tensin homolog (PTEN) deficiency (Barlaam et al., 2015).

2.5 Conventional cancer treatment

In the beginning of the 16th and 17th centuries, cancer was identified as a mortal disease. Removing the tumor mass through surgery was the earliest treatment for cancer therapy (Baum, Demicheli, Hrushesky, & Retsky, 2005). Even though over centuries the techniques of tumor removal surgery have remarkably improved, the severe side effects of surgery such as infection, pain, blood clots, bleeding, damage to other organs, damage to nearby tissues and lastly the long process of recovery of other body functions are the main disadvantages of surgery. Moreover, a recurrent tumor mass is observed in most of the cases which leads to the application of the other treatments besides surgery (DeSantis et al., 2014).

Chemotherapy and radiotherapy are applied to destroy and damage the remaining cancer cells after tumor removal surgery. The combination of surgery and chemotherapy increases cancer patients' survival chance. However, chemotherapy has intense side effects that render it unbearable for some patients. Chemotherapy drugs generally refer to a group of drugs with inhibition effect on cell division process. Hormonal therapy is a subgroup of chemotherapy. In hormonal therapy, the extracellular growth signals are blocked to inhibit the growth and division of the cell (Torre et al., 2015). Since chemotherapy drugs spread through the whole body, they can affect not only cancer cells but also the healthy cells. This leads to immunosuppression, cognitive impairment, organ damage, peripheral neuropathy, gastrointestinal distress, infertility, vomiting nausea, fatigue and anemia (DeSantis et al., 2014). On the other hand, radiotherapy that is applied for the same reason as chemotherapy and destroys the remaining cancer cells after surgery also affects the rest of the body severely. In radiotherapy, ionizing radiation is used to control or kill the malignant cells by damaging the DNA of cancerous tissue. The side effects of radiotherapy depend on the dose, time and length of ionizing radiation and the location of the body that receives the radiation (Donker et al., 2014). In general, the side

effects of radiotherapy contain damage to the epithelial surfaces such as stomach, mouth and throat, swelling, nausea and vomiting, intestinal discomfort and infertility. Since radiotherapy is mainly damaging cancer cells, in some cases, the radiation may cause DNA damage to other organs and result in secondary cancers (Kamada et al., 2015).

2.6 Herbal treatment

The conventional cancer treatment includes chemotherapy and radiotherapy that cause severe side effects and sometimes treatment failure (DeSantis et al., 2014). As an alternative, the use of synthetic agents from medicinal plants are proposed (Efferth, Li, Konkimalla, & Kaina, 2007). The use of plants to cure cancer started long ago (Kaur, Kapoor, & Kaur, 2011). Medical plants contain therapeutic value constituents which produce physical action in the human body (Petrovska, 2012). Recent phytochemical investigations and clinical studies have shown the potential anti-cancer benefits from natural compounds (Sharma, Parihar & Parihar, 2011; Bhanot et al., 2011).

From 1950 to 1970, plants contribute to modern therapeutics by providing about 100 new drugs for the USA drug market. Drugs such as vincristine, deserpidine and reserpine have been derived from plants. Drugs with plant base were introduced to the worldwide markets such as artemisinin, eguggulstrone, ectoposide and ginkgolides from 1971 to 1990. Thereafter, approximately 2% drugs were introduced from 1991 to 1995 including topotecan, irinotecan, gomishin, paclitaxel and others isolated from plant *Catharanthus rosesus* that has been used for the treatment of various types of cancers such as choriocarcinoma, nonhodgkins and Hodgkin's lymphomas, leukemia in children, testicular and neck cancer (Pandey, Debnath, Gupta, & Chikara, 2011).

Lastly, natural products are the important source of anticancer agent. More than 60% of anticancer agents have been derived from natural sources like organisms and microorganisms, marine and plants (Kinghorn et al., 2009). The search is ongoing and

many more plants with potential anticancer properties have yet to be explored. Based on the report of National Cancer Institute of the United States of America, around 114,000 extracts have been screened for anticancer activity (Shoeb, 2006).

2.7 *Litsea costalis*

Litsea costalis, with the official name *Litsea costalis* (Nee) Kosterm, belongs to the large family of the plants known as Lauraceae. It has been widely used in China and Malaysia to treat several diseases like stomach aches, infuenza, hypertension and diabetes (Gondwe, Kamadyaapa, Tufts, Chuturgoon, Ojewole & Musabayane, 2008; Hosseinzadeh, et al., 2013b). *Litsea costalis* contains structurally diverse and biologically active chemical constituents aporphine alkaloids (Wang et al., 2010), anolides (Hosseinzadeh, et al., 2013a), flavonoids, sesquiterpenes and phenylpropenes (Zhang et al., 2003). The bark of *Litsea costalis* has been shown in **figure 2.1.**

Figure 2.1: *Litsea costalis* bark (Hosseinzadeh, et al., 2013a)

2.8 Biseugenol B

In 2013, Hosseinzadeh et al. had extracted, isolated and purified 8 different compounds from *Litsea costalis* bark (Hosseinzadeh, 2013c). The chemical constituents of these compounds contain aldehyde, neolignan, biphenyl ether lignan, phenyl hydrazine, stilbene and oxyneolignan. Biseugenol B or 2,2'-oxybis (4-allyl-1-

methoxybenzene) is an oxyneolignan, a new compound that has been isolated from this plant and never been reported before as a phytochemical.

Oxyneolignan is a natural plant product, which belongs to a major class of "lignans and neolignans". It is basically derived from cinnamic acid derivatives and biochemically related to phenylalanine metabolism (Häusler, Ludewig, & Krueger, 2014).

From a chemical point of view, lignans and neolignans are characterized by the coupling of two C_6C_3 units. The bonding patterns of the two C_6C_3 units differentiate the lignans, neolignans and oxyneolignans.

The C_6C_3 unit or phenylpropanoid is an organic metabolism that consists of one aromatic ring (C_6) that is attached to an unsaturated 3-carbon chain (C_3) and is synthesized from amino acid phenylalanine by plants (Chikezie, Ibegbulem, & Mbagwu, 2015) (**Figure 2.2**). The C_6C_3 has been observed in most of the anti-cancer compounds such as pinoresinol (colorectal cancer) (Fini et al., 2008), podophyllotoxin (breast cancer) (Butt & Amjad, 2015) and steganacin (leukemia) (Hazra & Chattopadhyay, 2016).

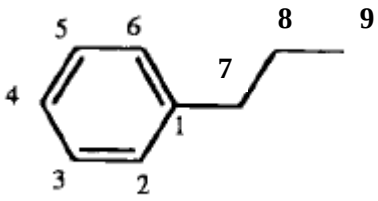


Figure 2.2: Chemical structure of C6C3 unit (Sainvitu et al., 2012)

From a phytochemical point of view, lignans and neolignans have served as antitumor, antiproliferation and anti-viral compounds. One of the best known antimitotic compounds, podophyllotoxin, is a lignan which exerts its anti-tumoral activity by binding to tubulin and inhibiting topoisomerase II. Compounds that inhibit tubulin polymerization are commonly used as chemotherapeutic agents against cancer (Lu, Chen, Xiao, Li &

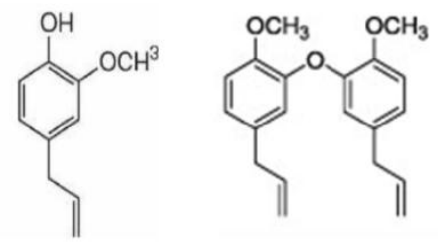
Miller, 2012). Additionally, podophyllotoxin has antiviral properties against HIV (Chen et al., 2007). The other lignan, nordihydroguaiaretic acid, was found to suppress HIV-1 (Barquero, Dávola, Riva, Mersich, & Alché, 2014). In some epidemiological studies, results showed that exposure to lignans is associated with lower risk of breast cancer (Boccardo, Puntoni, Guglielmini, & Rubagotti, 2006; Adlercreutz, 2007). Also, they revealed a potent cytotoxicity against colon cancer (Kim, Moon, Kim, Choi, & Lee, 2012). Moreover, neolignans have shown anti-proliferation and anti-tumor activities against prostate cancer (Song et al., 2005), breast cancer (Liao et al., 2012) and cervical cancer (Kma, 2013).

On the other hand, lignans and neolignans have shown antioxidant activity against biotic and abiotic factors in plants and play an anti-inflammatory and antioxidant role in some basic research models of human diseases (Potapovich et al., 2011). Lignans may also have anticarcinogenic activities.

Since the exploration of the compounds in lignans and neolignans family has already resulted in the characterization of many useful and interesting compounds in various therapeutic areas including cancer therapy, it can be expected that the new members of lignans family have the same potential.

The compound biseugenol B with IUPAC (International Union of Pure and Applied Chemistry) name of 2, 2'-oxybis (4-allyl-1-methoxybenzene) is available in two forms; in yellow brown oil and in bright yellow powder form, with molecular formula of $C_{20}H_{22}O_3$ and molecular weight of 309.20 g/mol (Hosseinzadeh, et al., 2013a).

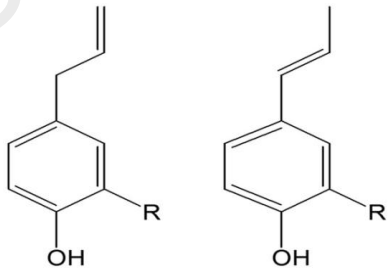
The reason of appellation of biseugenol B is its molecular structure. By comparing the chemical structure of “eugenol” and biseugenol B, it can be stated that biseugenol B is formed of two “eugenol”, linked together by an oxygen bond (**Figure 2.3**).



A **B**

Figure 2.3: Chemical structure of (A) Eugenol and (B) Biseugenol B (Munerato, Sinigaglia, Reguly, & de Andrade, 2005; Hosseinzadeh, et al., 2013a)

Eugenol and isoeugenol (**Figure 2.4**) are the subclass of phenylpropenes that are known as phenylpropanoids, chavicol and t-anol (Koeduka et al., 2006). Plants generally produce and store phenylpropenes to defend against herbivores, parasitic, bacteria and fungi. For instance, eugenol has shown a general active anti-microbial and anti-animal toxin with analgesic properties for humans (Kaufman, 2015). Moreover, eugenols have also shown anti-cancer properties. In 2007, Pisano and Pagnan et al. reported the anti-proliferative effect of eugenol, iseugenol and five other members of this family on melanoma (Pisano et al., 2007).



A **B**

Figure 2.4: Chemical structure of (A) Eugenol and (B) Isoeugenol (Munerato, Sinigaglia, Reguly & de Andrade, 2005)

2.9 Apoptosis

Apoptosis is a programmed cell death. It comes from the ancient Greek word "ἀπόπτωσις" meaning "falling off". Due to energy-dependent biochemical events,

apoptosis occurs in multicellular organisms, which leads to morphological and biochemical alterations that eventually result in cell death (Elmore, 2007).

Apoptosis is a crucial part of various vital processes in the body including function and development of immune system, normal cell repair, embryonic evolvement, hormone-dependent atrophy and chemical-induced cell death. Imbalanced apoptosis (either too little or too much) can result in many human disorders such as immune and neurodegenerative disease and many types of cancers (Elmore, 2007). The capability of controlling the life or death of the cell is a massive therapeutic potential. Hence, illumination and modulation of cell signaling pathways and cell cycle machinery for controlling apoptosis and cell cycle arrest are valuable due to their massive therapeutic potential (Butt & Amjad, 2015; Wong, 2011). Many proteins with the key role in cell apoptosis have been recognized up to now. However, their exact molecular mechanism remains to be elucidated. In 1972, the term apoptosis was used for the first time by Kerr, Wyllie and Currie. Apoptosis described a special form of cell death with distinct morphological alteration (Elmore, 2007). The turning point of our understanding of "programmed cell death" mechanism in mammalian cells transpired from the study of programmed cell death in nematode *Caenorhabditis elegans*, which occurs during its development (Yuan & Kroemer, 2010). In the process of formation, 1090 somatic cells of an adult worm were generated. 131 cells underwent programmed cell death or apoptosis. The death of these 131 cells is essentially constant among the worms that indicate a significant control in this system. Thereafter, apoptosis has been identified as a specific mode of "programmed cell death" for the elimination of cells (Debnath, Baehrecke, & Kroemer, 2005).

Apoptosis mainly occurs during development and homeostasis of the cell. It also takes place in the immune system as a defense mechanism when cells are impaired by diseases or toxic agents (Orrenius, Nicotera & Zhivotovsky, 2011). Apoptosis death occurs via

p53-dependent pathway in the cells with DNA damage from the usage of drugs for cancer chemotherapy (Chipuk & Green, 2006). In some other cells that express Fas or TNF receptor, apoptosis occurs via ligand binding (Galluzzi et al., 2011). In some cases, survival factors like growth factor or hormones block the death pathway in the cell. Finally, apoptosis is a consonant, energy-dependent process which is necessary for the activation of a group of enzymes called "caspases." The activation of caspases results in a cascade of events, which eventually leads to programmed cell death or apoptosis (Kumar, 2007).

2.9.1 Morphology of apoptosis

Apoptosis makes some morphological changes in the cell that are detectable with light and electron microscopes. Some samples of these changes are smaller cell size, cell shrinkage and chromatin condensation. "Pyknosis", the most characteristic feature of apoptosis is an irreversible condensation of chromatin in the nucleus and it is followed by fragmentation of the nucleus. As apoptosis progresses, plasma membrane blebbing and nuclear fragmentation occur (Orrenius, Nicotera, & Zhivotovsky, 2011; Kepp, Galluzzi, Lipinski, Yuan, & Kroemer, 2011).

2.9.2 Biochemical Features

In addition to morphological alterations, apoptosis causes several biochemical modifications including DNA breakdown, protein-cross-linking, phagocytic recognition and protein cleavage. Apoptosis cells broadly express caspases that initiate the protease cascade. Caspases possess proteolytic activity, which means they can cleave proteins. When caspases are activated, the irreversible death fate processes in the cell (Henry, Hollville, & Martin, 2013). Up to now, ten main caspases have been recognized and they are classified into three major groups: 1- initiator caspases (caspases-2, -8, -9, and -10), 2- executioners or effectors (caspases-3, -6, and -7) and inflammatory caspases (caspases-1, -4, and -5). The other characteristic of apoptosis cell is protein cross-linking that results

in DNA breakdown known as DNA fragmentation (Elmore, 2007). One of the other biochemical changes during apoptosis process is binding Annexin V protein to phosphatidylserine residues. Annexin V is a specific protein that specifically and strongly binds to phosphatidylserine residues and it is useful for apoptosis detection (Niu & Chen, 2010).

2.9.3 Mechanisms of Apoptosis

Apoptosis is a highly complex, energy-dependent cascade of molecular events. To date, two main apoptosis pathways have been identified: extrinsic (death- receptor) and intrinsic (mitochondrial) pathways. Recent findings have shown that the two pathways are linked (Wong, 2011).

2.9.3.1 Extrinsic pathway

Extrinsic apoptosis pathway is a signaling pathway that initiates in response to extracellular stimuli such as drugs, toxins, immune system, infections and hormones. It is activated by extracellular ligands binding to the surface cellular receptors that transfer the apoptosis signals into the cytoplasm. After that, the apoptosis pathways begin in the cell (Chan et al., 2011; Fulda & Debatin, 2006). **Figure 2.5** simplifies the extrinsic signaling pathway in which extracellular stimuli initiates the apoptosis pathway via surface cellular receptors which leads to activation of caspase-8 and then caspase-3, -6 and -7 that results in cell apoptosis.

In extrinsic apoptosis signaling pathway, transmembrane receptor-mediated interactions such as tumor necrosis factor (TNF) a receptor gene superfamily (Ashkenazi, 2008), initiate the apoptosis process. The family of TNF receptor share a similar extracellular domain, as their cytoplasmic domain also called “death domain,” contains 80 amino-acids (Croft et al., 2012). The transmitting of death signals from cell surface to the intracellular pathway is performed by cytoplasmic domain or death domain.

Apo2L/DR5, Apo3L/DR3, Apo2L/ DR4, FasL/FasR and TNF-α/TNFR1 are known as some of the best-characterized ligands and equivalent death receptors (Elmore, 2007; Napetschnig & Wu, 2013). The FasL/FasR and TNF-α/TNFR1 models characterize the sequence of events that can define the extrinsic apoptosis pathway. This model includes receptors and is bound to the homolog ligand. The binding of Fas and TNF ligands to Fas and TF receptors results in the binding of the adaptor proteins of FADD and TRADD, with the recruitment of FADD and RIP respectively (Oeckinghaus, Hayden, & Ghosh, 2011). The interrelation of FADD with procaspase-8 via the dimerization of the death effector domain which forms a death-inducing signaling complex (DISC), results in the activation of caspase-8. The activation of caspase-8 triggers the apoptosis execution (Gurung et al., 2014).

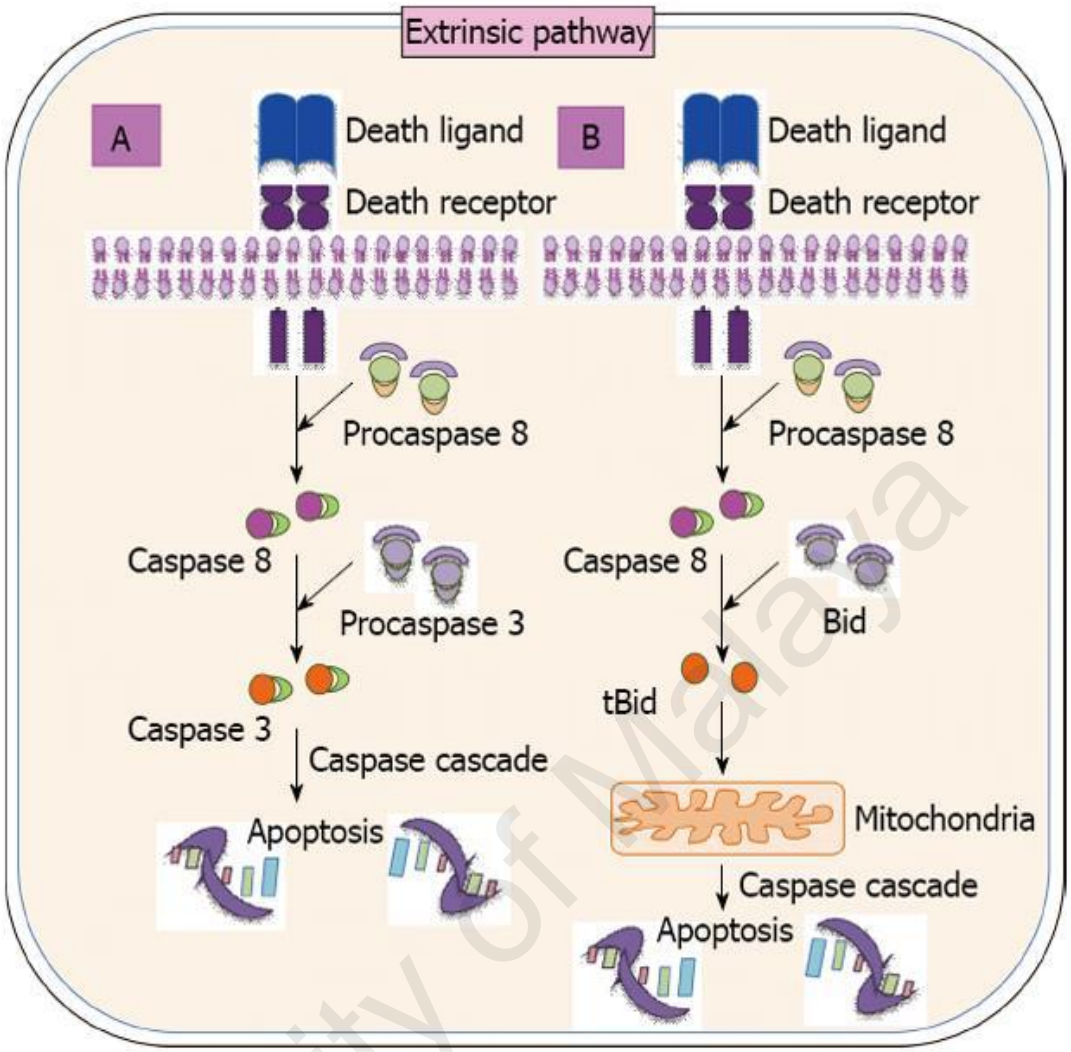


Figure 2.5: Schematic picture of the cell extrinsic apoptosis pathway showing the involvement of caspase-8 (Larrubia, Lokhande, García-Garzón, Miquel, Subirá, & Sanz-de-Villalobos, 2013)

2.9.3.2 Intrinsic apoptosis pathway

The intrinsic apoptosis pathway is a pathway that is initiated by internal cell stimuli such as DNA damage, cell division hang up, hypoxia and chromosome rearrangement. These intracellular signals pass through the cell and activate the apoptosis responses in the cell. Figure 2.6 simplifies the intrinsic signaling pathway in which intracellular stimuli initiates the apoptosis pathway via activation of pro-apoptosis proteins like Bax which leads to release of cytochrome c and activation of caspase-9, -3 and -7 that results in cell apoptosis.

In the intrinsic apoptosis pathway, intracellular signals can directly start apoptosis signals within the cell. This pathway is also known as the mitochondrial-initiated pathway (Elmore, 2007). There are several non-receptor-mediated stimuli that are involved in the apoptosis cascade events that are originally initiated in mitochondria. The intrinsic pathway is usually initiated by the stimuli that produce intracellular signals. These stimuli might have either positive or negative functions. In the case of negative signals, the absence of certain cytokines, hormones or growth factors may fail the suppression of the cell death program and hence trigger the apoptosis (Fulda & Debatin, 2006; Ghobrial, Witzig, & Adjei, 2005). The loss of apoptosis suppression and withdrawal of factors (certain cytokines, hormones or growth factors) lead to apoptosis initiation. On the other hand, there are stimuli that act in a positive fashion including radiation, hypoxia, free radicals, toxins and viral infections which can initiate intracellular apoptosis cascade (Valko et al., 2007).

Mitochondrial membrane potential or MMP plays an essential role in apoptosis signaling pathway. Cytochrome c is one of the most important pro-apoptotic proteins which is normally located inside the mitochondrial membrane. These stimuli cause alterations in the mitochondrial inner membrane, which in its place, can result in the opening of the mitochondrial permeability transition (MPT) pore. This subsequently leads to the release of pro-apoptosis protein from mitochondria into the cytosol (Saelens et al., 2004; Casares et al., 2005). Cytochrome c (one of pro-apoptosis) binds and activates procaspase -9, forms the "apoptosome" and initiates caspase-dependent mitochondrial pathway (Hill, Adrain, Duriez, Creagh, & Martin, 2004; MacFarlane & Williams, 2004). Activation of pro-caspase-9 eventually results in caspase-9 activation (Yaoxian et al., 2013).

Bcl-2 family of proteins control and regulate the mitochondrial apoptosis events and can be either pro-apoptotic or anti-apoptotic. For instance, Bcl-2 is an anti-apoptotic,

while Bcl-10 and Bax are pro-apoptotic (Garcia-Saez, 2012). In turn, Bcl-2 has been regulated by p53 which is a well-known tumor suppressor protein (Wu et al., 2013). The balance between Bcl-2 family protein member expressions can determine the fate of the cell: it either proceeds to apoptosis or aborts the process. The main mechanism of the action of the Bcl-2 family of proteins regulates the changes of the mitochondrial membrane permeability to control the release of cytochrome c from mitochondria into the cytosol (Weyhenmeyer, Murphy, Prehn, & Murphy, 2012) **(Figure 2.6)**.

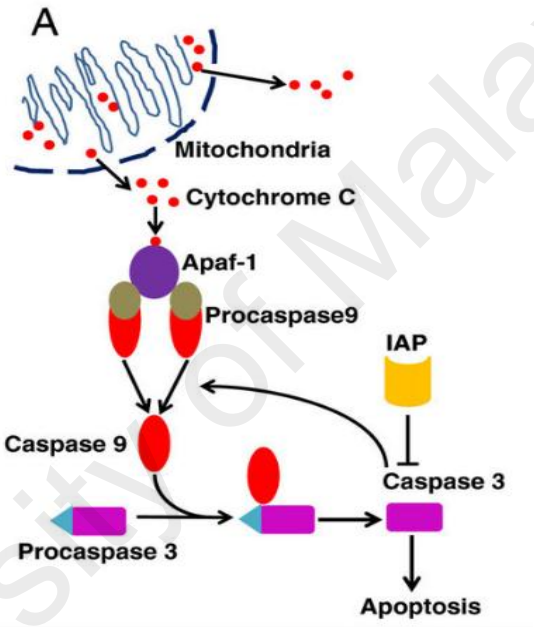


Figure 2.6: Schematic picture of cell intrinsic apoptosis pathway showing the involvement of Bcl-2, Bcl-X, Cytochrome c, Caspase 9 and Caspases 3 & 7 (Ooi & Ma, 2013).

2.9.4 NF-κB anti-apoptosis signaling pathway

In addition to apoptotic pathways in the cell, there are signaling pathways known as anti-apoptotic or survival pathways. Anti-apoptotic pathways are activated when the cell faces difficult conditions like high ROS (reactive oxygen species) or stress that might lead the cell to apoptosis. In such conditions, anti-apoptotic signaling pathways are activated to ensure the survival of the cell (Hoesel & Schmid, 2013). One of these survival pathways is NF-κB signaling pathway. Logically, in cancer therapy, inhibition of survival

pathways is one of the strategies to propel the cell to apoptosis in cancer cells (Signore, Ricci-Vitiani, & De Maria, 2013).

In 1986, NF-κB, a nuclear transcription factor was discovered. NF-κB adheres to enhancer factor of the light chain of immunoglobulin kappa in activated B-cells (Hoesel & Schmid, 2013). The protein that harbors NF-κB is expressed in almost all cell types. NF-κB regulates many target genes by binding to DNA. The other members of this transcription factor family are NF-κB1, NF-κB2, p65 (RelA), RelB and RelC (Oeckinghaus & Ghosh, 2009).

The active form of NF-κB is not a single protein, but dimers that bind to κB site, a common sequence motif (Gilmore, 2006). All members share the Re homolog domain which is essential for binding to DNA elements as well as dimerization (Israël, 2010). Different distinct NF-κB dimers can induce different target genes that influence a broad range of biological processes like inflammation, immune responses such as innate or adaptive immunity, stress responses, lymphoid organogenesis and B-cell development. In quiescent mode, these dimers are bound to the inhibitors of NF-κB proteins. These inhibitors contain ankyrin repeats that connect DNA-binding domain with the transcription factors to turn them to transcriptionally inactive forms. P105 and p100, which are the precursors of p50 and p52, also contain ankyrin repeats. Their ankyrin repeats were cleaved upon maturation. In contrast to other members of NF-κB, p105 and p100 do not have transcription domain and when bound to NF-κB elements act as transcriptional processors (Shih et al., 2012; Solan, Miyoshi, Carmona, Bren, & Paya, 2002). The binding between IκB molecules and NF-κB dimers can prevent the binding of NF-κB dimers to DNA and keep the NF-κB in the cytosol (Wu & Zhou, 2010). Generally, NF-κB activation occurs by either the release of IκB molecules or cleavage of the p100 and p105 inhibitory ankyrin domain. This happens at inhibitor proteasomal degeneration or the degeneration of precursors.

The activity of various types of NF-κB dimers is regulated by two main pathways. In the first pathway, also known as the canonical NF-κB activation, κB inhibitor proteins (IκB) captivates the dimer that contained RELA, REL-C and p50 in the cytosol. This pathway is usually triggered in response to the exposure to cytokines protein-inflammatory and viral or bacterial infections. These extracellular stimuli activate the IκB kinase (IKK) complex. This results in the liberation of NF-κB dimers and their translocation to the nucleus (Basseres & Baldwin, 2006).

In the second pathway, NF-κB generally dimerizes with REL (Gilmore, 2006). In this pathway, a certain member of TNF (tumour-necrosis factor) triggers the process that activates the IKKα subunit along with NIK (a protein kinase). Activated IKKα and NIK together induce the removal of the C-terminal domain of NF-κB2 which results in the translocation of the RELB-p52 to the nucleus (Yamamoto & Gaynor, 2004; Razani et al., 2010). The transcriptional function of NF-κB is modulated in the nucleus by phosphorylation. Even though each NF-κB dimmer possesses a different regulatory function, they have same target genes. The main target genes of NF-κB dimers are divided into four groups: anti-apoptosis genes, inflammatory and immunoregulatory genes, genes that induce cell-proliferation and finally genes that suppress NF-κB. Genes of all four groups have contribution in tumorigenesis (Hoesel & Schmid, 2013). In general, IκB proteins bind and inhibit NF-κB/Rel proteins. The inhibition of NF-κB anti-apoptosis pathway is an attractive target to induce apoptosis in cancer cells as a cancer treatment strategy. Some natural compounds like curcumin are well-known as NF- κB inhibitors (Zhu et al., 2014; Shakibaei, John, Schulze-Tanzil, Lehmann, & Mobasheri, 2007).

2.9.5 The role of anti-apoptotic protein, Hsp70

Hsp70 or heat shock proteins are a large group of proteins with strong anti-apoptotic properties (Lanneau, et al, 2008). Their main function is mostly described as protection of the cell in oxidative or thermal shocks. They are mainly being activated in toxic and

heat stress situation in the cell (Richter, Haslbeck & Buchner, 2010). The cytoprotective activity of Hsp70 protein may be explained by its anti-apoptotic property. Hsp70 protein family play a vital role in inhibition of apoptotic machinery in pre-mitochondrial as well as the post-mitochondrial level of apoptosis (Rérole, Jego & Garrido, 2011). Previous studies showed that the expression of Hsp70 in cancer cells is abnormally high that some studies have suggested the oncogenic role for Hsp70 (Garrido, et al, 2003). Therefore, one of the anti-cancer strategies may be the suppression or down-regulation of Hsp70. Accordingly, if a compound can show the down-regulation of Hsp70 property, it can potentially be a candidate for anti-cancer agent.

CHAPTER 3: METHODOLOGY

For obtaining results based on objective 1: assess cytotoxicity and selectivity of biseugenol B against human prostate cancer cell line using:

- Cell viability assay (MTT)
- Quantification of apoptosis using propidium iodide and acridine orange double staining
- Assay of the apoptotic rate by Annexin V (AV)-fluorescein isothiocyannate (FITC) staining
- Multiple cytotoxicity assay
- Measurement of reactive oxygen species generation

For obtaining results based on objective 2: determine apoptosis mechanism by biseugenol B using:

- Multiple cytotoxicity assay
- Measurement of reactive oxygen species generation
- Bioluminescent assays for caspase activity

For obtaining results based on objective 3: determine the modulation of apoptotic signaling pathway through cell cycle status, RT-PCR and western blotting using:

- Cell cycle analysis
- Analysis of mRNA expression by RT-PCR
- Western blot analysis
- NF-κB translocation

3.1 Materials

The isolation of the natural compound, biseugenol B from the bark of *Litsea costalis* was carried out by Hosseinzadeh and team (Hosseinzadeh, et al., 2013a) in the Chemistry Department, Faculty of Science, University Malaya. The crude extract has been obtained applying cold extraction technique by using methanol and dichloromethane following n-hexane. Chromatographic technique like preparative thin layer chromatography, column chromatography and higher performance liquid chromatography has been applied for isolation the pure compound from the raw extract. The process of extraction has been detailed in the thesis and related research paper (Hosseinzadeh, 2013c), (Hosseinzadeh, et al., 2013a).

The LC-MS result indicated the formula of $C_{20}H_{22}O_3$ with molecular weight of 309.20 g/mol . The detailed results of IR Spectrum, LC-MS spectrum, NMR spectra data, H-NMR spectrum, COSY spectrum, C-NMR/DEPT spectrum, HMQC spectrum, HMBC spectrum has been described in Hosseinzadeh's thesis (Hosseinzadeh, 2013c).

PC3 (Human prostate cancer cell line), RWPE-1 (non-tumorigenic human prostate cells), WRL68 (hepatic fetal human epithelial cell line), MCF7 (human breast adenocarcinoma cell line), MDA-MB-231 (human breast adenocarcinoma cell line) and CEMSS (cervical cancer) were obtained from (American Type Cell Collection (ATCC). RPMI 1640 medium, K-SFM media, FBS (fetal bovine serum), BPE (Bovine Pituitary Extract), streptomycin/penicillin- rhEGF (human Epidermal Growth Factor) and antibiotic/antimycotic solution were Gibco brand which purchased from Fisher Scientific (United States). BD Pharmingen ™ Annexin V-FITC Apoptosis Detection Kit, DNA reagent Kit BD Cycletest Plus were obtained from BD Bioscience (USA) while, Multiparameter Cytotoxicity 3 Kit purchased from Fisher Scientific (USA). Caspase-Glo® assay kits (Promega) was bought from ITS Interscience is a Malaysia laboratory

equipment and medical supplies company. Nuclear determinant kappa B (NF-κB) activation kit was purchased from Fisher Scientific (USA). The Hsp70, NF-κB/p65 (sc-398442), Bax (sc-20067) and β-actin (sc-130300) antibodies were purchased from Santa Cruz Biotechnology Inc (USA)., while Bcl-2 (ab38629) was purchased from Abcam (UK). T25 Flasks and 96-Well Cell Culture Plates were purchased from Thermo Fisher Scientific (USA).

3.2 Methods

3.2.1 Cell culture

Human prostate cancer cells (PC3) and non-tumorigenic human prostate cells (RWPE-1), the most prevalent type of normal prostate cells immortalized by human papilloma virus (HPV) 18 (Miki et al., 2007; Ali et al., 2011) were obtained from American Type Cell Collection (ATCC) and maintained in the incubator with 5% CO_2 at 37°C (Mohan et al., 2012). Human prostate cancer cells (PC3) were cultured in RPMI 1640 medium with 1% 100 unit/mL streptomycin/penicillin and 10% FBS (Anasamy et al., 2013), while, normal human prostate cells (RWPE-1) were cultured in concentration of 4×10^4 cells/well in K-SFM media with 0.2 ng/mL rhEGF (human Epidermal Growth Factor), 25µg/mL BPE (Bovine Pituitary Extract) (Nyberg & Espinosa, 2016) and 1% antibiotic/antimycotic solution. Cultured cells are maintained in the incubator with CO_2 at 37°C (Achanzar et al., 2001; Gnanasekar, Thirugnanam, & Ramaswamy, 2009; Gummadi et al., 2013).

3.2.2 MMT Cell viability assay

The cell growth determination was done by counting viable cell after coloring the cells with a yellow MMT dye. The viable cells can reduce the tetrazolium MMT dye to its purple color insoluble formazan. By measuring the absorbance of the colored cells at 570 nm by spectrophotometer the amount of viable cell can be calculated. Briefly, cells by the

number of 5×10^4 cells/well of PC3, WRL68, MCF7, MDA-MB-231, CEMSS and 1×10^5 cells/well of RWPE-1 cell line were treated with biseugenol B at different concentration (100, 50, 25, 12.5, 6.25, 3.125, 1.563 and 0 µg/mL) in 96 well plate and maintained in incubation for 24, 48 and 72 hours. For positive control docetaxel (the most famous chemotherapy medication for prostate cancer treatment) (De Bono et al., 2010) has been used. PC3 cells in concentration of 1×10^4 cells/well of PC3 were treated with different concentration of docetaxel (100, 50, 20, 10, 5 and 0 nM) for 24, 48 and 72 hours. Later, MTT dye (Invitrogen, Thermos Fisher Scientific, Waltham, Massachusetts, USA) has been added in the wells in an equal amount following incubating for 3 hours in the dark at 37°C. 20 µL of DMSO has been added. The colorimetric assay was measured and recorded at the absorbance of 570 nm. IC_{50} value was measured as the potency of cell growth inhibition for test agent. The plot of the data obtained based on absorbance against the number of cells has been transferred to excel to calculate the average of absorbance and provides a curve with a linear portion (Mohan et al., 2012).

3.2.3 Quantification of apoptosis using propidium iodide and acridine orange double staining

Nucleic acid binding dyes, acridine orange (AO) and propidium iodide (PI) can separately bind to nucleic acid of the cell and measure the cell viability. Acridine orange is an intercalating dye that is able to transmit to both live and dead cell and generate green fluorescent. On the other hand, propidium iodide (PI) can only pass through the dead cells or cells with damaged membrane and generate red fluorescent. In this method, all the cells were stained with both dyes. Viable cells which have total membrane integrity only allow acridine orange (AO) intercalate the cell and stain their nucleic acid. Thus, viable cells can be observed as green. Meanwhile, propidium iodide (PI) can intercalate early and late apoptosis cells which have poor membrane integrity and detected as orange-red fluorescent. Quantification of apoptosis was performed using acridine orange (AO) and

propidium iodide (PI) double staining. Cell death induced by biseugenol B in PC3 human prostate cancer cells was measured based on the regular process as they were being observed under a fluorescence microscope (Leica attached with Qfloro Software, Solms, Germany) (Mohan et al., 2012). Fluorescence microscope was used for counting the cells to investigate the population of viable cells, early apoptosis, late apoptosis and secondary necrosis. Concisely, PC3 cells by the number of 2×10^5 cells/well were treated with different concentration (0, 2, 4 and 8 µg/mL) of biseugenol B in a TPP Brand 25-mL culture flask, then incubated for 24 hours at 37°C in the humidity of 5% CO_2. Afterwards, for 10 minutes the cells were spun down at 1800 rpm resulting in eliminating supernatant. Then pellet was washed twice by using cold PBS to eliminate the media. At that time, the addition of ten microliters of mixed fluorescent dye PI (10 µg/mL) and AO (10 µg/mL) (Nexcelom Bioscience, Lawrence, Massachusetts, USA) to the cellular pellet was carried out at identical volumes. On a glass slide, stained cell suspension was freshly dropped and screened by a coverslip and observed under the fluorescent microscope 30 minutes prior to fading (Ali et al., 2011). The untreated PC3 cells were served as control.

3.2.4 Assay of the apoptotic rate by Annexin V (AV)-fluorescein isothiocyanate (FITC) staining

For quantitatively measuring the percentage of apoptosis cells FITC Annexin V technique was applied. This technique mainly relies on the loss of membrane asymmetry which happens in early stages of apoptosis. In this case, phosphatidylserine (PS) which is membrane phospholipid and normally is located on the inner side of membrane cell translocates to external leaflet of cell membranes. Annexin V is a phospholipid-binding protein with high affinity for phosphatidylserine (PS). When apoptotic cell exposed to annexin V, it binding to PS and if it conjugated to fluorochrome like FITC, the early apoptotic cell can be detected by flowcytometer. Meanwhile, late apoptosis cells with damaged membrane integrity are permeable to propidium iodide (PI). Hence, cells that

stained with none of annexin V and PI are viable cells, cells that stained with only annexin v and not PI are in early stage of apoptosis and cells that stained with both annexin V and PI are in late stage of apoptosis. The kit Annexin V-FITC Apoptosis Detection contains FITC Annexin V and Propidium Iodide (PI) dye, plus Annexin V Binding Buffer. Human prostate cancer cells (PC3) were cultured by 1×10^5 cells/well and exposed to different concentrations (0, 2, 4 and 8 µg/mL) of biseugenol B. By using the BD Pharmingen ™ Annexin V-FITC Apoptosis Detection Kit (APO Alert Annexin V, Clontech, Mountain View, CA, USA) (Ibrahim et al., 2014), the AV assay was executed. To eliminate the media, treated cells were centrifuged at 1800 rpm for 5 minutes. 1×binding buffer (provided by the manufacturer, BD Pharmingen ™ Annexin V-FITC Apoptosis Detection Kit) was used to wash the cells. Subsequently, the cells were suspended for the second time in 200 µL of binding buffer and prior to the incubation at 37°C that was carried out in the dark for 15 min in 5 µL of AV and 10 µL of PI (Sigma-Aldrich Co, St Louis, MO, USA) was added. The former caused a jump in the volume of the reaction to 500 µL for the analysis of flow cytometric that was carried out employing FACS Canto II (BD Biosciences, Sam Jose, CA, USA) (Ibrahim et al., 2014). The untreated PC3 cells were served as control.

3.2.5 Multiple cytotoxicity assay

Apoptosis is a complex process that affects multiple parameters in the cells, starting by the alteration in nuclear size and morphology and then, loss of mitochondrial membrane potential (MMP) which followed by the release of cytochrome c from mitochondria into the cytosol and alteration in membrane permeability. These six independent factors: nuclear size, changes in cell membrane permeability, morphological changes, release of cytochrome c, mitochondria membrane potential (MMP) changes and cell loss were quantified at the same time by applying a Multiparameter Cytotoxicity 3 Kit (Cellomics Technology, Halethorpe, MD, USA) (Ahmadipour et al., 2015; Mohan et

al., 2012). The kit contains Hoechst dye which detects the alterations in nuclear morphology and cell loss, cytochrome c primary and secondary antibody which monitors the translocation of cytochrome c, MMT dye which detects the mitochondrial membrane potential and permeability dye which assays the cell membrane permeability, plus, washing, blocking and permeabilization buffer. In brief, prior to treatment with 4 µg/mL of biseugenol B for one day, the PC3 cells were seeded and then maintained in the incubator overnight at 37°C with 5% CO_2. The cell permeability dye and the MMP dye were later added to the cells and incubation was carried out for half an hour at the same degree. Afterwards, fixing, permeabilizing and blocking of the cells were conducted with 1×blocking buffer and subsequently analyzed with initial antibody of cytochrome c and secondary DyLight 649 conjugated goat anti-mouse immunoglobulin G for 55 min. To stain the nucleus, Hoechst 33342 staining solution was employed and 1000 stained cells were studied by the ArrayScan™ high content screen system (Cellomocs Technology). Stained cells were identified by this system and the distribution of fluorescence and the intensity in individual cells were reported. A special filter was used for each fluorescence channel for obtained images. All images and data were stored in the Microsoft SQL database based on their intensity and wave of fluorescence in each individual cell. The average fluorescence of whole cell population in the well was stored as well. Later, all the factors were examined by Data Viewer version 3.0 software (Cellomics Technology) and ArrayScan II Data Acquisition (Ahmadipour et al., 2015). The untreated PC3 cells were served as control.

3.2.6 Measurement of reactive oxygen species generation

2',7'Dichlorofluorescein diacetate (DCFH-DA) (Signa-Aldrich, Mrek, Saint Louis, USA) is an intracellular de-esterified probe. In the normal situation it is not fluorescent but in the presence of ROS, it turns to highly fluorescent 2′,7′-dichlorofluorescein. DCFH-DA was used to measure the production of intracellular ROS (Ng et al., 2013; Zhang et

al., 2013). Briefly, 500-fold in HBSS (Thermo Fisher Scientific) was diluted with 10 mM DCFHDA stock solution in methanol. HBSS is defined as Hank's balanced salt solution (Thermo Fisher Scientific) which contains no additives. For producing a 20 µM working solution, 10 mM DCFH-DA stock solution in methanol was diluted 500-fold in HBSS (Ibrahim et al., 2014).

PC3 and RWPE-1 cells were exposed to different concentrations of biseugenol B (0, 2, 4 and 8 µg/mL) in a 96-well black plate and then washed twice with HBSS, followed by incubation at 100 µl working solution of DCFH-DA for 30 minutes at 37°C. The results were obtained using a fluorescence microplate reader at 485 nm excitation and 250 nm emission (Tecan Infinite M 200 PRO, Männedrof, Switzerland) (Arbab et al., 2013; Zahoor, Davies, Kirk, Rollinson, & Walker, 2009). The untreated PC3 cells were served as control.

3.2.7 Bioluminescent assays for caspase activity

Caspase-Glo® assay kits (Promega, Madison, WI, USA) has been designed to measure caspase enzyme activity in the cells. For this purpose, we used three different kits of Caspase-Glo® assay: caspase-3/7and caspase-8 and caspase-9. Caspase-3/7 and -9 play a vital role in intrinsic apoptosis pathway, while caspase-8 is essential for the extrinsic pathway. Caspase-Glo® assay kits are ideal system for luciferase activity, cell lysis and caspase activity by providing a luminogenic caspase- substrate. The kit contains Caspase-Glo® buffer, Caspase-Glo® substrate. We carried out a dose-dependent study of caspases-3/7, caspase-8 and caspase-9 in triplicate in a 96-well microplate (Lo, 2012). The RWPE-1 cells at in concentration of 1×10^5 cells per well and PC3 cells at the concentration of 1×10^4 cells/well was seeded. Then, the cells were incubated with biseugenol B at different concentrations (0, 2, 4 and 8 µg/mL) for 24 hours. The activity level of the caspases was studied and analyzed (Mohan et al., 2012; Ng et al., 2013). In brief, the reagent of 100 µL of caspase-Glo was added and later incubated for half an hour

at room temperature. Aminoluciferin labeled synthetic tetra-peptide was cleaved due to the existence of active caspases from apoptotic cells followed by discharging the substrate for luciferase enzyme. By using an Infinite 200 pro microplate reader (Tecan), caspase activity was measured (Paydar et al., 2014). The untreated PC3 cells were served as control.

3.2.8 Cell cycle analysis

The BD Cycletest™ Plus DNA kit was used to isolate and stain the cell nuclei, following by detecting the difference of DNA index (DI) by flow cytometer. This kit contains the set of reagents to dissolve the lipid of the cell membrane by nonionic detergent, remove the nuclear proteins and cell cytoskeleton by trypsin, use RNase enzyme to digest the cellular RNA and spermine to stabilize the nuclear chromatin. Propidium iodide (PI) is used to stain the DNA can be detected by flow cytometer. In wavelengths between 580 to 650 nm, the fluorescent light is omitted by PI-stained nuclei. Human prostate cancer cells (PC3) were cultured at the concentration of 2×10^5 cells/mL in medium RPMI 1640, was completed with 1% penicillin/streptomycin and 10% of fetal bovine serum (FBS) in a TPP brand 25-culture flask. Later, it was treated with various concentrations of biseugenol B (0, 2, 4 and 8 µg/mL) for 1 day by using DNA reagent Kit BD Cycletest Plus (BD Biosciences). Then, according to the protocol, the cells were harvested and exposed to cell cycle analysis using a Guava easyCyte 8HT benchtop flow cytometer (Merck, Whitehouse Station, TX, USA) (Achanzar et al., 2001). The untreated PC3 cells were served as control.

3.2.9 Analysis of mRNA expression by RT-PCR

Human prostate cancer cells, PC3 were cultured in 12-well plates and treated with different concentrations (0, 2, 4 and 8 µg/mL) of biseugenol B. The RNeasy mini Kit (Qiagen, Germany) (Mohan et al., 2012; Wang et al., 2012) was used to extract total mRNA. Polymerase chain reaction was performed on 1µL of transcribed cDNA using

specific primers Bcl-2, Hsp70 and Bax genes. β-actin mRNA was applied as the loading control. The primers for Bcl-2: sense, 5'-ATG AAC TCT TCC GGG ATG G-3' and antisense, 5'-TGG ATC CAA CAA GGC TCT AGG TG-3', Hsp70: sense, 5'-CGC AGC TGA ACA AGC TAA ACA ATC-3' and antisense: 5'- GAT TGT TTA GCT TGT TCA GCT GCG-3', Bax: sense, 5'-TTT GCT TCA GGG TTT CAT CC-3' and antisense, 5'-GCC ACT CGG AAA AAG ACC TC-3' AND β-actin: sense, 5'-CGG GAA ATC GTG CGT GAC-3' and antisense, 5'-GCC TAG AAG CAT TTG CGG TG-3'. The amplification of PCR was carried out through a thermal cycle in which the reaction was initiated in primary denaturation for 5 minutes at 95°C, succeeded by 30 denaturation cycles that were annealed and extended at 95°C, 60°C and 72°C for 30 seconds and 40 seconds and 1 minute, respectively. This was followed by the last extension which was carried out at 72°C and lasted for 10 minutes. The amplification product sizes were 515bp, 213bp and 166bp for β-actin, Bax and Bcl-2, respectively. The products of polymerase chain reaction were loaded into 1.5% agarose gel electrophoresis. The staining process was later carried out with ethidium bromide. With the help of UV light employing Gel Doc XR System (BioRad, USA), the products were featured (Mohan et al., 2012). The untreated PC3 cells were served as control.

3.2.10 Western blot analysis

Human prostate cancer cells, PC3 were cultured in a TPP brand 25-mL flask (TPP Techno Plastic Products AG, Trasadingen, Switzerland), followed by treating the cells with different concentration of biseugenol B (0, 2, 4 and 8 µg/mL) for 24 hours (Ibrahim et al., 2014). Lysis buffer (50 mM TrisHCL pH 8.0, 120 mM NaCl, 0.5% NP40, 1mM PMSF) was used to extract the total protein cell. The SDS-PAGE (10%) was used to separate the 40 µg of extracted protein. The separated proteins were transferred to polyvinylidene difluoride or PVDF membrane (Immun-Blot® PVDF membrane for Protein Blotting, BioRad, USA) and the proteins were blocked using blocking buffer with

5% non-fat milk in TBS Tween buffer 7 (0.12 M Trisbase, 0.1% Tween 20 and 1.5 M NaCl). This stage took 30 minutes and was performed at room temperature followed by incubation of primary antibody at 4°C overnight. Subsequently, proteins were incubated in alkaline phosphatase conjugated secondary antibody for half an hour at room temperature. The mixture of Tween 20 buffer and tris buffer saline was used for washing. The Hsp70 (heat shock protein), NF-κB/p65 (sc-398442), Bax (sc-20067) and β-actin (sc-130300) were purchased from Santa Cruz Biotechnology Inc., while Bcl-2 (ab38629) was purchased from Abcam plc (Cambridge, UK) and served as primary antibodies. For secondary antibodies, we applied conjugated goat anti-mouse or goat anti-rabbit alkaline phosphatase. Proteins were incubated in a ratio of 1: 5000 of secondary antibody at room temperature for 1 hour. Then it was washed three times by TBST using an orbital shaker for 10 minutes using BCIP®/NBT (Santa Cruz Biotechnology Inc.). The blots were developed for sensitive colorimetric detection to quantify the target protein band (Ye et al., 2005; Mohan et al., 2012). The untreated PC3 cells were served a control.

3.2.11 NF-κB translocation

Cellomics® NF-κB Activation HCS Reagent Kit (Thermo Scientific, Waltham, MA, USA) quantify the activation of NF-κB by measuring the translocation of NF-κB from the cytosol into the nucleus. The kit contains NF-κB primary antibody (rabbit), DyLight™ 488-Conjugated Goat Anti-Rabbit IgG and Hoechst Dye, plus, wash buffer I and II and Permeabilization Buffer (10X). NF-κB which normally presents in the cytoplasm can be activated by tumor necrosis factor and been translocated to the nuclei. In this situation, it can activate the transcription of anti-apoptosis genes. Accordingly, one of the most definitive measuring of NF-κB is its translocation from cytoplasm to nuclei. Concisely, PC3 cells were seeded at of 1×10^4 cells/well and then maintained in the incubator at 37°C overnight with 5% CO_2. The cells have been treated with 4 µg/mL of biseugenol B for three hours before stimulating with 10 ng/mL of TNF-α (tumor necrosis

factor) for 30 minutes. Later, eliminating the medium and fixing by the cells Permeabilization buffer followed by staining them with fixation solution were carried out based on the manufacturer's protocol. On an ArrayScan high content screening reader, the plate was analyzed. Measuring the intensity ratio of nuclear NF-κB, and the cytoplasmic NF-κB was performed using Cytoplasm to Nucleus Translocation BioApplication software. For 200 cells per well, the quantification of the average intensity was done followed by comparing the different ratio of TNF-α stimulated in untreated and treated cells (Arbab et al., 2012; Ahmadipour et al., 2015). Curcumin, a well-known NF-κB inhibitor was used as a positive control (Zhu et al., 2014; Shakibaei et al., 2007). The untreated PC3 cells were served as control.

3.2.12 Statistical analysis

Data analysis was performed using Statistical Package for Social Science (SPSS) version 19.0 (IBM Corp. Released 2010, IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp, USA). Independent data were expressed as the mean ± standard deviation. Paired sample T-test was used to compare the means of control and treated. The statistical significance was expressed as $*P < 0.05$ (Ahmadipour et al., 2015).

CHAPTER 4: RESULTS

4.1 Cytotoxicity activity of biseugenol B in different cancer cell lines

The viability assay of biseugenol B on 5 different cell lines: WRL68 (hepatic fetal human epithelial cell line), MCF7 (human breast adenocarcinoma cell line), MDA-MB-231 (human breast adenocarcinoma cell line), CEMSS (cervical cancer) and PC3 (human prostate cancer) were performed using MTT assay. The results demonstrated significant cytotoxicity effect of biseugenol B on PC3 compare to other cancer cell lines. The results summerized in **Table 4.1.**

Table 4.1: IC_{50} values of biseugenol B on different cell lines using MTT assay at 48 hours.

Cell line	IC_{50} (ug/mL) ± SD
WRL68	>100 (ug/mL)
MCF7	61.07± 2.59 (ug/mL)
MDA-MB-231	86.12± 1.02(ug/mL)
CEMSS	>100 (ug/mL)
PC3	2.1± 0.38 (ug/mL)

The MTT results in **Table 4.1** demonstrated the selective cytotoxicity effects of biseugenol B in human prostate cancer cells (PC3) (IC_{50} < 5 µg/mL), while insignificant selectivities (IC_{50} > 30 µg/mL) were noted on the other 4 cancer cell lines.

The evaluation of the effects of biseugenol B on PC3 (human prostate cancer cell line) and RWPE-1 (normal human prostate cell line) was performed using the MTT assay in a dose-dependent manner. The IC_{50} values of biseugenol B in human prostate cancer, (PC3) were documented to be the highest after 24 hours (2.42±1.05 µg/mL) and the lowest after 72 hours (1.33±0.47 µg/mL), while the IC_{50} of biseugenol B in normal human prostate cell line was noticeably higher after 72 hours (57.43±1.15 µg/mL) **(Table 4.2).** The IC_{50} results after 24, 48 and 72 hours of treatment with biseugenol B for PC3 were also demonstrated as growth curve lines **(Figure 4.1).**

Table 4.2: The IC_{50} values of biseugenol B against PC3 and RWPE-1 cells treated after 24, 48 and 72 hours.

Cell line	IC_{50} ± SD (µg/mL)		
	24 hours	48 hours	72 hours
PC3	2.42±1.05	2.1±0.38	1.33±0.47
RWPE-1	57.43±1.15	45.65±2.94	34.92±1.6

Note: The results are shown as mean ± standard deviation of three independent experiments. IC_{50}

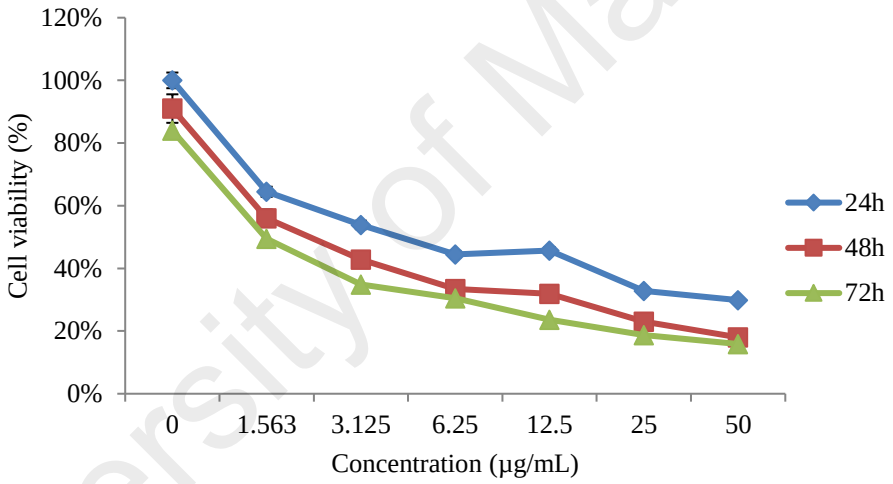


Figure 4.1: The MTT assay growth curve of PC3 cells treated with biseugenol B at 24, 48 and 72 hours. Note: MTT: (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)

The cytotoxicity effect of docetaxel has been evaluated as positive control. The IC_{50} value of docetaxel in the human prostate cancer cell, PC3 at 24 hours was the highest (93.01 ± 2.81 nM equal to 7.5 ± 2.2 µg/mL) and the lowest after 72 hours (14.31± 1.73 nM equal to 1.13± 1.4 µg/mL). The results have been shown in **Table 4.3.**

Table 4.3: The IC_{50} values of docetaxel in PC3

IC_{50} ± SD		
24 hours	48 hours	72 hours
7.5 ± 2.2 µg/mL	3.39± 1.7 µg/mL	1.13± 1.4 µg/mL

Note: The results are shown as mean ± standard deviation of three independent experiments.

4.2 Acridine orange (AO) and propidium iodide (PI) double-staining cell morphological analysis of biseugenol B in PC3 cells

The control cells were illustrated by the green nuclear structure which were shown to be intact (**Figure 4.2A**). After a subsequent treatment with 2 early apoptosis cells were distinguished with a bright green fluorescence using PI within the fragmented DNA. After a subsequent treatment with 4 µg/mL biseugenol B, blebbing and nuclear chromatin condensation were detected in bright orange color which represents a moderate apoptosis, (**Figure 4.2B**). Furthermore, after treatment with 8 µg/mL of biseugenol B in the later phases of apoptosis, due to the binding of PI to the denatured DNA, the reddish-orange color was observed (**Figure 4.2C and 4.2D**). In the 200-cell population, a statistically significant ($P < 0.001$) increase was noted in early apoptosis cell (**Figure 4.3**). This results clearly show the different stages of apoptosis which have been induced by biseugenol B in PC3 cells in a dose-dependent manner.

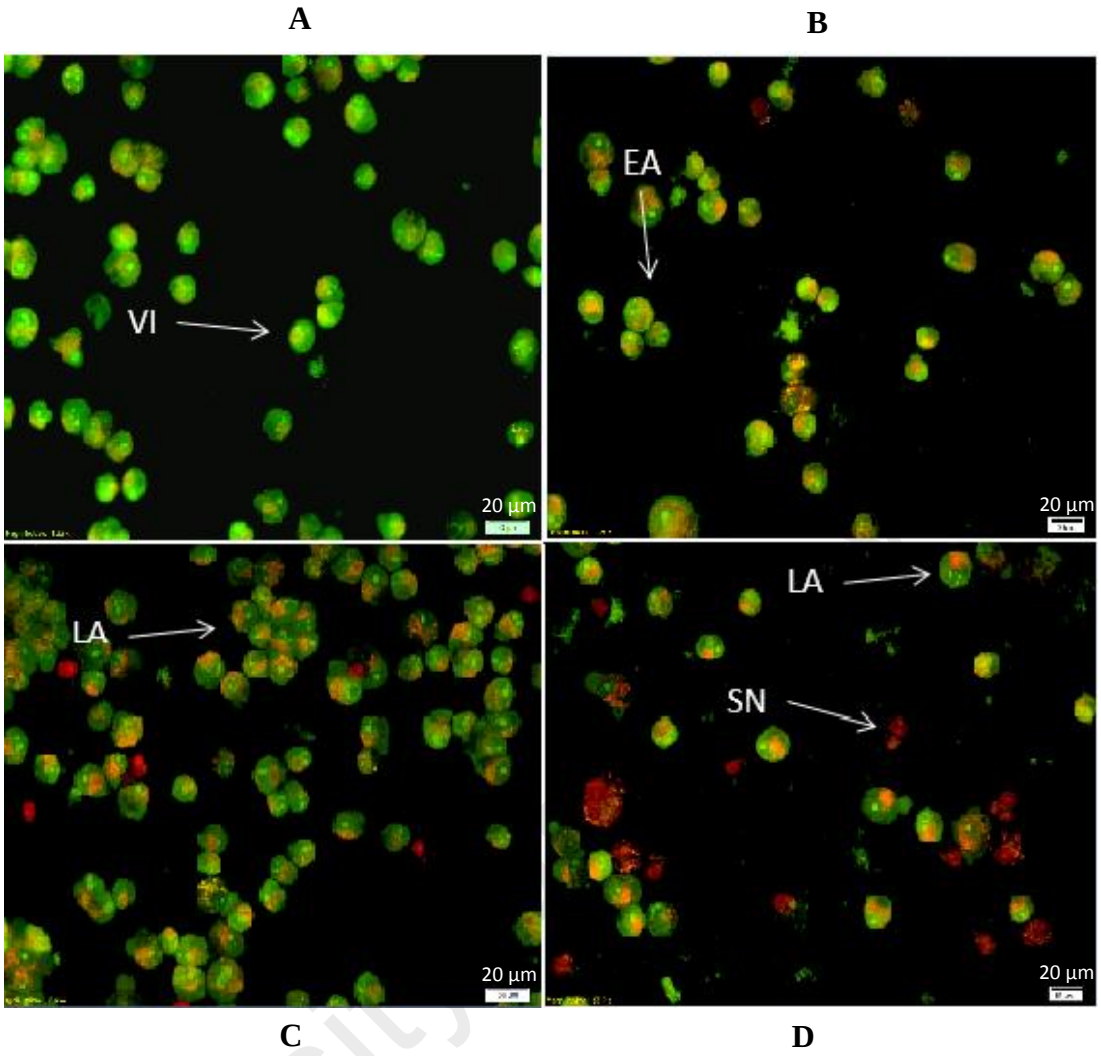


Figure 4.2: Acridine orange (AO) and propidium iodide (PI) double-staining cell morphological analysis in untreated and treated PC3 cells with biseugenol B (magnification 40×). Image **A**: untreated PC3 cells (control), **B**: PC3 cells treated with 2 µg/mL of biseugenol B. **C**: PC3 cells treated with 4 µg/mL of biseugenol B and **D**: PC3 cells treated with 8 µg/mL of biseugenol B for 24 hours. VI= viable cells, LA= late apoptosis, EA= early apoptosis, SN= secondary necrosis

Normal structure without noticeable apoptosis or necrosis were shown in untreated cells (**A**); after treatment with 2 µg/mL, early apoptosis features were observed with intercalated Propidium Iodine (bright green) among the fragmented DNA (**B**); in 4 µg/mL treatment the hallmark of moderate apoptosis were detected represented by blebbing and orange color (**C**); after treatment with 8 µg/mL, secondary necrosis were visible and represented by bright red color (**D).**

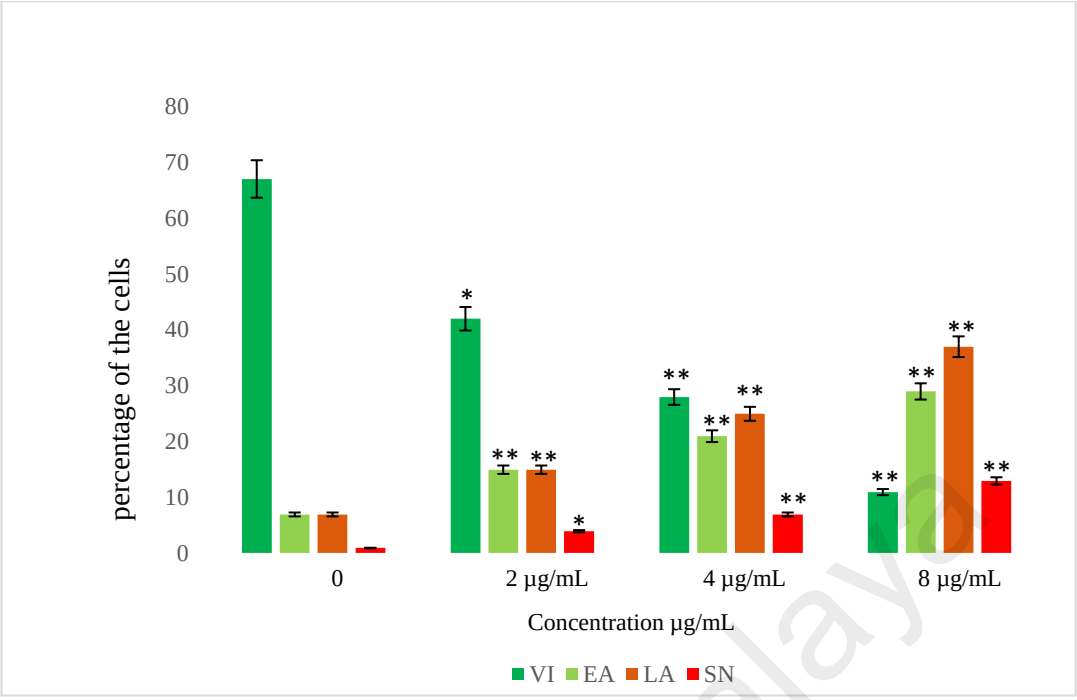


Figure 4.3: Percentages of viable, early apoptotic, late apoptosis and secondary necrotic cells after biseugenol B treatment.

The number of early and late apoptosis cells significantly increase at 2, 4 and 8 µg/mL of biseugenol B, while viable cells decrease. Note: The results are shown as mean ± standard deviation of three independent experiments. VI= viable cells, LA= late apoptosis, EA= early apoptosis, SN= secondary necrosis. * $P < 0.05$, ** $P < 0.005$ indicates a significant difference

A significant increase in early apoptosis cells was observed in PC3 cells with exposure to the different dose of biseugenol B, 2 µg/mL ($P < 0.01$), 4 µg/mL ($P < 0.001$) and 8 µg/mL ($P < 0.001$).[1]

4.3 Determination of the ratio of apoptotic cells by AV-FITC staining

For confirmation of the apoptotic effects of biseugenol B on PC3 cells, double staining flow cytometric analysis with AV/PI was used. AV^{-}/PI^{-} staining represents viable cells, while $AV+/PI^{-}$ staining represents early apoptosis and AV+/PI+ staining was marked as late apoptotic cells. In contrast, $AV^{-}/PI+$ staining represents the necrotic cells, since PI can only pass through the damaged membrane of the dead cells or late apoptotic cells and binds to the nuclei (**Figure 4.4A-D**). The PC3 cells were treated with biseugenol B at

[1] VI= viable cells, LA= late apoptosis, EA= early apoptosis, SN= secondary necrosis

different concentration. The cells were analyzed after staining with FITC-conjugated Annexin V and PI by flow cytometer. The untreated cells were served as control (**A**) (AV^- /PI^-), the early apoptosis events ($AV+/PI^-$) shown in the lower right quadrant (Q4-1) and late stage of apoptosis/dead cells (AV+/PI+) was shown in quadrant (Q2-1). The effects of 0 (A), 2 (B), 4 (C) and 8 µg/mL (D) exposures of PC3 cells to biseugenol B. **Figure 4.5** shows the perecntage of the cell in different stages of apoptosis, induced by bieugenol B.

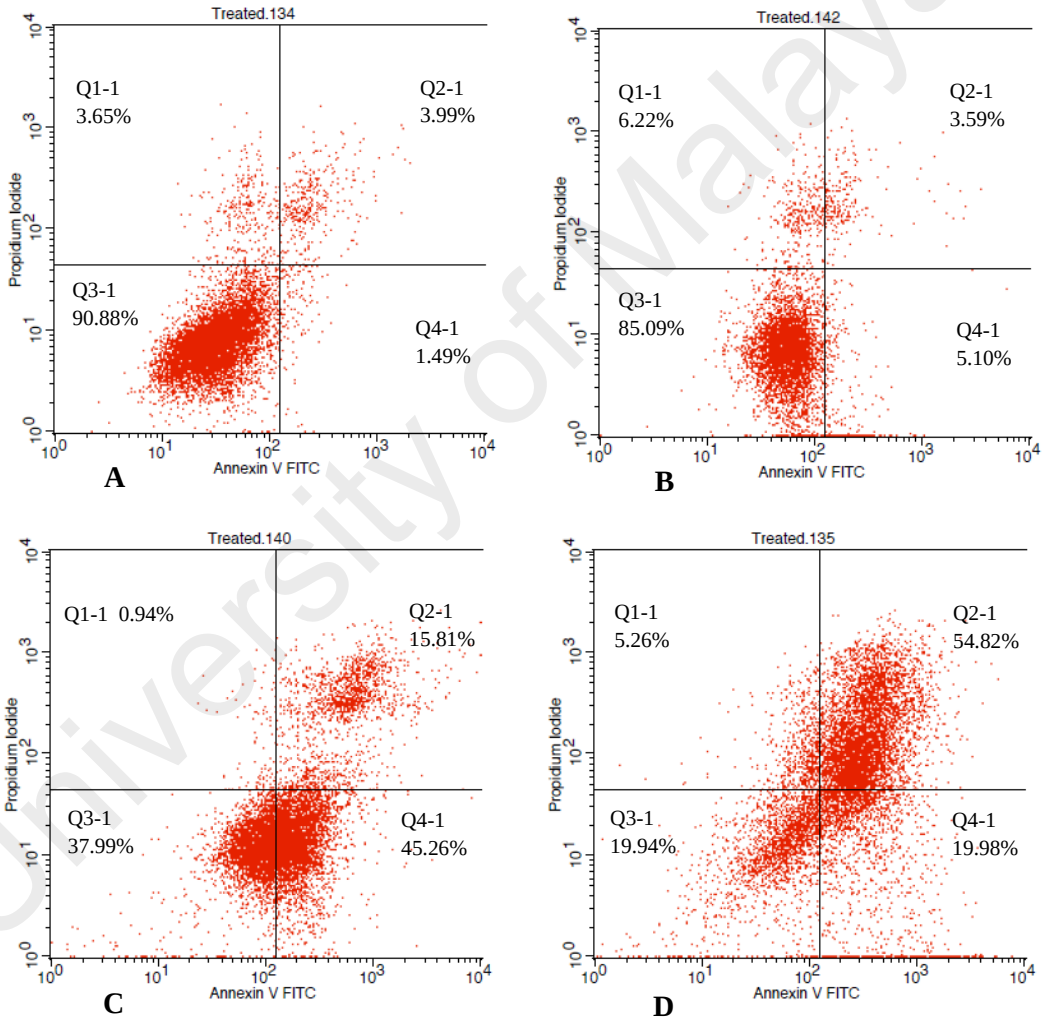


Figure 4.4: Effects of biseugenol B on the ratio of apoptotic cells by AV-FITC staining.

The characteristic of dot plots of the flow cytometric assessment of apoptosis after treatment with 0 (A), 2 (B), 4 (C) and 8 (D) µg/mL of biseugenol B. The movement of the dot plots indicated that early apoptosis significantly increased in PC3 cells with exposure to different dose of biseugenol B, 2 µg/mL ($P < 0.01$), 4 µg/mL ($P < 0.001$) and 8 µg/mL ($P < 0.001$). Moreover, the biseugenol B treatment resulted in a decrease of viable cells at 4 and 8 µg/mL. AV: Annexin V, FITC: fluorescein isothiocyanate, PI: propidium iodide.

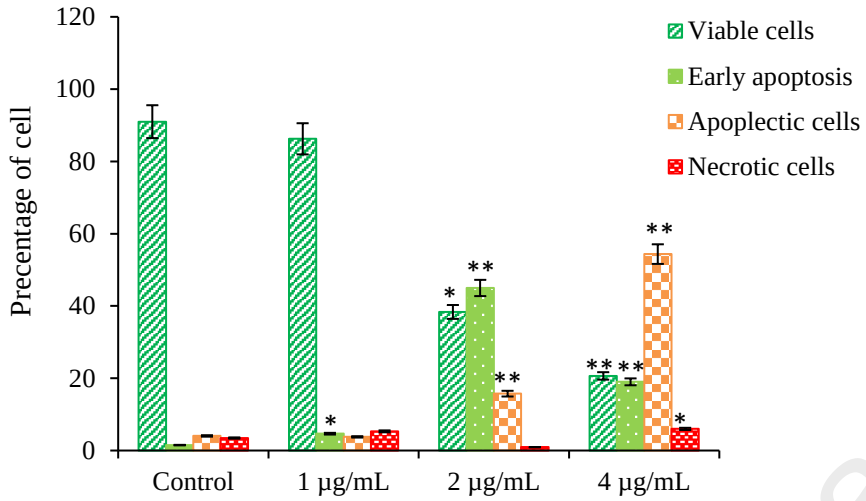


Figure 4.5: The effects of biseugenol B on the ratio of apoptotic cells by AV-FITC staining

The bar chart represents the percentage of viable cells, early and late apoptosis and necrotic cells at the different concentration of biseugenol B in PC3 cells. The number of early and late apoptosis cells increase at 2 and 4 µg/mL of biseugenol B, while viable cells decrease. The results were shown as mean ± standard deviation of three independent experiments. AV: Annexin V, FITC: fluorescein isothiocyanate, PI: propidium iodide. * $P < 0.05$, ** $P < 0.005$ indicates a significant difference

4.4 Effect of biseugeol B on MMP disruption and release of cytochrome c

The effect of biseugenol B on the MMP of PC3 cells is shown in **Figure 4.6**. A significant increase in cell permeability was observed in PC3 cells with exposure to biseugenol B ($P < 0.001$). A noteworthy increase in nuclear fluorescence intensity was observed in PC3 cells with exposure to biseugenol B ($P < 0.001$). In addition, a notable increase in the translocation of cytochrome c from mitochondria into the cytosol was observed in PC3 cells with exposure to biseugenol B ($P < 0.001$) **(Figure 4.7).**

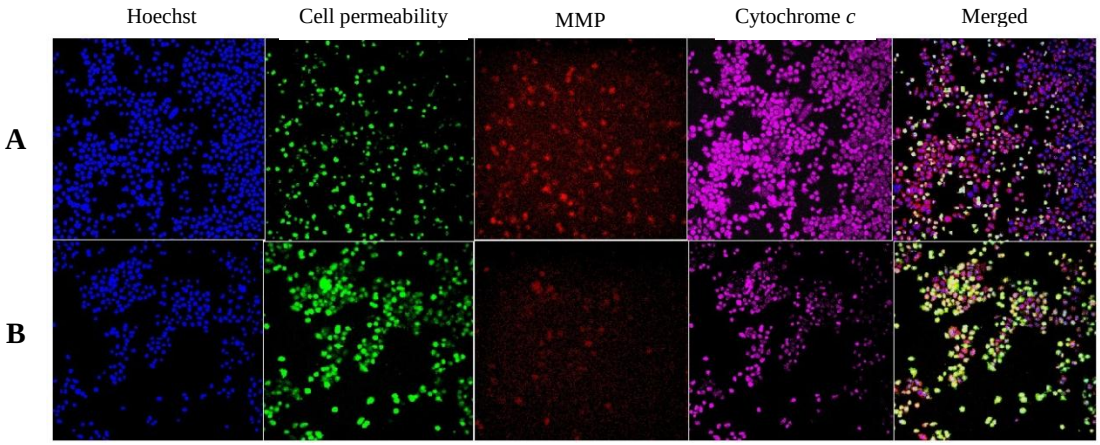


Figure 4.6: Representative images of effect of biseugeol B on MMP disruption and release of cytochrome c in PC3 by multiple cytotoxicity assay

(magnification 20×) (**A**) Untreated PC3 cells (control), (**B**) treated with 4 µg/mL biseugenol B, were stained with Hoechst 33342 (nuclear cell dye), cell membrane permeability, cytochrome c and MMP (mitochondrial membrane potential). The increase in total nuclear intensity, cell permeability, cytochrome c release from mitochondria and an obvious decline in mitochondrial membrane potential (MMP) were observed.

PC3 cells produced a marked decrease in MMP and a noteworthy elevation in total nuclear intensity, membrane permeability and cytochrome c.

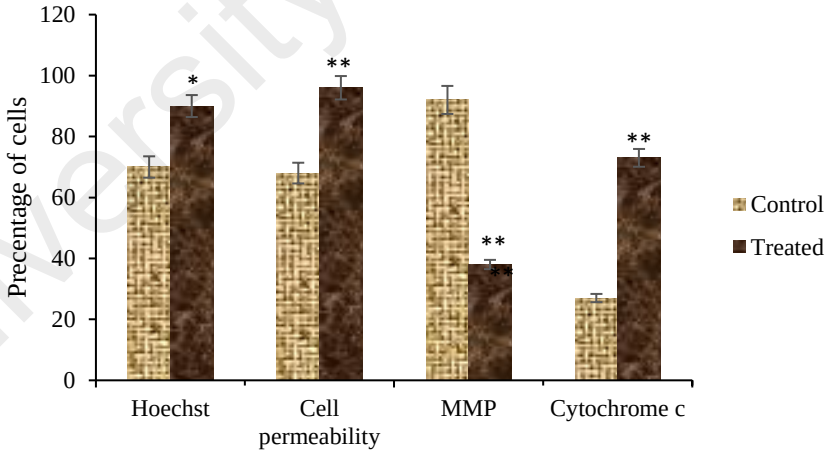


Figure 4.7: The effect of biseugenol B on nuclear intensity, cell membrane permeability, MMP and cytochrome c release with biseugenol B (4 µg/mL)

Simultaneous alterations in total nuclear intensity, cell permeability mitochondrial membrane potential (MMP) and cytochrome c release were quantified in PC3 cells. After treatment with biseugenol B (at concentration of 4 µg/mL), statistically significant loss of mitochondrial membrane potential and an obvious increase in total nuclear intensity, cell permeability and cytochrome c release from mitochondria were observed. The data represent the means ± standard deviations (SDs) of 3 independent tests. Statistical analysis is defined as significant if * $P < 0.05$, ** $P < 0.005$

4.5 ROS generation in PC3 cells treated with biseugenol B

ROS (reactive oxygen species) generation was significantly increased in treated PC3 cells compared to untreated PC3 cells in a dose-dependent manner. The ROS generation was noted to increase significantly with exposure to 1 µg/mL, 2 µg/mL, 4 µg/mL and 8 µg/mL of biseugenol B.

An immediate and considerable formation of ROS (more than twice that of control) was detected with 1 and 2µg/mL treatment while increasing to approximately three times more than control with the higher concentration at 4 and 8µg/mL (**Figure 4.8A**). In contrast, no significant increase of ROS generation was detected in normal prostate cells (RWPE-1) and after 24 hours treatment with biseugenol B (**Figure 4.8B**)

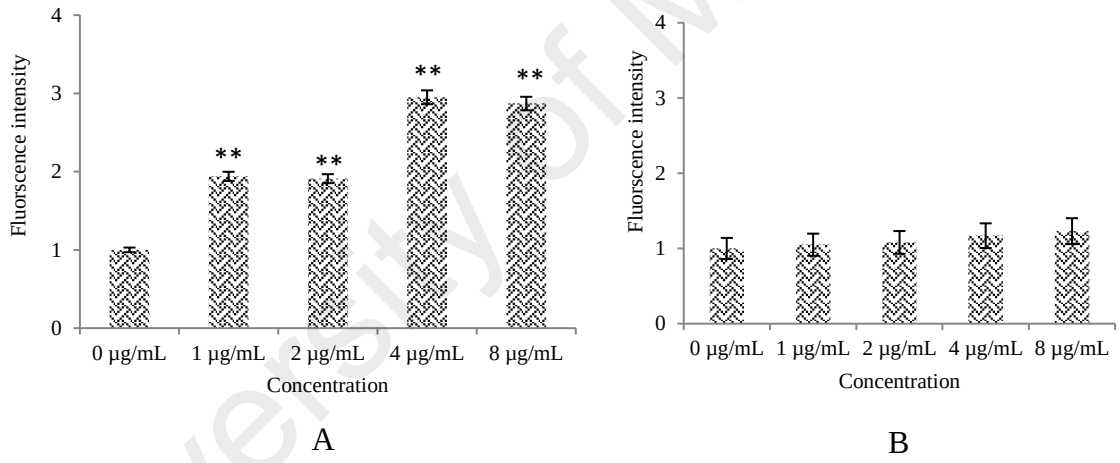


Figure 4.8: ROS generation after treated with 0, 1, 2, 4 and 8 µg/mL exposure of biseugenol B exposure for 24 hours in PC3 (**A**) and RWPE1 (**B**).

The significant increase can be observed in ROS generation in PC3 cell after treatment with 1, 2, 4 and 8 µg/mL of biseugenol B, while the alteration of ROS generation is neglectable in RWPE-1 cells after treatment with 1, 2, 4 and 8 µg/mL of biseugenol B. The data represent the means ± standard deviations (SDs) of 3 independent tests. Statistical analysis is defined as significant if $*P < 0.05$, $** P < 0.005$

The increase of ROS production in PC3 and RWPE-1 were shown after 24 hours treatment with different concentration of biseugenol B (**Figure 4.9**).

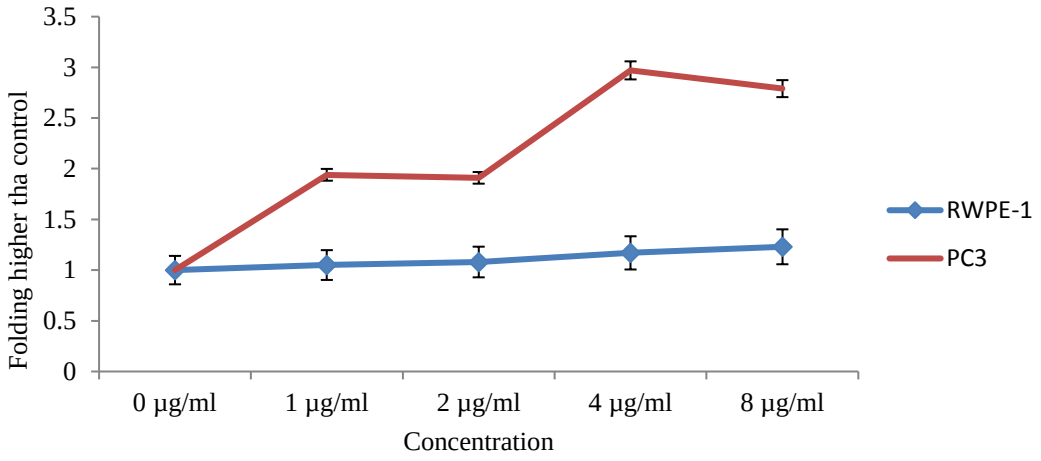


Figure 4.9: Comparison of ROS formation in PC3 and RWPE-1 cells with biseugenol B.

Note: The significant increase of the induction of ROS generation at 1, 2, 4 and 8 µg/mL of biseugenol B in PC3 cells were compared to the insignificant effect of biseugenol B on ROS generation in RWPE-1. The results are shown as mean ± standard deviation of three independent experiments.

4.6 Analysis of caspases-3/7, -8 and 9 by bioluminescent assays on PC3 and RWPE-1 cells treated with biseugenol B

The activity of caspases-3/7, -8 and -9 were measured in the PC3 and RWPE-1 cells treated with biseugenol B in different concentration for 24 hours. As shown in **Figure 4.10**, caspases-3/7 level was significantly increased with 1µg/mL ($P < 0.001$), 2 µg/mL ($P < 0.001$), 4 µg/mL ($P < 0.01$) and 8 µg/mL ($P < 0.001$) of biseugenol B. Meanwhile, caspase-8 level was significantly increased with 1µg/mL ($P < 0.01$), 2 µg/mL ($P < 0.01$), 4 µg/mL ($P < 0.001$) and 8 µg/mL ($P < 0.001$) of biseugenol B. Simultaneously, caspase-9 level was also meaningfully increased with 1µg/mL ($P < 0.01$), 2 µg/mL ($P < 0.01$), 4 µg/mL ($P < 0.001$) and 8 µg/mL ($P < 0.001$) biseugenol B.

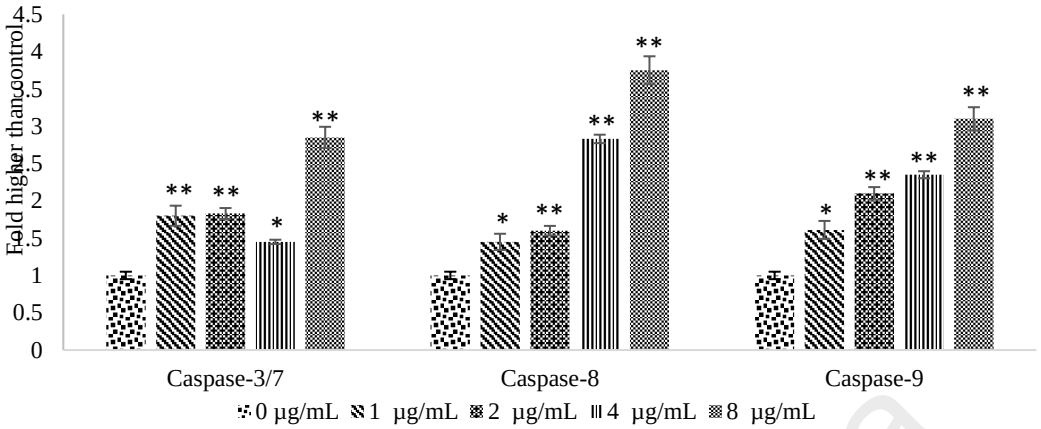


Figure 4.10: Relative bioluminescence expression of caspases-3/7, caspase-8, and caspase-9 in PC3 cells treated with biseugenol B at different concentration.

Note: The results are shown as mean ± standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.005$ indicates a significant difference

In contrast, the normal prostate cells (RWPE-1) were less sensitive to biseugenol B, showing a mild increase in caspases-3/7, caspase-8 and caspase-9 level with exposure to different dose of biseugenol B. Caspases-3/7 level of RWPE-1 cells were not significantly increased with 1 µg/mL (P=0.231), 2 µg/mL (P=0.178), 4 µg/mL (P=0.078) and 8 µg/mL (P=0.091) of biseugenol B, respectively. At the same time, caspase-8 level was slightly increased with 1 µg/mL (P=0.19), 2 µg/mL (P=0.092), 4 µg/mL (P=0.88) and 8 µg/mL (P=0.073) of biseugenol B, respectively. Meanwhile, caspase-9 level was not significantly increased with 1 µg/mL (P=0.18), 2 µg/mL (P=0.12), 4 µg/mL (P=0.06) and 8 µg/mL (P=0.08) of biseugenol B, respectively (**Figure 4.11)**.

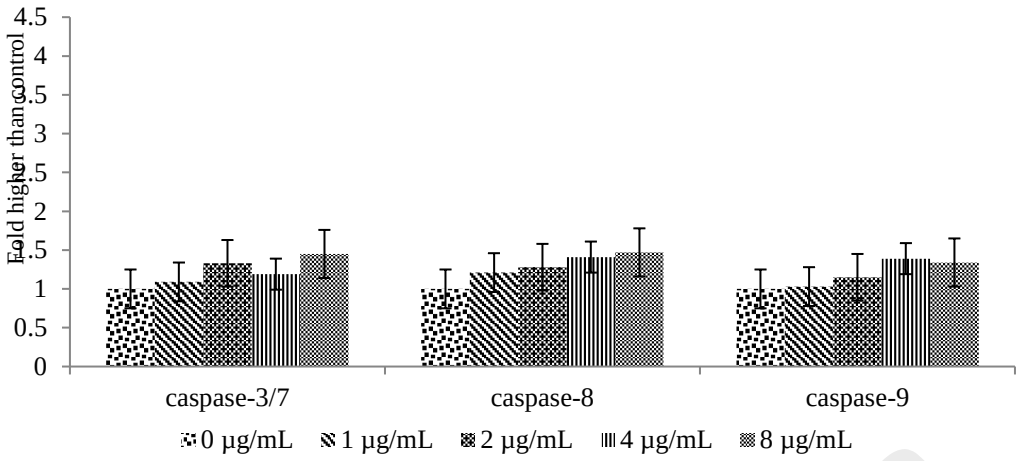


Figure 4.11: Relative bioluminescence expression of caspases-3/7, caspase-8, and caspase-9 in RWPE-1 cells treated with biseugenol B at different concentration.

Note: The results are shown as mean ± standard deviation of three independent experiments.

The results of caspases-3/7, caspase-8 and caspase-9 expression in PC3 (human prostate cancer cell line) and RWPE-1 (normal prostate cell line) after treatment with 2, 4 and 8 µg/mL have been combined in **Figure 4.12** as to make a comparison.

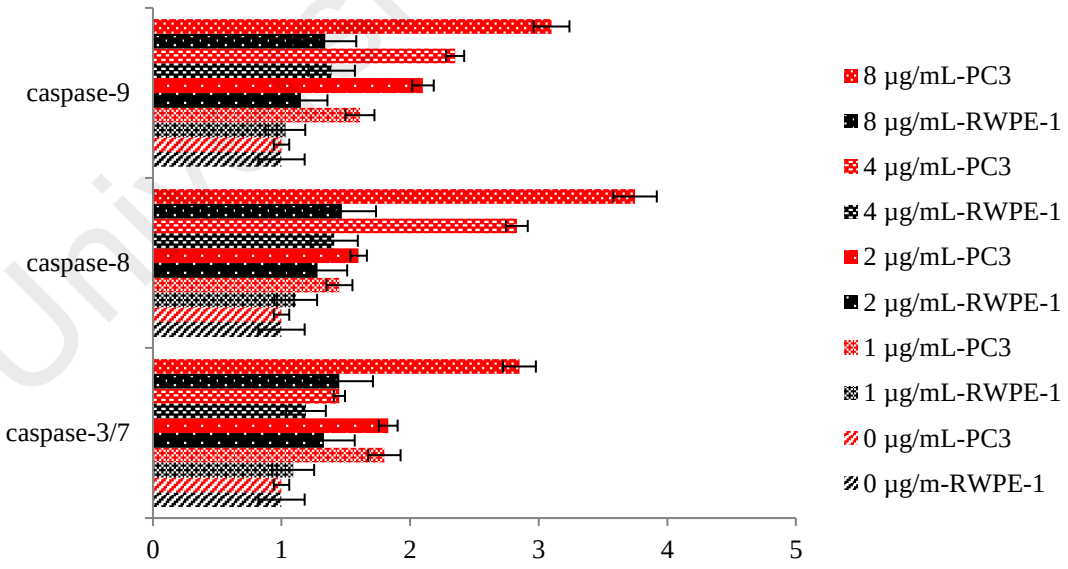


Figure 4.12: Comparison of relative bioluminescence expression of caspases-3/7, caspase-8 and caspase-9 between PC3 and RWPE-1 cells treated with biseugenol B at different concentration.

4.7 Cell cycle analysis in PC3

The results indicated that biseugenol B arrested the cell cycle progression in the G0/G1 phase with 2 µg/mL of biseugenol B, sub-G0 phase arrest was significantly increased ($P < 0.001$), followed by 4 µg/mL ($P < 0.001$) and 8 µg/mL ($P < 0.001$). The results demonstrated a significant G0/G1 phase arrest in a dose-dependent manner in the PC3 cells (**Figure 4.13** and **Table 4.4**) which account for 66.88%, 78.06% and 64.93% of cells after treatment for 24 hours with 2, 4 and 8 µg/mL, respectively (**Table 4.4**). In the meantime, in both S and G2/M phases, cell number was reduced significantly in dose-dependent manner. The results have been also shown in bar chart form (**Figure 4.14**).

Table 4.4: Effect of biseugenol B on cell cycle phases

Concentration	Sub-G0-G1	G0-G1	S	G2/M
0 µg/mL	1.94±0.32	58.84±1.15	16.45±2.02	8.81±1.65
2 µg/mL	2.47±0.46	66.88±1.58**	18.73±1.38*	10.22±1.06**
4 µg/mL	1.75±0.42	78.06±1.37**	7.77±0.94**	11.22±1.67**
8 µg/mL	24.73±0.24**	64.93±1.04**	3.59±0.55**	5.94±1.48**

The table summarized the percentages of cells in each phase of the cell cycle after treatment with biseugenol B. Data in the same vertical column but different rows refer to the same phase of the cell cycle and various biseugenol B concentrations. Note: The results are shown as mean ± standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.005$ indicates a significant difference

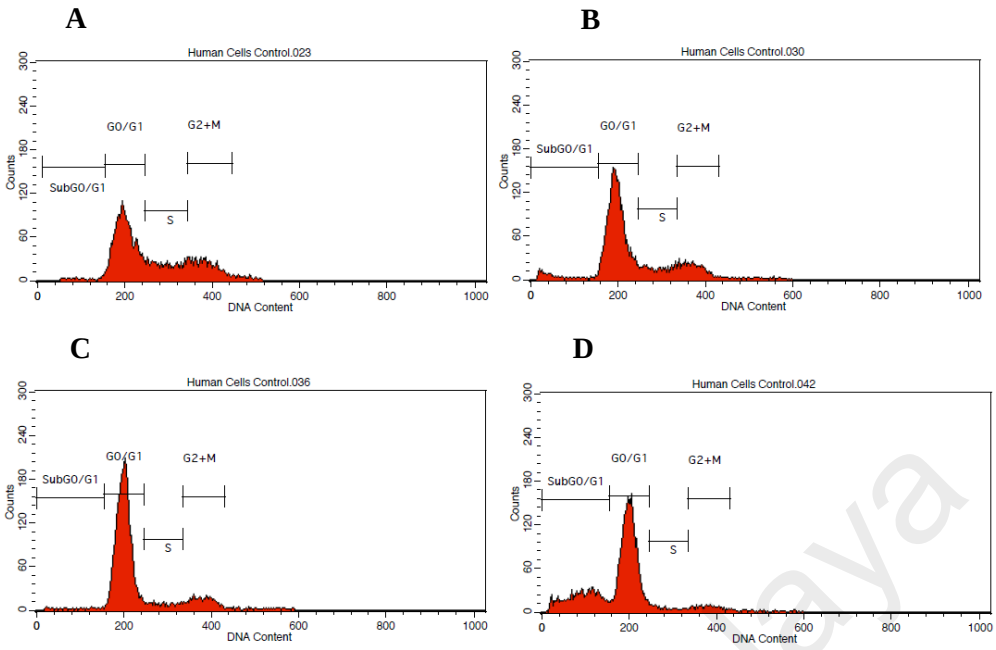


Figure 4.13: Cell cycle histogram from analysis of PC3 cells treated with biseugenol B. Results are representative of one of three independent experiments. Distribution of different stages of cell cycle in PC3 cells before treating with biseugenol B (**A**) treated with 2, 4 and 8 µg/mL for 24 hours (**B**), (**C**) and (**D**), respectively.

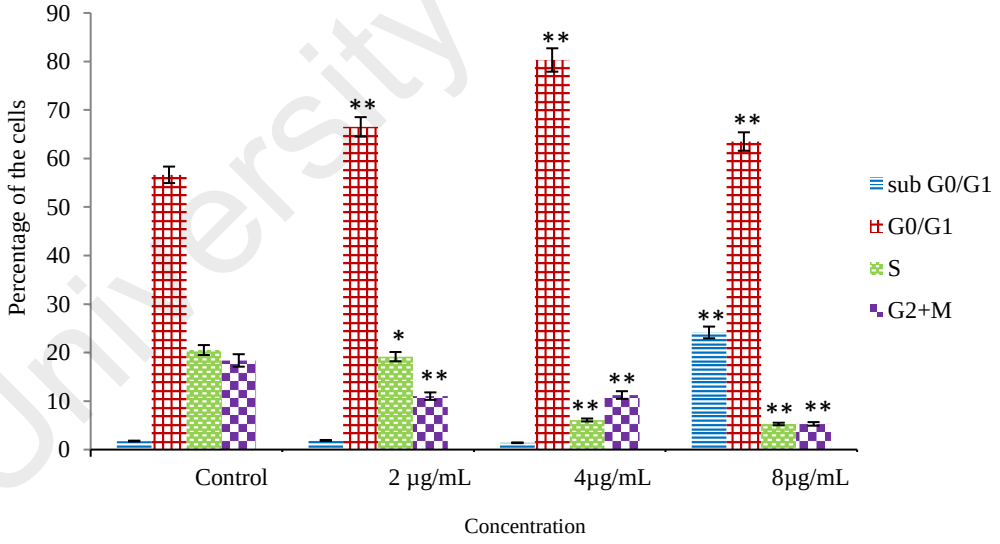


Figure 4.14: Bar chart represents the cell cycle progression for control and biseugenol B-treated PC3 cells.

Note: The results are shown as mean ± standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.005$ indicates a significant difference

4.8 RT-PCR analysis of apoptotic markers

The expression levels of apoptotic markers: Bax (pro-apoptotic), Bcl-2 and Hsp70 (anti-apoptotic) were evaluated by reverse transcriptase-PCR (RT-PCR). β- actin was used as the internal control (**Figure 4.15A**). The images represent the alteration in the expression of apoptotic markers in treated and untreated cells. A significant down-regulation of Bcl-2 and Hsp70, as well as, up-regulation of Bax were observed (Bcl-2 was significantly down-regulated with 2 µg/mL ($P < 0.01$), 4 µg/mL ($P < 0.001$) and 8 µg/mL ($P < 0.001$) of biseugenol B. Meanwhile, Bax was significantly up-regulated with 2 µg/mL ($P < 0.01$), 4 µg/mL ($P < 0.001$) and 8 µg/mL ($P < 0.001$) of biseugenol B, respectively. Hsp70 was also significantly down-regulated with 2 µg/mL ($P < 0.01$), 4 µg/mL ($P < 0.001$) and 8 µg/mL ($P < 0.001$) of biseugenol B, respectively (**Figure 4.15B-D**).

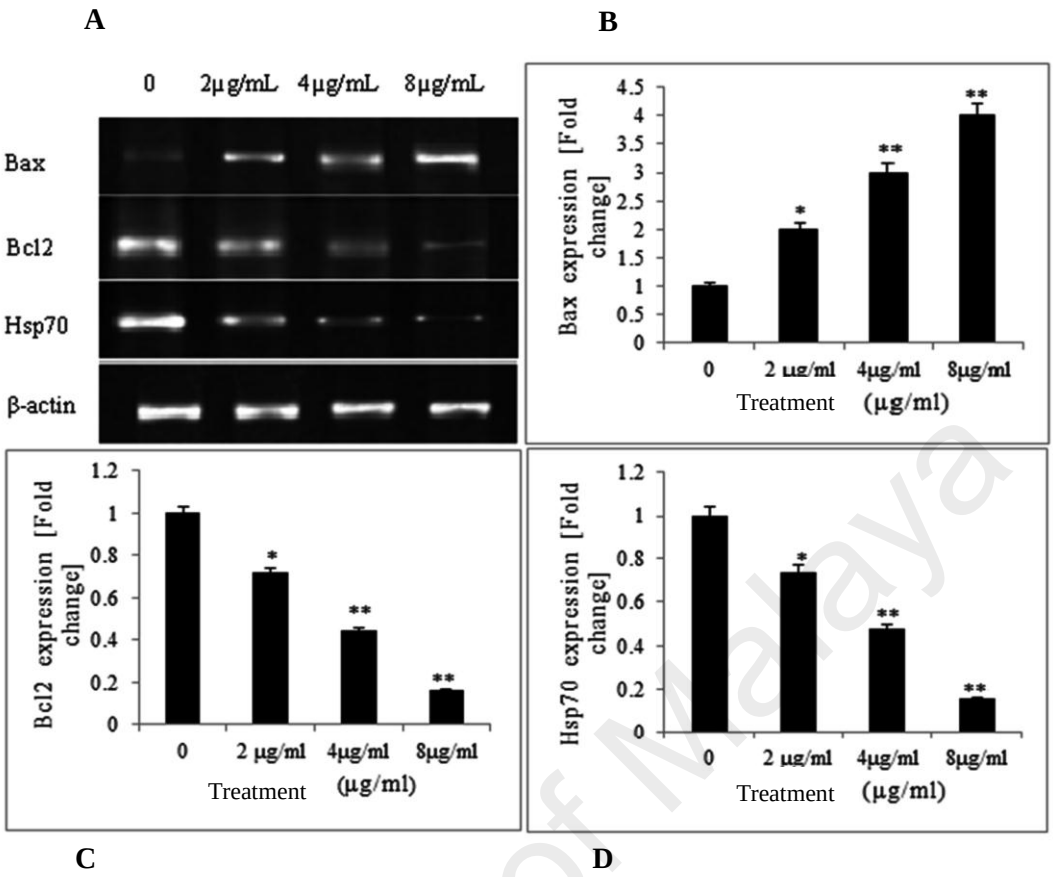


Figure 4.15: RT-PCR analysis of apoptotic markers.

The blot densities were expressed as fold of control for Bax, Bcl-2 and Hsp70 at 2, 4 and 8 µg/mL of biseugenol B, untreated PC3 cells (0 µg/mL) were considered as control (**A**). Bar chart represents a dose-dependent up-regulation of Bax. (**B**) Bar chart represents dose-dependent down-regulation of Bcl2 (**C**) and Hsp70 (**D**). Note: The results are shown as mean ± standard deviation of three independent experiments.

* $P < 0.05$, ** $P < 0.005$ indicates a significant difference. Note: The results are shown as mean ± standard deviation of three independent experiments.

4.9 Western blot analysis of apoptotic markers

The results obtained from the western blot analysis (**Figure 4.16A**) confirmed that biseugenol B induced up-regulation of Bax significantly (**Figure 4.16B).** A significant down-regulation of Bcl-2 and Hsp70 were observed. Bcl-2 was significantly down-regulated with 2 µg/mL ($P < 0.01$), 4 µg/mL ($P < 0.001$) and 8 µg/mL ($P < 0.001$) of biseugenol B. Meanwhile, Bax was significantly up-regulated with 2 µg/mL ($P < 0.01$), 4 µg/mL ($P < 0.001$) and 8 µg/mL ($P < 0.001$) of biseugenol B, respectively. Hsp70 was

significantly down-regulated with 2 µg/mL ($P < 0.01$), 4 µg/mL ($P < 0.001$) and 8 µg/mL ($P < 0.001$) of biseugenol B (**Figure 4.16-C, D**).

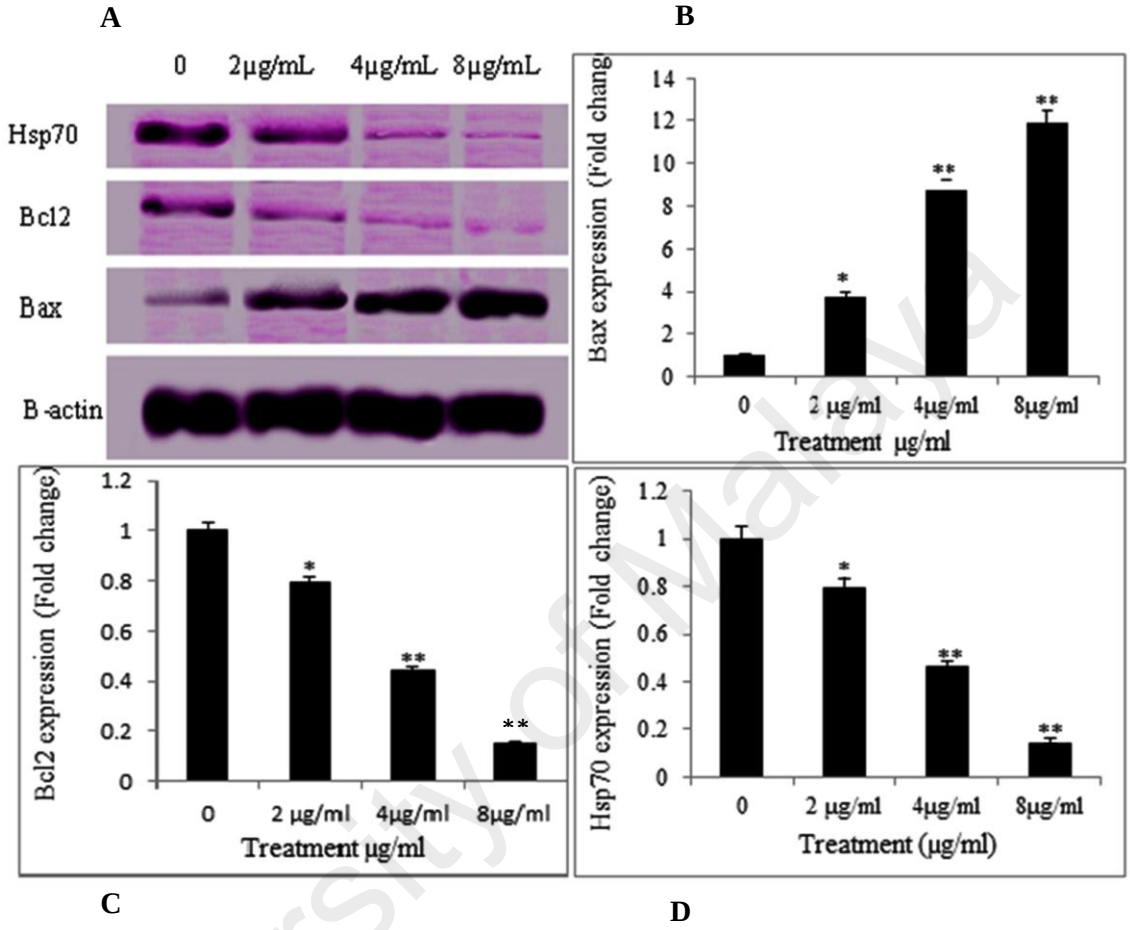


Figure 4.16: Western blot analysis of apoptotic markers. Western blot analysis of biseugenol B in selected apoptotic signaling markers.

The blot densities were expressed as fold of control. The obvious increase can be observed in the density of the of Bax and decrease of Bcl-2 and Hsp70 after treatment with 0, 2, 4 and 8 µg/mL of biseugenol B. β-actin was served as control (**A**). Bar chart represent dose-dependent up-regulation of Bax (**B**) and down-regulation of Bcl-2 (**C**) and Hsp70 (**D**). **Note:** The results were shown as mean ± standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.005$ indicates a significant difference

4.10 Effect of biseugenol B on NF-κB translocation in PC3

In the control cells, a high intensity of NF-κB fluorescent was detected in cytoplasm (**Figure 4.17A**). In the control cells, the deficiency of NF-κB activation resulted in the nuclei faint color. A considerable NF-κB fluorescent intensity was observed in the nuclei when 10 ng/mL of TNF-α was added to PC3 cells (**Figure 4.17B**). Biseugenol B

demonstrated a significant triggering of the inhibition of the NF-κB (**Figure 4.17C**). In the cells treated with well-known NF-κB inhibitors such as curcumin, a well-known suppressor of translocation of nuclear NF- κB has been detected. The NF-κB-related fluorescence intensity of nuclei was reduced in PC3-TNF-α stimulated cell treated with 8 µg/mL of biseugenol B. In addition, immunofluorescence staining demonstrated the morphological changes of NF-κB translocation, indicated the inhibitory effect of biseugenol B on TNF-α-induced NF-κB translocation (**Figure 4.18C**).

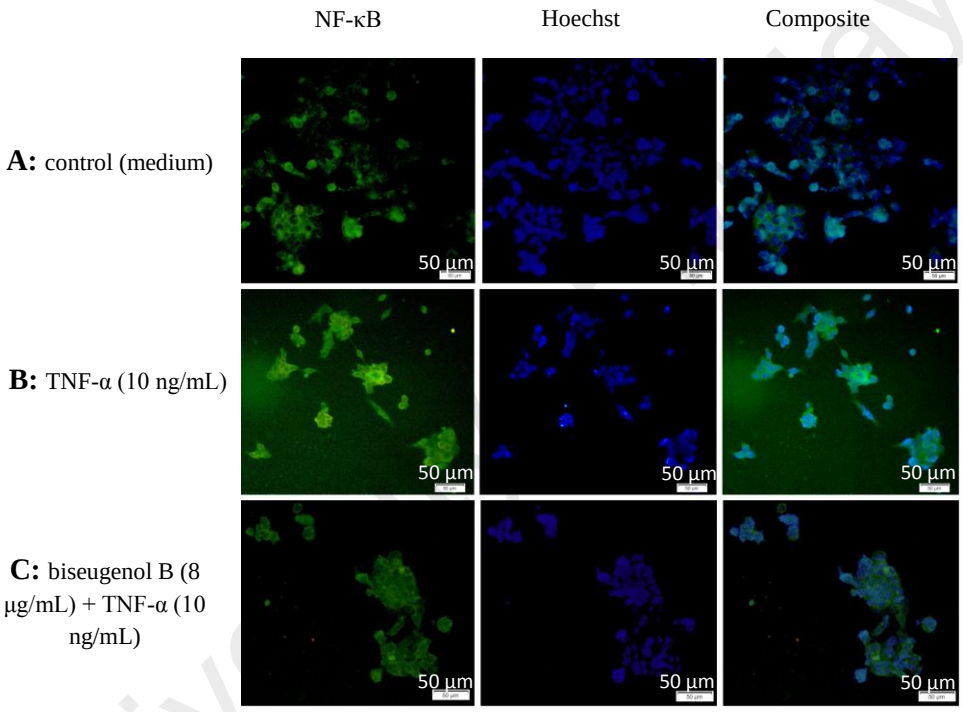


Figure 4.17: Inhibition of TNF-α-induced NF-κB nuclear translocation by biseugenol B.

Images of intracellular targets in stained PC3 cells with medium alone as control (**A**). PC3 treated with biseugenol B for 3 hours and then, NF-κB-activation by stimulating with 10 ng/mL TNF-α for 30 minutes (**B**). The average intensity of fluorescent was declined, indicating the inhibitory effect of biseugenol B 8 µg/mL on TNF-α-induced translocation of NF-κB from the cytoplasm to the nucleus (**C**). Scale bar represents 50 µm.

Nuclear content of NF-κB p65 immunoblot was measured to affirm the immunofluorescence results (**Figure 4.18A, B**). Treatment with different dose of biseugenol B significantly decreased the escalation of NF-κB p65, induced by positive

control (TNF-α) in a dose-dependent manner with 2 µg/mL ($P < 0.05$), 4 µg/mL ($P < 0.001$) and 8 µg/mL ($P < 0.001$).

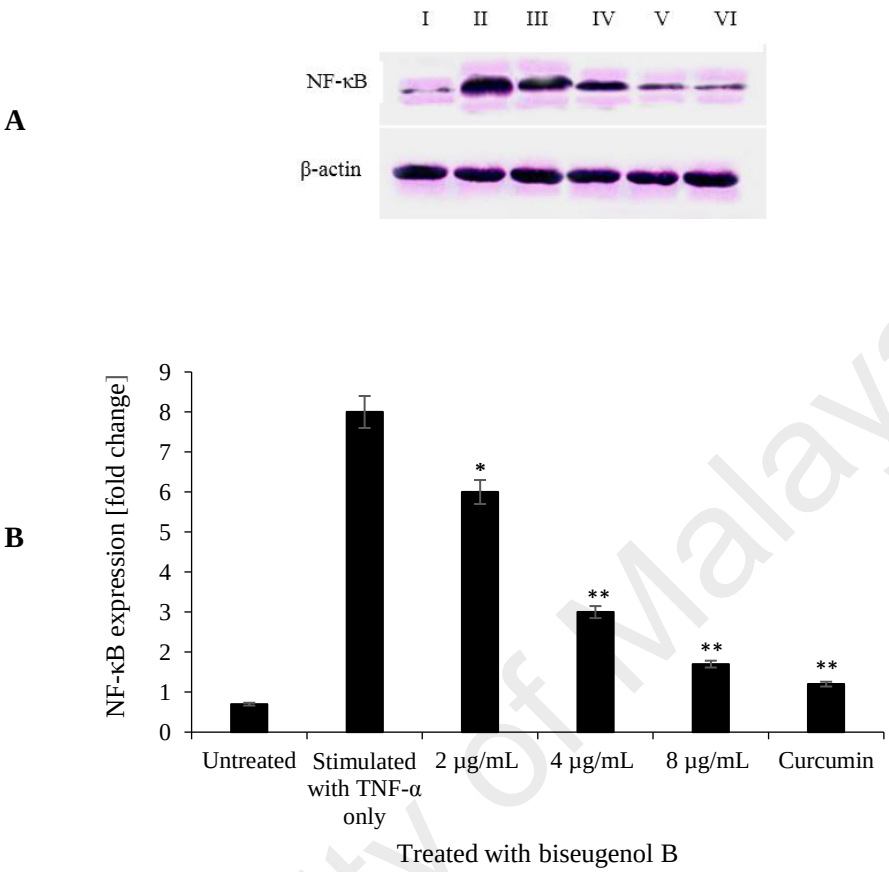


Figure 4.18: Immunoblot analysis of nuclear NF-κB p65.

(**A**) Immunoblot analysis of nuclear NF-κB p65. I: Untreated PC3; II: Stimulated with TNF-α only; III, IV, V: PC3 treated with 2, 4 and 8 µg/mL of biseugenol B, respectively; VI: PC3 treated with curcumin (**B**) Representative bar chart indicating a significant decline of nuclear NF-κB p65 are expressed as folds of control. Note: The results are shown as mean ± standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.005$ indicates a significant difference.

CHAPTER 5: DISCUSSION

Apoptosis is an extremely controlled process that plays an essential role in cell destruction and has a crucial role in various cell functions from fetal development to adult tissue homeostasis (Jiang, Akhtar, Bradbury, Zhang, & Isman, 2009). Tumors occur through reduction of cell apoptosis as well as unrestrained cell proliferation. Thus, one of the most logical methods of cancer therapy is using cytotoxic drugs that activate the apoptosis pathway to destroy cancer cells (Shi et al., 2013). Herbal medicine is the major source of apoptosis-inducing agents (Di Maro et al., 2013). As stated by several reports, many natural compounds may be associated with human cancer therapy which can induce apoptosis in cancer cells (Pan et al., 2013). Apoptosis is related to several biochemical changes in cells including nuclear fragmentation, change in the MMP and regulation of caspases (Hunter, LaCasse, & Korneluk, 2007). The current study is the first *in vitro* report of the effects of biseugenol B, a natural compound derived from plant *Listea costalis*, against human prostate cancer cells (PC3).

Litsea costalis is a well-known plant (Chong et al., 2016) that contains biologically active and structurally diverse aporphine alkaloids (Yang et al., 2005) which has been used to treat various diseases (Wang et al., 2010). The natural compound biseugenol B is an oxyneolignan isolated from *Listea costalis* (Hosseinzadeh, et al., 2013a). The present study elucidates the mechanism of apoptosis triggered by biseugenol B in PC3 cells. According to Shier, who modified and established MTT assay as a bioassay for the measurement of cytotoxicity of the compounds (Ahmadipour et al., 2015), compounds with the IC_{50} value of more than 30 µg/mL are not considered potentially cytotoxic, whereas compounds with IC_{50} value of less than 5.0 µg/mL are considered highly cytotoxic. The viability assay was performed in five different cancer cell lines to compare the results and to select the most sensitive cancer cell lines to biseugenol B. The results

showed that biseugenol B has significant cytotoxicity effects on the human prostate cancer cell, but not on the other cancer cell lines. This selectivity and specificity of biseugenol B toward PC3 cell line is remarkable. Docetaxel is one the most famous chemotherapy drug which has been widely used for prostate cancer treatment. The IC_{50} of docetaxel was evaluated in prostate cancer cell line, PC3 as a positive control (page. Table). The results showed that the cytotoxicity of biseugenol B in PC3 at 72 hours is very close to docetaxel which indicates that biseugenol B could potentially be a candidate for chemotherapy purposes. The cytotoxicity effect of biseugenol B was determined in the non-tumorigenic human prostate cell line (RWPE-1) and the results were then compared with the human prostate cancer cell line PC3 (page 43, table 4.2). Even though the detection of the cytotoxicity of biseugenol B in normal prostate cell line is not the aim of this study, the MTT results of biseugenol B treatment in the normal prostate cell line (RWPE-1) has been performed to compare the toxicity of biseugenol B in normal and cancer cell line. The goal of finding new natural compounds which could be used in chemotherapy is to detect the compound that could minimize damage to normal cells and to alleviate the toxic effect in cancer cells. In line with the objective, most of the researchers have investigated the potential effects of anti-cancer compounds performed via MTT in both normal and cancer cells (Alabsi et al., 2012; Mondal, Panigrahi, & Khuda-Bukhsh, 2014; Teerasripreecha et al., 2012; Darakhshan & Ghanbari, 2013). This method has been widely used in numerous studies to evaluate the toxicity of certain compounds in a distinct cell line (Graidist, Martla, & Sukpondma, 2015; Shafagh, Rahmani, & Delirezh, 2015; Al-Sheddi et al., 2015). To the best of our knowledge, since the first publication of isolation of a new compound, biseugenol B from *Litsea costalis* in 2013 (Hosseinzadeh, et al., 2013a), this is the first time that anti-cancer effects of biseugenol B have been investigated.

The reason of highly cytotoxicity effect of biseugenol B on prostate cancer cells (PC3) and less cytotoxicity effect on normal prostate cells (RWPE-1) can be explained in different ways, although the exact mechanisms are still unknown and further research may be needed to discover the exact chemical interactions in each apoptosis signaling pathway. Generally, the group of anti-cancer drugs or compounds that act as apoptosis inducers mostly target the cells with very rapid cell divisions like cancer cells. This explains the effects of chemotherapy drugs on hair loss and epithelium cells in skin and intestine, due to their capability to divide faster than the other normal cells in the body (Avendaño & Menendez, 2015). As cancer cells undergo rapid cell division, they are generally more susceptible to anti-proliferation effect of these drugs than normal cells. In addition, cancer cells often have impaired ability to recognize and/or repair DNA damage which increase the chances of not properly replicating their DNA, eventually causing cell death by mitotic catastrophe or apoptosis mechanisms (Abbotts, Thompson, & Madhusudan, 2014). Furthermore, proteins that are present in cancer but not normal cells, or that are more abundant in cancer cells can be the other factor as to why cancer cells have been targeted by anti-proliferative drugs more than normal cells. For instance, CXCR5 a transmembrane receptor, which positively correlates with PCa (prostate cancer cell line) progression and mediates PCa cell migration and invasion was highly expressed in cancer cells, while its expression in normal cells is poorly detected (El-Haibi et al., 2013). These were some hypothesis to explain the potential mechanisms. Nevertheless, further molecular studies about potential receptors or cellular protein which exclusively express in cancer cells and targeted by apoptosis induced compounds like biseugenol B are needed before such confirmation can be made.

Since biseugenol B has a strong potential cytotoxic effect on prostate cancer cells, we applied AO and PI fluorescent dyes to observe the various stages of apoptosis, starting with chromatin condensation until the apoptotic body formation with biseugenol B

treatment. Although AO/PI was clearly featured in the morphological changes associated with apoptosis, we conducted Annexin V assay to quantify the population of apoptotic cells. The current study established that treatment with biseugenol B can induce cell death in PC3 cells through apoptosis. In addition, the results documented a significant dose-dependent increase in both early and late stages of apoptosis. The utilization of AO/PI and annexin V techniques for modulation of apoptosis induction effect of a certain compound on distinct cell line is an established technique. These techniques are broadly applied in a number of studies to assess the apoptosis induction effect of various anti-cancer compounds on different cancer cell lines (Focaccetti et al., 2015; Hajrezaie et al., 2015; Namvar et al., 2015; Li et al., 2015; Rahman et al., 2016).

Previous studies suggest that oxidative stress has a role in mitochondria change and apoptosis (Ham et al., 2012). We measured the reactive oxygen species (ROS) levels upon biseugenol B treatment on the PC3 cells. The results accentuate this significant relation. The results showed a threefold increase in intracellular ROS with 4 µg/mL and 8 µg/mL biseugenol B treatment in PC3 cells which could be due to the generation of free radicals during cytotoxicity, while no significant increase ($P > 0.05$) in reactive oxygen species was observed in non-tumorigenic prostate cell line (RWPE-1). This indicated that biseugenol B effectively induces the generation of reactive oxygen species (ROS) which represents the level of oxidative stress in mitochondria that subsequently demonstrate the induction of apoptosis in PC3 cells. The negligible increase of oxidative stress in mitochondria by biseugenol B in normal prostate cell line (RWPE-1) evidenced the inefficiency of biseugenol B on normal prostate cells which was in accordance to the MTT results. This finding concurred with most of the published evidence that a remarkable increase of ROS level was seen in different cancer cell lines after treatment by an effective anti-cancer compound (Qiu et al., 2015; Chen et al., 2015; Hubaux et al., 2015).

The apoptosis mode which can be caused by various natural compounds is closely associated with the cell cycle arrest (Mohan et al., 2010; Park et al., 2012). Cell cycle control has been proven to ensure accurate cellular division. Many carcinogenic processes have been noted to cause cell cycle deregulation. Thus, one of the chemotherapy targets in cancer therapy is the alteration of cell cycle regulators in cancer cells (Khan et al., 2013; Xiao et al., 2005). With various concentrations of biseugenol B, the cell cycle examination of PC3 revealed a significant increase in the number of cells in the sub-G0 phase, with the highest concentration of biseugenol B and a larger number of cells in the G0/G1 phase. Alternatively, the number of cells decreased in the S and G2/M phases as compared to untreated cells. The results indicated that the inhibitory ability of biseugenol B in cellular proliferation is through G0/G1 phase arrest. The similar effect on cell cycle modulation has been observed in previous studies (Ho, Yazan, Ismail, & Ismail, 2009; Guo, Chen, Lam, & Zhang, 2015; Murad et al., 2016; Wang et al., 2015).

Although both extrinsic and intrinsic pathways are associated with apoptosis, the intrinsic pathway is more frequently involved in the tumor occurrence (Mohana-Kumaran, Hill, Allen, & Haass, 2014). Mitochondria can directly initiate the apoptosis cellular pathway, so they are considered as the core organelles for the intrinsic apoptosis pathway. Mitochondria can execute multiple cellular functions, but their primary involvement is in cell redox status (Circu & Aw, 2010).

The fluorescence-based high content screening analysis revealed the effect of biseugenol B on the mitochondria. A significant increase in cell permeability after treatment with biseugenol B was noted. Simultaneously, a noteworthy decline was shown in fluorescence intensity which reflected the break-down of MMP, while the release of cytochrome c from mitochondria into the cytosol was also increased. Outer membrane permeabilization and mitochondrial transmembrane potential alteration are necessary to initiate the apoptotic cascade and release of pro-apoptotic proteins, including cytochrome

c which eventually results in the activation of caspase-9 and -3 (Anatole et al., 2013). The decrease of mitochondria membrane potential (MMP) is essential for the beginning of the apoptosis process. Similar results have been seen in different cancer cells lines after treatment with a compound with significant anti-cancer effects (Ahmadipour et al., 2015; Ibrahim et al., 2014; Ahn, Kang, Shin, & Chung, 2012). Thus, our results supported the hypothesis that biseugenol B can induce alterations in outer membrane permeabilization and mitochondrial transmembrane potential which can lead the cell to apoptosis.

The key regulatory factor in apoptosis is a caspase activation (Czabotar, Lessene, Strasser, & Adams, 2014). In the intrinsic pathway, the release of cytochrome c from mitochondria into the cytosol leads to the formation of apoptosome and activates caspase-9, resulting in activation of effector caspases such as caspase-3, caspase-6 and caspase-7 (Li et al., 2010). Moreover, previous studies showed that Bcl2 protein family members mediate the release of cytochrome c in the context of apoptotic stimuli (Green, 2006). Additionally, movement of Bax into the mitochondria causes the cytochrome c release into the cytosol, MMP loss and then results in the induction of mitochondrial permeability transition events (Westphal, Kluck, & Dewson, 2014). Biseugenol B clearly triggered the cytochrome c release and increased the activity of caspases 3-7 and caspase-9 which obviously demonstrate that apoptosis occurred via intrinsic apoptosis pathway. Even so, in extrinsic apoptosis pathway, caspase activation occurs via transduction signals that transit through cell death receptor like, TNF-a and fas, causing caspase-8 to be activated which accordingly results in activation of downstream effector caspases such as caspase-3 and caspase-7 (Mohan et al., 2012). The activation of caspase-8 is indicative of the extrinsic signaling pathway (Li et al., 2010; Schug, Gonzalvez, Houtkooper, Vaz, & Gottlieb, 2011). Remarkably, biseugenol B increased the activation of caspase-8 which suggests that apoptosis occurs via extrinsic pathway as well. In many previous studies, the modulation of caspases by certain compounds or drugs is the reliable approach to

assess the apoptosis induction effect, thus, compounds and drugs that induce apoptosis through intrinsic pathway demonstrate the higher level of caspase-3/7 and caspase-9 (Ahmadipour et al., 2015; Druškovič, Šuput, & Milisav, 2006). On the other hand, extrinsic apoptosis pathway is detected by higher level of caspase-8, while higher level of caspases-3/7, caspase-9 and caspase-8 together indicates the involvement of both intrinsic and extrinsic pathways in apoptosis induction (Kim, Kim, Lee, & Song, 2009; Winter et al., 2014; Khaw-On & Banjerdpongchai, 2012; Elumalai et al., 2012). The number of studies that assessed the level of caspases in normal cell lines is very limited (Sarzaeem, Mirakabadi, Moradhaseli, & Sayad, 2013). Nevertheless, in this study, we have evaluated the effect of biseugenol B on caspases level in normal prostate cell line (RWPE-1). We found that the results concurred with MTT and ROS tests: biseugenol B had negligibly increased the caspases level in RWPE-1. The alteration of caspases-3/7, caspase-8 and caspase-9 were insignificant in comparison to untreated RWPE-1 cells. The combined results of MTT, ROS and caspase assays in RWPE-1 indicates the less toxic effects of biseugenol B on normal cells. This supports the hypothesis that biseugenol B could be a potential anti-cancer drug that induces apoptosis in cancer cells with minimum damage on normal cells.

Based on the evidence, members of the Bcl-2 protein family mediate the release of cytochrome c, which can stimulate apoptosis (Martinou & Youle, 2011; Azad et al., 2010). Subsequently, Bax moves into the mitochondria, consequently resulting in the cytochrome c release, MMP loss and induction of mitochondrial permeability transition events (Kroemer, Galluzzi, & Brenner, 2007). Bax is an apoptotic activator that contracts with Bcl-2 and increases the opening of the anion channel of mitochondrial which results in mitochondrial membrane potential loss and the cytochrome c release (Alluri et al., 2014). Bcl-2 is mainly known as an important anti-apoptotic protein, hence is identified as an oncogene (Czabotar, Lessene, Strasser & Adams, 2014). The decrease of Bcl-2

could be a sign of apoptosis induction. Also, Hsp70 has an anti-apoptotic function that could inhibit the key effectors of the apoptotic machinery at the pre- and post-mitochondrial level (Rérole, Jego, & Garrido, 2011). Considering the key role of Bax, Bcl-2 and Hsp70 genes in apoptosis, we performed PCR and western blot to detect the change of these key factors in apoptosis. The PCR results showed a significant increase in expression of Bax (an apoptosis activator) after treatment with biseugenol B, while Bcl-2 and Hsp70 (both considered as anti-apoptosis) were detected to be significantly decreased after 24 hours treatment with biseugenol B. The up-regulation of Bax along with down-regulation of Bcl-2 and Hsp70 can support the apoptosis-induction effect of biseugenol B. It is worth mentioning that Bcl-2 and Bax are involved in intrinsic pathway, so these results could be another finding to support that biseugenol B plays a role in intrinsic apoptosis pathway. In addition, Hsp70 is involved in the mitochondrial intrinsic pathway by preventing Bax translation (Stankiewicz, Lachapelle, Foo, Radicioni, & Mosser, 2005) and the decrease of Hsp70 indicates the induction of apoptosis in the cell. The majority of studies to evaluate the effect of certain compound or drug on apoptosis induction usually detect the expression of pro and anti-apoptosis genes (Leisching, Loos, Botha, & Engelbrecht, 2015; Sagar et al., 2014; Fallahian, Aghaei, Abdolmohammadi, & Hamzeloo-Moghadam, 2015). Concomitantly, certain members of the Bcl-2 family such as Bcl-2 and Bax have been identified to control the apoptosis process (Czabotar, Lessene, Strasser & Adams 2014). The balance between the anti-apoptotic and pro-apoptotic properties of the Bcl-2 family is crucial in actuating the cell toward apoptosis (Adams & Cory, 2007). Previous studies have shown that the up-regulation of Bax and down-regulation of Bcl-2 cause susceptibility to mitochondria-mediated apoptosis (Lee, Jung, Jeong, Yoon, & Kim, 2012; Zhu et al., 2013). Therefore, we inspected the effect of biseugenol B on the expression of the Bcl-2 family. Bcl-2 is an anti-apoptosis protein

located in the cytoplasm and it plays a significant role in apoptosis inhibition (Ferenc, Solár, Kleban, Mikeš, & Fedoročko, 2010).

The PCR and Western blot results in this study indicated that the Bcl-2 protein was regulated in PC3 cells after treatment with biseugenol B in a dose-dependent manner which can explain the apoptosis-inducing effect of biseugenol B on PC3 cells. In addition, the down-regulation effect of biseugenol B on PC3 cells could be associated with apoptosis factors that are produced in mitochondrial and ultimately resulted in apoptosis (Jin, Zhang, Kang, Wang, & Zhao, 2009).

NF-κB is a protein complex that has a role in regulating DNA transcription and is considered as an apoptosis inhibitor (Naugler & Karin, 2008; Verfaillie, Garg, & Agostinis, 2013). Therefore, repressing the activity of NF-κB can induce apoptosis. In this study, we demonstrate that biseugenol B can repress the apoptosis-inhibitor activity of NF-κB by preventing its translocation from the cytoplasm to the nucleus of the PC3 cells. This finding suggests that biseugenol B can induce apoptosis by suppressing the TNF-α-induced NF-κB anti-apoptosis signaling pathway (Rahman et al., 2007; Rahman & Sarkar, 2005; Bhuiyan et al., 2006; Mohammad, Banerjee, Aboukameel, Kucuk & Sarkar, 2006; Haffner, Berlato, & Doppler, 2006).

The selectivity and specificity of biseugenol B toward human prostate cancer cell line (PC3) is considerable and it is worth discussing. In chapter 2, the chemical structure of biseugenol B was reviewed: biseugenol B is an oxyneolignan that belongs to the plant natural compounds “lignans and neolignans”, which are mainly biochemically related to phenylalanine metabolism. Phenylpropanoids are a group of natural compounds that are mainly composed of C_6C_3 units (Yasukawa & Tabata, 2015). Previous studies have shown the significant anti-cancer effect of phenylpropanoids on different cancers such as pinoresinol (colorectal cancer) (Fini et al., 2008), podophyllotoxin (breast cancer)

(Chattopadhyay, Bisaria, Panda, & Srivastava, 2004) and steganacin (leukemia) (Lee, 2010). Thus, biseugenol B is also expected to possess anti-cancer activities. Nevertheless, the specificity and selectivity of bisugenol B for prostate cancer cannot be explained by its chemical structure.

In other chemical classification, biseugenol B has a unique structure among the entire family of eugenol. The search of the chemical structure of the other members of this family and similar compounds revealed that biseugenol B has two eugenols that are connected by an oxide bonding. This leads to the main reason the compound was named as a biseugenol. For a better understanding of the chemical structure of biseugenol B and its anti-proliferative effect, we briefly study its sub-structure: eugenol. In 2007, Pisano, et al. reported the anti-proliferative effect of eugenol and iseugenol and five other members of this family on melanoma. The findings showed the efficient anti-proliferative activity effect of the dimeric forms (biphenyls) as observed in biseugenol B that contains two phenyl units (Pisano et al., 2007). This fact can be related to anti-proliferative and anti-cancer effect of biseugenol B as it is structurally similar to dimer eugenol. Nevertheless, there is still no evidence that biseugenol B structure is associated with the anticancer properties exhibited in prostate cancer. One of the most obvious differences between human prostate cancer cells and the other types of cancer cell lines is hormone (androgen) receptors on prostate cells surface. Therefore, these receptors might play a role in the specificity of biseugenol B for human prostate cancer cell line.

The other attempt for finding a reasonable explanation for the specificity of biseuganol B toward human prostate cancer is the reviewing of previous natural compounds that have been effective on prostate cancer. Numerous attempts have been made to investigate the effects of various natural compounds in prostate cancer. Among the compounds are polyphenol (Adhami, Siddiqui, Ahmad, Gupta, & Mukhtar, 2004) and epigallocatechin-3-gallate (EGCG) extracted from green tea (Adhami et al., 2007), silibin extracted from

milk thistle (Silybummarianum) (Rajamanickam, Velmurugan, Kaur, Singh, & Agarwal, 2010), sulphoraphane extracted from cruciferous vegetables (Warin, Chambers, Potter, & Singh, 2009), genistein extracted from soy (Singh-Gupta et al., 2010) and apigenin extracted from plant flavonoids. The compounds have different chemical structure and their anti-prostate cancer effects were found to be not specific (Lattanzio, Lamolinara, Iezzi, & Piantelli, 2013). None of these compounds has a similar chemical structure to biseugenol B.

Overall, we can conclude that biseugenol B is a novel and unique compound that has a potential therapeutic activity against human prostate cancer cells. The findings of this study could provide a primary evidence that biseugenol B can be used as a natural anti-cancer drug. However, further studies are needed before biseugenol B could be claimed to be an effective and safe agent for treatment of prostate cancer.

CHAPTER 6: CONCLUSION

Based on the observations from this study, biseugenol B is capable of inducing apoptosis in PC3 cells through apoptosis signaling pathway that regulates MMP via the down-regulation of Bcl-2 and up-regulation of Bax which can cause the release of cytochrome c from mitochondria to cytosol. Upon the release of cytochrome c into the cytosol, caspase-9 is activated which in turn activates the downstream executioner caspase-3/7. Thereafter, the apoptosis cascade occurs in the cell by slicing specific substrates. Meanwhile, the increase of caspase-8 reveals the apoptosis mechanisms of biseugenol B through extrinsic pathways. These findings suggest that apoptosis occurs through both intrinsic and extrinsic pathways with regulation of NF-κB, Bax, Bcl-2 and Hsp70 protein modulation.

6.1 Study limitation

In this study, we tried to assess the potential apoptosis induction activities of biseugenol B from *Litsea costalis* in human prostate cancer cell lines *in vitro*. Although PC3 cell line cells closely emulate the human prostatic cancer cells, the isolated and cultivated primary cells usually differ from the corresponding cell type in an organism. Moreover, the environmental factors which generally affect the organs in *in vivo* system are absent in *in vitro* experiments. Hence, for to confirm the current results, *in vivo* experiment seems inevitable in future.

6.2 Future recommendations

In this study, results showed that the biseugenol B compound can induce apoptosis in PC3, human prostate cancer cell line via both intrinsic and extrinsic apoptosis pathway and by inhibition of anti-apoptosis NF-κB pathway. However, the confirmation of these results in the animal model (*in vivo*) is required. In addition, the primary MTT results revealed the selectivity and specificity of biseugenol B in human prostate and no other

type of cancers. Further chemical and structural study of biseugenol B might uncover the reason of this specificity.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. Receptor tyrosine kinase (c-Kit) inhibitors: a potential therapeutic target in cancer cells.

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2. Apoptotic induction and inhibition of NF-κB signaling pathway in human prostatic cancer PC3 cells by natural compound 2,2'-oxybis (4-allyl-1-methoxybenzene), biseugenol B, from *Litsea costalis* : an *in vitro* study.

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