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Investigating the Anticancer Effects of *Ficus religiosa* Plant Leaves

A dissertation

Submitted to the Council of College of Science, Al-Nahrain University, in partial fulfillment of the Requirements for the Degree of Doctorate of Philosophy in Science, Biotechnology

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Summary

Ficus religiosa plant is an important medical plant and traditionally used to treat various diseases including mastitis, otitis media, pharyngolaryngitis, urethritis, dysmenorrhea and diabetis. The present study was conducted to evaluate anti-tumour properties of *Ficus religiosa* leaves collected from Baghdad, Iraq.

1- Test different extracts of *Ficus religiosa* plant (chloroform, butyl alcohol, absolute methanol and a crud aqueous extracts) to determine the cytotoxic effect on breast cancer (MCF7), prostate cancer sensitive to docetaxel (PC3) and resistance to docetaxel (PC3-TxR), leukemic cancer cells sensitive to daunorubicin (K562) and resistance to daunurobicin (K562Dox) cancer cell lines using sulforhodamine B assay. Then choose the best extract caused cancer cell inhibition to study chemosensitizing effect on cancer cell lines using sulforhodamine B assay. HPLC-DAD was used to determinate the active compounds in plant extract and study its ability for inhibition *P-gp, EGFR, NrF2, Id1, Id2, Id3, Patch1* and *Gli2* genes expression on PC3-TxR cells using RT-PCR and Western blot.

2- Investigate the toxic effect of extract *in vivo* by determination of LD_{50} and evaluate anti-tumour (histopathologically and immunohistochemically for *P-gp* gene expression) in SCID mice.

The *in vitro* results of inhibition concentration (IC_{50}) against cell lines demonstrated that treatment of cell cultures with four *Ficus religiosa* extracts decreased inhibitor concentrate (IC_{50}) of the cells significantly ($P \leq 0.05$) and the effect was dose. The chloroform extract with low IC_{50} caused high toxic against PC3-TxR while a crud aqueous extract had less toxicity against cancer cell lines. The chloroform extract revealed that the extract exhibited strong chemosensitizing effect (CE) on PC3-TxR cell line than other cancer cell lines.

Chemical analysis of chloroform extract was carried out using high performance liquid chromatography (HPLC-DAD) analysis. Accordingly, five

different compounds (quercitin, myrecitin, tannic acid, gallic acid and serotonin) were characterized. The results displayed tannic acid and serotonin in the extract, while the other pure compounds have not been reported and there were many other compounds in the extract were considered unknown. The highest concentration of tannic acid was observed in the extract while serotonin was less concentration. Moreover, the chloroform extract had potent inhibitory cytotoxic effected against the proliferation of PC3-TxR cancer cells, through down-regulation of *P-gp, Id1* and *Id2* genes expression and the effect was markedly detect after 24 hours of treatment. The Western blot result confirmed chloroform extract inhibit *P-gp* protein production in PC3-TxR cancer cell lines.

Lethal dose (LD_{50}) of chloroform extract of *Ficus religiosa* leaves was 11.3 $g.Kg^{-1}$ and the results showed the chloroform extract was significantly effective in reducing tumour size in a dose-dependent manner, when compared with mice intravenous (i.v) with docetaxel (20 $mg.Kg^{-1}$) once weekly which associated with adverse side effects as well as observed reduction in mice weights more than 30% compared with negative and positive groups. Histopathological examinations of tumour sections from treated mice revealed decreased of mitosis and increased vascularization of cells. The *P-gp* gene showed a positive expression in tumour cells treated with docetaxel alone (positive control), while it showed a decreased expression in tumour sections of animals treated orally with the dose 3.8 $g.kg^{-1}$ and at the dose 7.5 $g.kg^{-1}$.

The results revealed that *Ficus religiosa* extract has anticancer activity against cancer cell *in vivo* and *in vitro*.

List of Contents

Chapter Three: Materials and Methods

Chapter Four: Results

Chapter Five: Discussion

Conclusions and Recommendations

List of Tables

List of Figures

List of Abbreviations

ACHE	Acetylcholinesterase
AGS	Gastric Cancer cell lines
ARE	Antioxidant Responsive Element
bax	Bcl2-associated X Gene
bcl-2	B Cell Leukemia/Lymphoma 2-like Gene
BrdUrd	5-Bromo-2-deoxyuridine
BVS	Blood Vasseles
CADRE	Cancer for Advancement of Drug Resistance Evaluation
CAT	Catalase
CE	Chemosensitizing Effect
CML	Chronic Myelogenous Leukemia
CV	Coefficient of Variation
DAR	Daunorubicin
Dhh	Desert hedgehog
DMH	1,2 Dimethylhydrazine
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DPPH	2, 2-Diphenyl-1-Picryl-Hydrazyl
Dtx	Docetaxel
DXR	Adriamycin
EDTA	Ethylene Diamine Tetra Acetic Acid
EEFR	Ethanol Extract of *Ficus religiosa*
EGFR	Epidermal Growth Factor Receptor
EPI	Epirubicin
ER	Estrogen Receptor
ErbB	Epidermal growth factor receptor
FAE	*Ficus religiosa* Aceton Extract
FBS	Fetoel Bovine Serum
FR	*Ficus religiosa*
GA	Gallic Acid
GSH-Px	Glutathione Peroxidase
HER	Human Epidermal Growth factor
Hh	Hedgehog pathway
HLH	Helix- Loop-Helix
HPLC-DAD	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase

HT-29	Human Colon cancer
i.v.	Intravenous Administration
IC	Inhibitory Concentration
ICCMGR	Iraqi Center for Cancer Medical Genetic Research
Id	Inhibition of differentiation/DNA
IDA	Idarubicin
IFN	Interferon
IHC	Immunohistochemistry
Ihh	Indian hedgehog
IL	Interleukin
IMR-32	Human neuroblastoma
K562	Chronic Leukemic cancer cell line
K562Dox	Chronic Leukemic cancer cells resistance to daunorubicin
LC	Lethal Toxicity
LD	Lethal Dose
LLOQ	Lower limit of Quantification
LPS	Lipopolysaccharide
MAD	Malanodialdehyde
MAPK	Mitogen Activated Protein Kinases
MCF-7	Michigan cancer foundation-7
MDA-MB-4435s	Breast cancer cell
MDR	Multidrug Resistance
MYR	Myrecitin
MF	Mitotic factor
M-H	Pseudo-Molecular Ion (Negative Mode)
MTD	Maximum Tolerance Dose
NIH3T3	Healthy Mouse Fibroblast
NK	Natural Killer Cells
NO	Nitric Oxid
Nrf2	Nuclear related factor2
p.o.	Oral Administration
p53	Tumour Suppressor Gene
PBS	Phosphate Buffered Saline
PC3	Prostate cancer cell line
P-gp	P-glycoprotein
PGR	Progesterone Receptor
PI	Propidium Iodide

PRP	Prostate Resistance Protein
Ptch1	Patch1
PTZ	Pentylenetetrazol
QC	Quality Control
QE	Qurecitin
R	Linearity
ROS	Reaction Oxygen Species
RT	Retention Time
RT-PCR	Real Time-Polymerase Chain reacton
SCID	Sever Combined immunodefficiency
SER	Serotonin
shh	Sonic hedgehog
Smo	Smoothened
SOD	Superoxide dismutase
SPF	Specific Pathogen Free
TA	Tannic acid
TGF	Transforming Growth Factor
TIC	Total Ion Current
TNF	Tumour Necrotic Factor
TPA	Total Peak Area
V-FITC/PI	Fluorescein Isothiocyanate/ Propidium Iodide
WHO	World Health Organization

1. Introduction

1.1 Introduction

Cancer is a leading cause of death worldwide and a diverse group of diseases (Mell *et al*., 2012). According to the World Health Organization (WHO), it was estimated 12.7 million people globally were diagnosed with cancer and about 7.6 million people died in 2008. As estimated in this report, more than 21 million new cancer cases and 13 million deaths are expected by 2030. Although cancer accounts for around 13% of all deaths in the world, more than 30% of cancer deaths can be prevented by modifying or avoiding key risk factors (World Health Organization, 2011).

Due to the societal and economical implications of this pathology, tremendous efforts have been made over the past decades to improve the available therapeutic options. Although a large number of potent chemotherapeutic anticancer agents have been identified and successfully used in clinical practice, while minimising their toxic side effects. Indeed, most anticancer agents display a narrow therapeutic window due to their lack of selectivity against cancer cells. Besides, the ability of the anticancer compounds to actually reach their target is often impaired by a number of physiological barriers (Wooster and Bachman, 2010), as well as the chemoresistance, is considered to be the responsible for treatment failure in over 90% of patients with metastatic cancer (Yu *et al*., 2012).

Herbal plants and plant-derived medicines have been widely used as natural alternatives to produce new potential therapeutic compounds for treatment combating diseases (Shoeb, 2006). The health promoting effects of plant constituents and extracts are being increasingly studied and their consumption is on the rise (Aggarwal and Shishodia, 2006). Many herbs have

been evaluated in clinical studies and are currently being investigated phytochemically to understand their tumoricidal actions against various cancers (Shishodia *et al*., 2006).

The flora of Iraq, the ancient Mesopotamian land of civilization are interesting, about thousands medicinal plants species have been recorded in Iraq. Tens of studies in Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), on the properties of local plant wealth against cancer have been curial act. Among these studies some plants and herbs possess a cytotoxic or antitumor activities, for instance, the studies made by Ibrahim (2005) on *Salvia triloba*; AL-Tymimie (2007) on *Apium graveolens*; Abdul-Hameed (2008), on *Lactuca serriolal*; *Cyperus rotundu;* (Zaid 2009) and Abd AL-Redha (2012) on *Citrus limonnum Risso*.

Pharmacological studies carried out on the plant materials of *Ficus religiosa* provide a pragmatic support for its numerous traditional uses. Its bark, fruits, leaves, adventitious roots, latex and seeds are medicinally used in different forms, sometimes in combination with other herbs (Aiyegoro and Okoh, 2009). Singh and his colleges (2011) suggested a detailed investigation for its potential against cancer, cardiovascular, neuro inflammatory and parasitic infections. Most of the pharmacological studies were aimed on validating its traditional uses for wound healing, anti-bacterial, anti-diabetic, anti-inflammatory, and anti-anxiety activity (Sheetal *et al*., 2008 ; Taskeen *et al*., 2009; Naira *et al*., 2009; Rucha *et al*., 2010).

1.2 Aims of study

Based on the forthcoming presentation, the present study was planned with the aim to evaluate the anti-tumour potential of *F. religiosa* leaves extract (*in vitro* and *in vivo*), the following assessments were achieved:

A- In vitro Investigating

Assessment cytotoxic effect of *Ficus religiosa* leaves extracts on different human cancer (MCF7, PC3, PC3-TxR, K562 and K562Dox) cell lines. Then determination chemosensitizing effect of *F. religiosa* chloroform extract on four human cancer (PC3, PC3-TxR, K562 and K562Dox) cell lines. Assessment the effect of *F. religiosa* chloroform extract on (P-gp, EGFR, Nrf2, Id1, Id2, Id3, Patch1 and Gli2) genes expression using RT-PCR. And assessment the effect of chloroform extract on Pgp-protein using western blot assay. Finally, Chemical analysis of *F. religiosa* chloroform extract using high performance liquid chromatography (HPLC-DAD).

B- In vivo Investigating

Single and multiple maximum tolerance doses (sMTD and mMTD) of *F. religiosa* chloroform extract were detected on normal CD-1 mice. And anti-tumour effect was further extended on SCID/nude mice a xenograft with PC3-TxR cancer cells, and the immunohistochemical was performed to evaluate of Pgp gene expression.

2. Literature Review

2.1 Medicinal Plants

Over the past decades, herbal medicine has become a topic of global importance, making an impact on both world health and international trade. Medicinal plants continue to play a central role in the health care system of large proportions of the world's population.

Medicinal plants have been used as a source of medicine to treat illness since time immemorial. For a long time, plants have provided a source of emerging modern medicines and drug compounds, as plant derived medicines have made large contributions to human health. Their role is twofold in the development of new drugs and/or a phytomedicine to be used for the treatment of diseases (Prasad *et al*., 2012).

Active compound present in the medicinal plants provide the bountiful resource of active compounds for the pharmaceutical, cosmetics and food industries, and more recently in agriculture for pest control (Cseke *et al*., 2006). Chemically the active components in bioactive herbs may be classified as isoprenoid derivatives, phenolic compounds, carbohydrate derivatives, fatty acid and structural, amino acid derivatives, microbes (probiotics, prebiotics) and minerals (Ca, Zn, Cu, K, Se) (Butterweck *et al*., 2001).

The biochemical mechanism of cancer prevention in tissues by herbs is proposed. Broadly herbs are rich in alkaloids, flavones, antioxidants, xanthones, omega-3 fatty acids, vitamins, minerals and fibers. Most of the herbs are derived from plants and they act as regulatory biochemical metabolites either by direct intermediary metabolism or regulating cancer pathways and stimulating immunity (Sharma *et al*., 2010).

Accordingly, there is a considerable scientific and commercial interest in the continuing discovery of new anti-cancer agents from all natural product

sources. Following this lead, *Ficus religiosa* is a further subject of medicinal plants that may have the potential to act as antimutagenic and/or anticarcinogenic natural product.

2.2 *Ficus religiosa* plant

Common names of *Ficus religiosa* are Peepul tree, Pipal tree and sacred fig (pullaiah, 2006; Khare, 2007). The genus Ficus (Moraceae) constitutes one of the largest genera of angiosperms with more than 800 species of trees, epiphytes and shrubs in the tropical and subtropical regions worldwide. It is one of the most diverse plant genera in regard to its growth habit with both deciduous and evergreen free standing trees, stranglers, climbers, creepers, small shrubs, lithophytes and rheophytes (Loutfy *et al*., 2005; Ronsted *et al*., 2008). Ficus spp. commonly known as fig, is a small or moderately size deciduous tree indigenous to Persia, Asia, Minor, Syria, Iraq and Mediterranean region, and widely found in tropical and subtropical regions of India (AL-Yousuf, 2012).

Ficus religiosa tree begins its life epiphytically and then strangle the host by its far growing roots that extend to the ground, establishing it as an independent tree, and because it was found having an annual rainfall varying from 50 to 500cm during the monsoon season and tolerates a wide variation in temperature (below 0 ◦C and above 40 ◦C) (Pullaiah, 2006), according of this growth condition *F. religiosa* widely cultivate in Iraq.

It is the most sacred tree of South Asia, to both Hindus and Buddhists. The specific epithet “religiosa” and synonym “bodhi tree” alludes to the religious significance attached to this tree (Kala *et al*., 2006; Sitaramam *et al*., 2009).

2.2.1 Taxanomy of *Ficus religiosa* (Sirisha *et al*., 2010)

Domain: Eukaryota

Kingdom: plantae

SubKingdom; Viridaeplantae

Phylum: Tracheophyta

Subphylum: Euphyllophytina

Infraphylum: Radiatopses

Class: Magnoliopsida

Subclass: Dilleniidae

Superorder: Urticanae

Order: Urticales

Family: Moraceae

Genus: Ficus

Species: religiosa

2.2.2 Botanical description

Ficus religiosa is a large deciduous tree up to 35m in height (figure 1A), epiphytic when young, and crown wide when mature. Its dropping branches bear coriaceous, stipulate leaves that are dark green in color (figure 1B), 10–18×7.5–10 cm, ovate-rotund, narrow upward and the apex produces into a linear lanceolate tail about half of the main portion of the blade, base broadly cuneate to cordate, margin entire or undulate; secondary veins five to seven on each side of the midvein, lateral veins eight pair with finely reticulate venation (figure 1C); petioles 7.5–10cm long, slender; stipules minute ovate, acute. Figs (figure 1D) axillary on leafy branchlets, paired or solitary, red when mature (figure 1E), (Zhekun and Gilbert, 2003; Warrier *et al*., 1995; Kirtikar and Basu, 1993). The bark (figure 1F) is flat or slightly curved with thin or membranous flakes, often covered with crustose lichen patches. The outer bark is grayish or ash colored, exfoliated with irregular rounded flakes of 2–2.5cm thickness. The middle bark sections appear as brownish or light reddish brown in color. The inner part consists of the layers of light yellowish or orange brown colored granular tissue. The bark is odorless and its taste is astringent. The plant bears few adventitious roots (Figure 1G) (Koilpillai *et al*., 2010; Ali, 2007; Warrier *et al*., 1995).

Figure 2-1: *Ficus religiosa* plant parts (Koilpillai *et al*., 2010).

2.2.3 Phytochemistry

Phytochemical research carried out on *F. religiosa* had led to the isolation of phytosterols, amino acids, furanocoumarins, phenolic components, hydrocarbons, aliphatic alcohols, volatile components and few other classes of secondary metabolites from its different parts. Phenolic components from tannins and flavonoids (Taskeen *et al*., 2009), and amino acids are present in almost all the parts of *F. religiosa*.

2.2.4 Constituents of leaves

Phytochemical research carried out on *F. religiosa* had led to the isolation of phytosterols, aminoacids, furano cumarins, phenolic compounds, hydrocarbons, aliphatic alcohols, volatile components and few other classes of secondary metabolites from its different parts. Phenolic components (tannins, and flavonoids (Taskeen *et al*., 2009)), (Singh *et al*., 2011).

Phytosterols (2.8%) like, campesterol, stigmasterol, sitosterol, 28-isofucosterol and triterpene alcohols (28.5%) like, amyrin and lupeol have been isolated from the non saponifiable fraction of light petroleum leaf extract of *F. religiosa*. Along with phytosterols and triterpene, 7.1% of long chain hydrocarbons and 7.9% of aliphatic alcohols have also been isolated from the

same fraction (Williamson and Hooper, 2002). Fibers have been identified in the leaves of *F. religiosa*. The leaves contain around 1.5% of total tannin content, which comprises tannic acid and condensed tannins (Niranjan *et al*., 2007).

The leaves are rich in minerals like, calcium, phosphorous, iron, copper, manganese, magnesium, zinc, potassium and sodium (Niranjan *et al*., 2007; Williamson and Hooper, 2002).

A variety of proteins and carbohydrates are present in the leaves, making them a good fodder (Bamikole *et al*., 2003; Bhadauria *et al*., 2002).

2.2.5 Contemporary uses

Ficus religiosa has been extensively used in traditional medicine for a wide range of ailments. Its bark, fruits, leaves, adventitious roots, latex and seeds are medicinally used in different forms, sometime in combination with other herbs. The Ayurvedic properties of *F. religiosa* include astringent, heavy dry, cold and pungent (Panda, 2005; Williamson and Hooper, 2002). It has coloring or pigmenting action, ability to arrest pain, remove edematous swellings and conserves blood (Krishnamurthy, 2001). It was reported to have numerous therapeutic uses in folk medicine; leaf juice has been used for the treatment of asthma, cough, sexual, disorders, diarrhea, haematuria, ear ache and toothache, migraine, eye trouble gastric problems and scabies (gulecha and Sivakma, 2011).

2.2.6 Pharmacological Reports

2.2.6.1 Antiinflammatory and analgesic activities

Sreelekshmi and his colleges (2007), studied the anti-inflammatory, analgesic and *in vitro* antilipid peroxidative effect of the stem bark methanolic extract of *F. religiosa*. Determination of *in vitro* antilipid peroxidative effect was based on the spectrophotometric estimation of malanodialdehyde (MDA) per milligram of protein in the rat liver tissue

homogenate. As well as suggested the antilipid peroxidative effect of the stem bark extract to be the responsible mechanism for its antiinflammatory effect. Jung *et al*., 2008 investigated the effects of the methanolic leaf extract of *F. religiosa* on lipopolysaccharide (LPS) induced production of nitric oxide (NO) and proinflammatory cytokines tumour necrosis factor-alpha (TNF), interleukin-beta (IL-1) and IL-6 in BV-2 cells of mouse microglia cell lines. The extract prevented the cellular toxicity; inhibited LPS induced NO, TNF, IL-1 and IL-6 production in a dose-dependent manner. Inhibitory effect of the extract on NO production was suggested due to the inhibition of inducible nitric oxide synthase (iNOS) up regulation during microglial activation by LPS, as evidenced by the attenuation of mRNA expression of iNOS in BV-2 cells. Since the role of microglia derived proinflammatory factors, such as inflammatory cytokines have been suggested in the pathogenesis of various neurodegenerative disorders like, multiple sclerosis, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, tauopathies and age related maculardе generation (Frank-Cannon *et al*., 2009).

Therefore, the study of Jung *et al*., 2008 indicates the leaves of *F. religiosa* to be a potential candidate for the treatment of neuro inflammatory disorders.

2.2.6.2 Antimicrobial and antiviral activitie

Ficus religiosa has been used as an ethnic remedy for the cure of numerous infectious disorders of bacterial, fungal, viral, mycobacterium and protozoal origin. Valsaraj *et al*., 1997 investigated the antibacterial and antifungal effects of the leaves of *F. religiosa* against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus nige*.

The antibacterial effect of the aqueous, ethanolic and methanolic leaf extracts of *F. religiosa* was investigated against *Bacillus subtilis, Pseudomonas aeruginosa, E. coli* and *Salmonella typhi*. In an *in vitro* study,

the ethanolic, methanolic and aqueous leaf extracts of *F. religiosa* exhibited inhibitory effect on methanogensis caused by methanogens (methane producing microorganisms) ((Preethi *et al*., 2010; Kamra *et al*., 2008).

The ethanolic leaf extract of *F. relgiosa* inhibited the growth of all the tested bacterial strains, but was found to be infective against both tested fungal strains. Aqil and Ahmad, 2003 studied the effect of the ethanolic leaf extract of *F. religiosa* on the growth of bacterial strains (*Staphylococcus aureus, Salmonella typhimurium, Salmonella paratyphi, Staphylococcus typhi, Escherichia coli, Shigella dysenteriae* and *Pseudomonas aeruginosa*), filamentous fungi (*Aspergillum niger, Alternaria alternata, Fusarium chlamydosporum, Rhizoctonia bataticola* and *Trichoderma viride*) and yeast (*Candida albicans*). The extract inhibited the growth of *Staphylococcus aureus, Salmonella paratyphi, Shigella dysenteriae, Salmonella typhimurium, E. coli, Salmonella typhi* and *Candida albicans* with zone of inhibition. Apart from the crude extracts and isolated components, fumes of the different parts of *F. religiosa* have shown excellent antimicrobial effect against several bacterial and fungal strains (Negi, 2012; Prabhu *et al*., 2009; Nautiyal *et al*., 2007).

2.2.6.3 Antioxidant activity

Antioxidant activity of ethanolic extract of *F. religiosa* Linn leaf was investigated for its free radical scavenging activity by adopting various *in vitro* models. Ethanolic extract of *F. religiosa* showed maximum scavenging of DDPH radical 91.20% at 250 μg/ml concentration and hydrogen peroxide and reducing power were also dose dependent (Hemant *et al*., 2011).

Aqueous *F.religiosa* extract decreased the exaggerated activity of superoxide dismutase SOD. The antioxidant potential of different parts of *F. religiosa* can be further explored in ameliorating the oxidative stress related disorders (Pandit *et al*., 2010; Kirana *et al*., 2009; Sreelekshmi *et al*., 2007).

2.2.6.4 Immunostimulant activity

Mallurwar and Pathak, (2008), investigated the immunostimulant activity of the ethanolic bark extract of *F. religiosa*. Pyrogallol induced immunosupression model was employed to induce immunosupression in mice. The investigators found that the extract stimulates the humoral and cell mediated immune response. However more preclinical and clinical studies are required to make use of the bark as a future therapy for immunological disorders.

2.2.6.5 Antidiabetic activity

The ethnomedical use of *F. religiosa* in treatment of diabetes has been validated in several experimental studies, Firstly in 1962, Brahmachari and Augusti (1962) evaluated the hypoglycemic effect of the aqueous root bark extract of *F. religiosa* in male albino rabbits. Later on Ambika and Rao (1967) isolated phytosterolin (sitosteryl-d-glucoside) from the bark of *F. religiosa*, and investigated its hypoglycemic effect. The study suggested phytosterolin to be the responsible bioactive metabolite for the hypoglycemic effect of the bark (Deshmukh *et al*., 2007).

Type 2 diabetes in rats was induced by administration of STZ (90 mg/kg; i.p.) in 2 days old neonatal rats. After 6weeks of STZ injection the rats were treated with the extract (100 and 200 mg/kg; p.o.) for next 4 weeks through oral catheter. Treatment with the extract increased the body weight and decreased the fasting blood glucose level in type 2 diabetic rats. Moreover, the extract treatment attenuated the STZ-induced oxidative stress by modulating the enzymatic activity of catalase, glutathione peroxidase and superoxide dismutase (Kirana *et al*., 2009).

Therefore, the studies of Kirana *et al*., 2009 and Pandit *et al*., 2010 suggested the antioxidant effect of the aqueous bark extract to be the possible mechanism for its antidiabetic activity. The results of above mentioned preclinical investigations showed that *F. religiosa* show promise as a

medication for both type 1 and 2 diabetes mellitus but more studies are needed to prove clinical efficacy and reveal the exact mechanism of action.

2.2.6.6 Nootropic effect

Amnesia is a type of cognitive disorder that refers to a specific, acquired difficulty in learning new information (anterograde amnesia) and/or retrieving information from the past (retrograde amnesia) (Kritchevsky *et al*., 2004). Several factors have been suggested that are responsible for the genesis of clinical amnesia. Among these, the brain serotonergic system is known to play a vital role in the learning and memory processes and have been implicated in various human cognitive disorders (Tagliaferro *et al*., 2003; Buhot *et al*., 2000). Since the fruits of *F. religiosa* contain high serotonin content, therefore it was hypothesized that their treatment might modulate the brain serotonergic functions and has therapeutic usefulness in amnesia. Hence the antiamnesic effect of the methanolic fruit extract was studied using scopolamine induced anterograde and retrograde amnesia model in mice (Kaur *et al*., 2010).

Vinutha and his colleges (2007), selected 37 traditional medicinal plants (including the stem bark of *F. religiosa*) known to be useful in treating cognitive decline, improving memory or related CNS activities, and screened for their acetylcholinesterase AChE inhibitory activity.

2.2.6.7 Wound healing activity

Wound healing activity of the hydroalcoholic leaf extract of *F. religiosa* has been investigated by Roy and his collogues (2009) in rats. Leaf powder was extracted with 70% hydroalcoholic solvent, dried under reduced pressure to get a semisolid extract (yield 32.5%, w/w). Phytochemical screening showed the presence of glycosides and tannins in the extract. The activity of the extract was determined using excision and incision rat wound models. Treatment with 5 and 10% extract ointment promoted the healing of

wound in a dose-dependent manner, indicated by increased rate of wound contraction, decrease in the period for epithelialisation and high skin breaking strength. A similar study proving the wound healing effect of the hydroalcoholic leaf extract has also been reported (Awad *et al*., 2012; Nayeem *et al*., 2009).

The stem-bark of *F. religiosa* has been evaluated for its wound healing activity in combination with other herbs as a polyherbal formulation (Jain *et al*., 2009).

2.2.6.8 Anticonvulsant activity

Based on the Ayurvedic reports, the effect of *F. religiosa* leaves was investigated in the pentylenetetrazol (PTZ) induced convulsion model. The animals pretreated with the leaf extract 30 min prior to PTZ (60 mg/kg; i.p.) exhibited 80–100% seizures protection (Vyawahare *et al*., 2007). However a limited text is available that could substantiate the anticonvulsant potential of the leaves of *F. religiosa*. It is well recognized that the serotonergic neurotransmission in the brain modulates a wide variety of experimentally induced seizures. Generally elevation of the extracellular serotonin level increases and its depletion decreases the epileptic seizures threshold. The role of serotonergic components on epilepsy has been suggested due to indirect modulation of glutamatergic and/or GABAergic functions (Singh, 2012; Singh and Goel, 2010).

2.2.6.9 Antihyperlipidemic activity

Extensive clinical and experimental studies have shown that the dietary fiber influences the lipid level of the blood and tissues to different extent, depending on their nature and quantity. *F. religiosa* fruits, being rich in fiber were evaluated for their hypolipidemic activity in male albino rats. The study suggested the negative impact of the fruit fiber of *F. religiosa* on the absorption of lipids. Hypercholesteremia and hypertriglyceridemia are the

secondary complications of diabetes (Ananthan *et al*., 2003).

2.2.6.10 Antiasthmatic activity

The antiasthmatic potential of *F. religiosa* was first experimentally studied by Malhotra and his collegues (1960). Patel and Patel, 2000 invented a composition made from the powdered interior bark of *F. religiosa*, admixed with a rice pudding containing milk, sugar, rice and cardamom. The composition claimed to suppress all the symptoms of asthma in a human. Lee and Yang (2007) invented another health food composite that claims to alleviate asthma. Kapoor and his worker (2011), concluded that *F. religiosa* leave is effective on histamine and acetylcholine induce bronchospasm in guinea pigs. In addition *F. religiosa* can potential the number of intact cells in mast cell stabilizing model (Kapoor *et al*., 2011).

2.2.6.11 Parasympathetic modulatory effects

Several studies suggesting the modulatory effect of the bark of *F. religiosa* on parasympathetic activities have been reported in literature. In an *in vitro* study, the alcoholic bark extract decreased the tone and amplitude of contraction in isolated rabbit ileum rat ileum, guinea pig ileum and rat uterus and found the extract blocked the cardio inhibitory effects of acetylcholine in isolated guineapig's heart. The study suggested the parasympatholytic properties of the bark (Malhotra *et al*., 1960). In another study, Gupta and Gupta, 1997 investigated the cholinesterase activity of the leaves and stem of *F. religiosa*. But neither leaves, nor stem showed significant cholinesterase activity. The controversial parasympathetic effects shown by the bark can be due to the variation in dose and/or evaluating model, or due to some other reason. Therefore, more studies are required to confirm the exact reasons for its divergent cholinergic effects. The bark extract exhibited *in vitro* acetylcholinesterase inhibitory activity (previously mentioned), indicating its parasympathomimetic potential (Vinutha *et al*.,

2007).

2.2.6.12 Anti-tumour activities

Ficus spp. ancient and modern source for enthnopharmacological specifically for employment against malignant disease and inflammation. The close connection between inflammatory/infectious and concerous diseases is apparent both from the medieval/ancient merging of these concepts and the modern pharmacological recognition of the initiating and promoting importance of inflammation for cancer growth (Lansk *et al*., 2008).

Methanol extract of *F. religiosa* leaves showed cytotoxicity effect on human cancer cell lines (gastric: AGS; colon: HT-29 and breast; MDA-MB-4435s) using MTT assay (Uddin *et al*., 2011), While Gulecha and Sivakuma, 2011 showed anticancer potential of *F. religiosa* fractions in MCF7 cancer cell lines.

Choudhari and his colleges (2011) suggested that *F. religiosa* could be explored further for its anticancer potential with special reference to cervical cancer.

The investigated potential effect of acetone extract of *F. religosa* leaf in multiple apoptosis signalling in human breast cancer cells, the results showed significantly induced irreversible inhibition of breast cancer cell growth with moderate toxicity to normal breast epithelial cells. Interestingly, acetone extract accelerated cell death in a mitochondrial dependent manner in continuous live cell imaging mode indicating its possible photosensitizing effect. Intracellular generation of reactive oxygen species (ROS) by acetone extract played a critical role in mediating apoptotic cell death and photosensitizing activity. Acetone extract induced dose and time dependent inhibition of cancer cell growth which was associated with Bax translocation and mitochondria mediated apoptosis with the activation of caspase 9 dependent caspase cascade. Acetone extract also possessed strong photosensitizing effect on cancer cell line that was mediated through rapid

mitochondrial transmembrane potential loss and partial caspase activation involving generation of intracellular ROS (Haneef *et al*., 2012; Shoeb, 2006).

2.2.7 Toxicology

The long history of traditional use, with no reports of any serious side effect suggests that *F. religiosa* can be considered as safe. In majority of toxicity studies carried out on *F. religiosa*, no sign of toxicity was observed. In acute toxicity studies carried out on male Swiss albino mice, the methanolic fruit extract of *F. religiosa* was found to be safe at 10 times of its effective doses (up to 1000 mg/kg; i.p.). The extract showed no neurotoxic effect in mice at its therapeutic doses (25, 50 and 100 mg/kg; i.p.), when assessed (Singh and Goel, 2009). The aqueous bark extract of *F. religiosa* was found to be safe (up to 2000 mg/kg; p.o.) dose in acute toxicity studies carried out using OECD guidelines on Swiss female albino mice (Pandit *et al*., 2010). Deshmukh *et al*., 2007 carried out acute oral toxicity studies on the alcoholic leaf extract and found it to be safe up to (5000 mg/kg; p.o.) dose. Few reports of toxicity at higher dose of *F. religiosa* have been documented in literature. Large dose of the powdered bark or its aqueous extract has been reported to cause catharsis/allergies (Balachandran and Govindarajan, 2005).

In the brine shrimp test, chloroform fruit extract of *F. religiosa* showed toxicity with LC50 of 400 µg/ml (Mousa *et al*., 1994), whereas the aqueous and alcoholic extracts of the leaves were found to be safe (Krishnaraju *et al*., 2006). Generally a component is considered to be non-toxic when LC50 > 100 µg/ml in brine shrimp test (Moshi *et al*., 2010), since fruit extract showed toxicity at LC50 400 µg/ml, hence can be considered as safe. The overall toxicity studies carried out on *F. religiosa* accounts for its safety at the recommended therapeutic doses (Balachandran and Govindarajan, 2005).

2.3 Cancer

The progression from normal to malignant cells involves multiple steps,

during which several distinguishable properties are acquired. Pending normal development and throughout adult life, intricate genetic control systems regulate the balance between cell birth and death in response to growth signals, growth inhibiting signals, and death signals (Cavuoto and Fenech, 2012).

Most importantly, cancer cells lose the ability to control growth, and can proliferate almost inevitably. They are able to promote angiogenesis and thus guarantee an unlimited supply of nutrients and oxygen. Furthermore, the telomerase enzyme is reactivated and maintains stable telomerase during repeated cycles of cell proliferation (Akouchekian, 2008).

The cancer forming process, called oncogenesis or tumourigenesis, is the interplay between genetic and environmental factors. Most cancer arise after genes are altered by carcinogens or by errors in the copying and repair of genes, and even if the genetic damage occurs only in one somatic cell, the division of cell will transmit the damage to the daughter cells giving rise to clone of altered cells. In this regard, cancer cells no longer respond to apoptotic signals (Wooster and Bachman, 2010), and in the final of progression, they are able to detach from their original site, migrate through blood vessels or lymphatics to new destinations, and form new colonies; a process called metastasis (Bayani *et al*., 2007).

At the tissue level, cancer can be categorized on the basis of their origin. For example; sacromas are derived from soft tissues and bones, whereas leukemias originate from blood cells (Beckman and Loeb, 2005). Carcinoma, derived from epithelial tissues, is the most common type cancer. It typically starts within a benign, well differentiated tumour, which has a structure resembling that of normal tissue. The next stage is carcinoma in situ (local tumour), and after reaching the invasive stage, cancer is able to penetrate into the basal membrane, and infiltrate the underling tissue. Finally, the ability to metastasize is acquired (Vijg and Dolle, 2002).

Normal cells require mitogenic growth signals (GS) before they can move from a quiescent state into an active proliferative state. These signals are

transmitted into the cell by transmembrane receptors that bind distinctive classes of signaling molecules: diffusible growth factors, extracellular matrix components, and cell-to-cell to adhesion/interaction molecules. To knowledge, no type of normal cell can proliferate in the absence of such stimulatory signals. Many of the oncogenes in the cancer catalog act by mimicking normal growth signaling in one way or another (Fedi *et al.*, 1997).

Dependence on growth signaling is apparent when propagating normal cells in culture, which typically proliferate only when supplied with appropriate diffusible factors and a proper substratum for their integrals. Such behavior contrasts strongly with that of tumor cells, which invariably show a greatly reduced dependence on exogenous growth stimulation. The conclusion is that tumor cells generate many of their own growth signals, thereby reducing their dependence on from their normal tissue microenvironment (Maslov and Vijg, 2009).

The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell death apoptosis represents a major source of this attrition. The term apoptosis was first used by John kerr in 1972 (kerr, 2002). It is derived from the ancient Greek and means ' the falling of petals from a flower' or 'of leaves from a tree in the autumn' (Holdenrieder and stieber, 2004). The term programmed cell death is often used interchangeable with apoptosis. However, it is important to note that other forms of programmed cell death have been described and other forms of programmed cell death may yet be discovered (Debnath *et al*., 2005).

The apoptotic machinery can be broadly divided into two classes of components sensors and effectors. The sensors are responsible for monitoring the extracellular and intracellular environment for conditions of normality or abnormality that influence whether a cell should live or die. These signals regulate the second class of components, which function as effectors of apoptotic death. Intracellular sensors monitor the cell’s well being and activate the death pathway in response to detecting abnormalities, including DNA

damage, signaling imbalance provoked by oncogene action, survival factor insufficiency, or hypoxia (Evan and Littlewood, 1998). Further, the life of most in part maintained by cell matrix and cell adherence based survival signals whose abrogation elicits apoptosis (Ishizaki *et al*., 1995).

As well as, apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues, and occurs as a defense mechanism such as in immune reactions or when cells are damaged by a disease or noxious agents. Although there are a wide variety of stimuli and conditions, both physiological and pathological, that can trigger apoptosis, not all cells will necessarily die in response to the same stimulus (Rath, 2001).

In oncology, the interest in apoptosis originates from the observations that ionizing radiation or interactions of antitumour drugs with their respective intercellular targets often result in this mode of cell death, and that the efficacy of several antitumour drugs correlates with their ability to induce apoptosis. The possibility, therefore, of modulating this process can open new strategies for improving chemotherapy. Also, the ability to monitor early signs of apoptosis in samples from patient tumour may be predictive of the outcome of treatment protocols (Portugal *et al*., 2009).

2.4 Treatment of Cancer

2.4.1 Hormonal Therapy

Hormonal therapy use in clinical practice is based on a positive estrogen receptor (ER) and/ or progesterone receptor (PGR) status of the primary tumor or, if at all possible, of an easily accessible metastasis (Mueller *et al*., 2008; Glass *et al*., 2003). This type of therapy is usually the first choice when the risk of rapid disease progression is low, i.e., there is no life threatens disease (Bernard-Marty *et al*., 2004).

2.4.2 Radiotherapy

Radiotherapy is a treatment which uses radiation to destroy cancer cells (NSW Breast Cancer Institute, 2004). Radiation therapy uses high-intensity x-rays to prevent cancer cells from reproducing; in this process it also kill off healthy cells, depresses the immune system, causes very unpleasant side effects, and greatly increases the risk of developing leukemia later (Larsen, 1996). Ionizing radiations have been used in the curative and palliative treatment of patients with breast cancer since shortly after the discovery of X-rays by Roentgen in 1895 (Gu *et al*., 2005). Currently, radiation therapy has major roles in conservation management after excision of the primary tumor (Parker, 2001).

2.4.3 Immunotherapy

The status of the patient's immune system is the key physiological factor affecting the outcome of cancer immunotherapy, however, each individual's immune status is in turn affected by several factors (including age, tumor induced and surgery associated immunosuppression, and nutritional status) that need to be assessed, and some require continuous monitoring for the successful application of immunotherapeutic regimens. Immune cells play a central role in mediating the effects of immunotherapy, and specific nutritional supplements that enhance immune cell function can be effective in preparing patients for immunotherapy or vaccination (Kantoff *et al*., 2010; Wurzenberger and Cornelia, 2009).

2.4.4 Chemotherapy

Chemotherapy is the least valuable and most dangerous of all conventional treatment modes, it uses a variety of highly toxic drugs to kill the cancer cells. Perhaps the greatest problem with chemotherapy is that it seriously weakens the immune system (Larsen, 1996). Drugs used in chemotherapy have devastating side effects and patients undergoing

chemotherapy, apart from being thoroughly miserable, frequently die of pneumonia, common infections or other cancer (Walters, 1993). The most active drugs are the anthracyclines and the taxanes, followed by alkalating agents, antimetabolites, and vinca alkaloids. They produce objective response rates of 20%-80% (Bernard-Marty *et al.*, 2004; Ellis *et al.*, 2000; Hortobagyi, 1998), anti-angiogenesis drug therapy (Endostar) (Wuhan GuangCi Cancer Center, 2009). Tamoxifen (IBCSG, 2006). However, the rare complete responses are short lived, and progression of disease is almost inevitable (Cardos *et al.*, 2002; Greenberg *et al.*, 1996).

2.4.4.1 Docetaxel

Docetaxel (Taxotere, TXT), is a second generation taxane derived from the needles of the European yew tree. *In vitro and in vivo* studies revealed that docetaxel has a wide spectrum of antitumor activity. Docetaxel a semisynthetic antineoplastic agent (Frederike and Jaap, 2005; Wilson *et al*., 2001; Clarke and Rivory, 1999) has primary mechanism of action by promote microtubulin assembly and stabilize the polymers against depolymerization, thereby inhibiting microtubule dynamics (Domingo-Domenechetal, 2012). The consequences of blocking microtubule dynamics are complex: a number of vital cellular functions in which microtubules play a critical role are compromised. Impairment of mitotic progression leading to cell cycle arrest is considered to be a principal component of docetaxel's mechanism of action. This blocks progression of a cell through its natural division cycle and, consequently, inhibits cell proliferation (Poppel, 2005). Because their ability to inhibition microtubule it has a broad antitumor activity against a variety of solid tumors, including breast (Qin *et al*., 2011), non-small cell lung cancer (Nga, 2008), ovarian as well as gastric, head and neck and prostate carcinomas (Jemal *et al*., 2011; Liu, 2011; Michael *et al*., 2009; Lyseng-Williamson and Fenton, 2005).

Docetaxel has ability to inhibit angiogenesis both *in vitro* and *in vivo* (Sweeney *et al*., 2001). Inhibition of angiogenesis is a potential strategy in antitumor drug development, with a number of agents currently undergoing clinical investigation (Rosen, 2000).

However, docetaxel has been associated with a significant increase in neutropenia, febrile neutropenia, leucopenia, stomatitis, edema, fatigue and/or asthenia, and diarrhea (Qin, 2011). These effects are due in part to the high doses used to achieve the desired antitumour effect, which are necessary because of the non specific distribution of both novel and traditional chemotherapies, with only a small fraction of drugs reaching the tumour. The drugs can accumulate in healthy organs, and there is a fine line between tolerability and severe morbidity, e.g., in the case of doxorubicin, a DNA intercalator that produces cardiotoxicity (Sánchez-Moreno, 2012; Olson, 1990).

Many studies showed discoveries of activation of signaling molecules by paclitaxel and paclitaxel-initiated transcriptional activation of various genes indicate that paclitaxel initiates apoptosis through multiple mechanisms. It is involved in p53 status, bcl-2 phosphorylation and caspase-3 (Tan *et al*., 2002).

Docetaxel, (taxane derivate), promoted the formation of reactive oxygen species (ROS) in mitochondria and elicited reduction of mitochondrial membrane potential, and release of cytochrome c to cytosol, and activated caspase-9 and -3 (Taniguchi *et al*., 2005). As well as docetaxel's ability to induce signaling aberrations is likely to trigger numerous messages within tumour cells. An example of a signaling pathway that feeds into processes affected by docetaxel is the epidermal growth factor receptor (EGFR) signaling pathway. Members of the EGFR family (e.g., the human epidermal growth factor receptors HER-1 and HER-2) and their signaling pathways influence cell cycle regulation, angiogenesis, and apoptosis (Woodburn, 1999; Nagy *et al*., 1999).

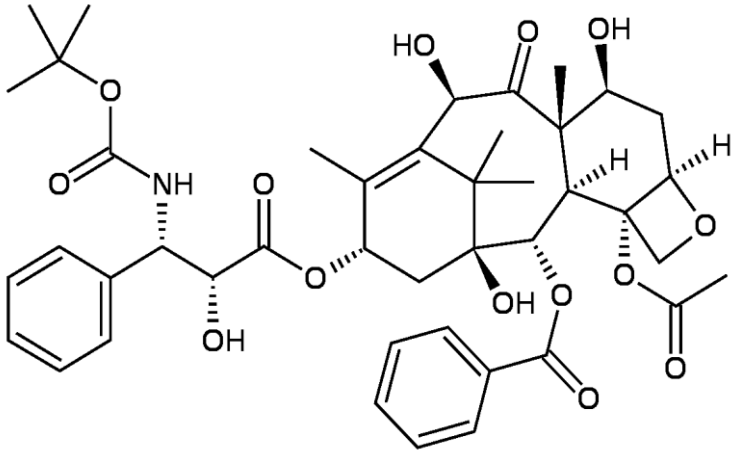


Figure 2-2: docetaxel structure (Fu *et al*., 2001).

2.4.4.2 Daunorubicin drug

Anthracyclines were isolated from a pigment producing *Streptomyces*. They have been used for more than 50 years and they can still be considered among the most useful anticancer agents ever developed: most patients treated with systemic cancer chemotherapy during the treatment (Lcurs *et al*., 2012).

At the present the most used anthracyclines clinically are: daunorubicin (DNR: daunomycin, rubidomycin), doxorubicin (DXR: adriamycin), epirubicin (EPI) and idarubicin (IDA), are among the most potent anticancer drugs in cancer chemotherapy (Xiao *et al*., 2012).

The first anthracycline antibiotics, daunorubicin and doxorubicin, have been isolated early in the 1960s and are still widely used for cancer chemotherapy (Simunek *et al*., 2005). Daunorubicin is used mainly in treatment of acute lymphoblastic or myeloblastic leukemias. Carminomycin (carubicin, 4-demethyldaunorubicin) (Bai *et al*., 2011).

Mechanism of action of anthracyclines were considered: 1) intercalation into DNA, leading to inhibited synthesis of macromolecules (DNA, RNA, proteins); 2) DNA damage via inhibition of topoisomerase II; 3) generation of free radicals, leading to DNA damage or lipid peroxidation; 4) direct membrane effect; 5) induction of apoptosis. A critical evaluation of the

proposed mechanisms has been made, according to which the mechanism of drug action at plasma concentration after drug administration is likely to be through the interaction with topoisomerase II by stabilizing a reaction intermediate, in which DNA strand are cut and covalently linked to tyrosine residues of the protein (Arozal *et al*., 2011; Xu *et al*., 2011). This action poisons the enzyme, transforming it into a DNA-damaging agent. The toxic effects of therapy with anthracyclines include nausea and vomiting, alopecia totalis and myelosuppresion (Taghisi *et al*., 2011). The most serious effect of multiple anthracycline administration is myocardial degeneration, causing congestive cardiac failure which has limited the therapeutic potential of these drugs (Egiel *et al*., 2002). However, the clinical effectiveness of anthracycline is limited by their side effects and development of multidrug resistance (MDR) (Egiel *et al*., 2002).

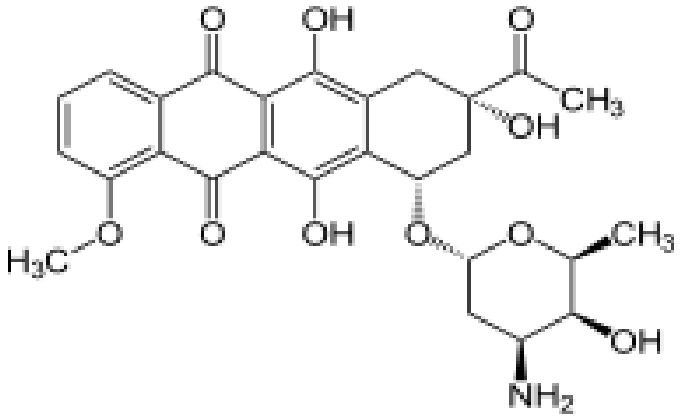


Figure 2-3: daunorubicin structure (Taghisi *et al*., 2011).

2.5 Molecular Mechanisms

Cellular signaling forms a complex network of gene interactions involving multiple signal transduction pathways. Each pathway ultimately increases or decreases the expression of its target genes resulting in alteration of cellular processes. Changes in target gene expression suggest signaling pathway activation or inhibition. However, the researcher used main pathways: *P-glycoprotein, epidermal growth factor receptor (EGFR), nuclear related factor (Nrf2), patch1, Gli2 and Inhibitor DNA* pathway *(Id1, Id2 and Id3)* genes expression which play important role in cancer cells.

2.5.1 P-glycoprotein

P-glycoprotein is located mainly in the plasma membrane where it actively extrudes drugs from the cell. It was originally discovered in 1976 in drug-resistant ovary cells from Chinese hamsters (Juliano and Ling, 1976). Humans possess one gene (originally named *MDR1* but today also denoted as *ABCB1*) encoding drug transporting *Pgp* whereas rodents have two (*mdr1a* and *mdr1b*). The combined tissue distribution of these two genes in rodents roughly coincides with that of the single *MDR1* in humans, indicating that *mdr1a* and *mdr1b* together fulfill the same function as the human *MDR1* (Schwab *et al*., 2007). The human *P-gp* consists of approximately 1280 amino acids and weighs around 170 kDa (Smyth *et al*., 1998).

Multidrug resistance (*MDR*) is a major obstacle to treating patients with cancer, *P-gp* belong to the superfamily of ATP-binding cassette transporters and actively efflux a wide range of structurally diverse amphipathic drugs used to treat cancer (Jeynes and Provias, 2013).

A role for *P-gp* (*MDR1*) as a drug flippase or phospholipid translocator has been strengthened, and protected vital cells against toxins has been postulated on the basis of *P-gp* expression in the apical membranes of gut epithelia, in the canicular membrane of liver cells, in kidney tubules, at blood tissue barriers (Zuben *et al*., 2007); and located in the placenta (Young *et al*., 2003). High *P-gp* expression also is found in hematopoietic pluripotent stem cells and specific lymphocyte lineages, including NK cells and mature single positive thymocytes (Miller *et al*., 2008). Significantly, *P-gp* is expressed in developing organs of the early fetus where the ordered process of cell differentiation and death is necessary for correct organogenesis.

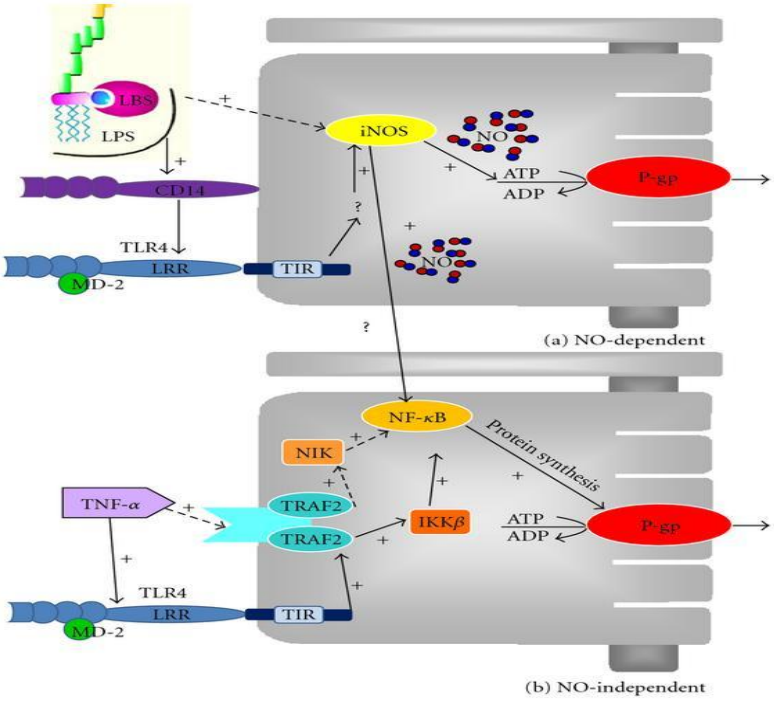


Figure 2-4: P- glycoprotein pathway (Del moral *et al*., 1998).

2.5.2 The epidermal growth factor receptor Pathway

The epidermal growth factor receptor (*EGFR*) is a transmembrane tyrosine kinase receptor that plays a central role in regulating cell division and death. The epidermal growth factor receptor is a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4). Mutations affecting EGFR expression or activity could result in cancer (Ou, 2012). The HER receptors are known to be activated by binding to different ligands, including EGF, TGFA, heparin-binding EGF-like growth factor, amphiregulin, betacellulin, and epiregulin. After a ligand binds to the extracellular domain of the receptor, the receptor forms functionally active dimers (EGFR-EGFR (homodimer) or EGFR-HER2, EGFR-HER3, EGFR-HER4 (heterodimer), EGFR dimerization stimulates its intrinsic intracellular protein-tyrosine kinase activity (Baas *et al*., 2012).

The pathways mediating downstream effects of EGFR had been well

studied and three major signaling pathways have been identified. The first pathway involves RAS-RAF-MAPK pathway. The second pathway involves PI3K/AKT pathway, which activates the major cellular survival and anti-apoptosis signals via activating nuclear transcription factors such as NFKB. The third pathway involves JAK/STAT pathway which is also implicated in activating transcription of genes associated with cell survival (D'Ambrosio *et al*., 2011; Kanae *et al*., 2005).

Mutations that lead to *EGFR* over expression (known as upregulation) or over activity have been associated with a number of cancers, including lung cancer ,prostate cancer, anal cancers and glioblastoma multi forme. In this latter case a more or less specific mutation of *EGFR*, called *EGFRvIII* is often observed. Mutations, amplifications or misregulations of *EGFR* or family members are implicated in about 30% of all epithelial cancers. Mutations involving *EGFR* could lead to its constant activation, which could result in uncontrolled cell division a predisposition for cancer. Consequently, mutations of *EGFR* have been identified in several types of cancer, and it is the target of an expanding class of anticancer therapies (Vecchione *et al*., 2011; Berasain *et al*., 2011). Natural inhibitors play acritical role in suppression of the epidermal growth factor receptor (Annette *et al*., 2011)

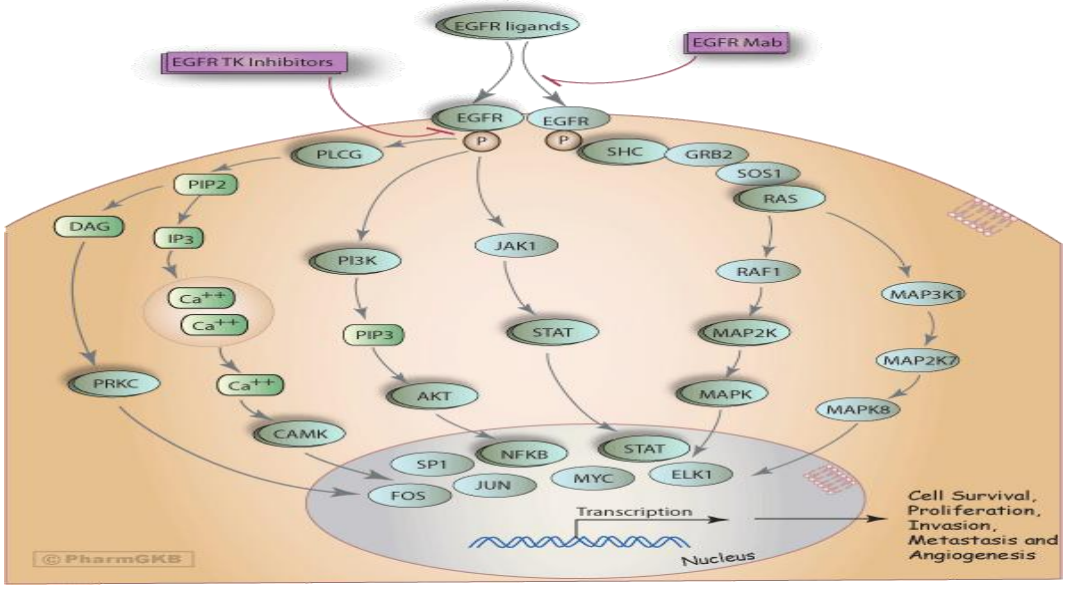


Figure 2-5: EGFR pathway (Whirl-Carrillo *et al*., 2012).

2.5.3 The nuclear related factor-2 signalling pathway

Since eukaryotic cells constantly encounter various environmental insults, they have evolved defense mechanisms to cope with toxicant and carcinogen induced oxidative stress or electrophiles. One of the most important cellular defense mechanisms against oxidative stress or electrophiles is mediated by the transcription factor called Nuclear related factor2 (*Nrf2*) (Osburn, 2008). Under the basal condition, *Nrf2-dependent transcription* (nuclear factor erythroid 2-related factor 2) is repressed by a negative regulator *Keap1* (Kelch ECH associating protein 1). When cells are exposed to oxidative stress, electrophiles, or chemopreventive agents, *Nrf2* escapes *Keap1*-mediated repression and activates antioxidant responsive element (ARE) dependent gene expression to maintain cellular redox homeostasis (Ane *et al*,. 2012; He *et al*., 2011). Beyond its antioxidant function, *Nrf2* has been recognized as a key factor regulating an array of genes that defend cells against the deleterious effects of environmental insults. Since this *Nrf2*-dependent cellular defense response is able to protect multi-organs or multi-tissues, activation of *Nrf2* has been implicated in conferring protection against many human diseases, including cancer, neurodegenerative diseases, cardiovascular diseases, acute and chronic lung injury, autoimmune diseases, and inflammation (Cui *et al*., 2013; Lee *et al*., 2013; Tsai *et al*., 2011).

Although, activation of the *Nrf2-Keap1* complex results in the induction of cellular defense mechanisms, including phase II detoxifying enzymes, phase III transporters, anti-oxidative stress proteins, and other stress defense molecules that protect normal cells from reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) and reactive metabolites of carcinogenic species. These protective mechanisms that block the initiation of carcinogenesis can be classically defined as chemoprevention, a concept that was originally introduced by (Chen *et al*., 2012).

Drug resistance is a major obstacle in the successful treatment. A role for

Nrf2 drug resistance is suggested based on its property to induce detoxifying, drug transport, and antioxidant enzyme. The loss of *keap1* function is shown to lead to nuclear accumulation of *Nrf2*, activation of metabolizing enzymes and drug resistance. Studies reported that somatic mutations also occur in the coding region of *Nrf2*. This leads to cancer cell survival and undue protection from anticancer treatment (Lee *et al*., 2013; Jaiswal, 2010).

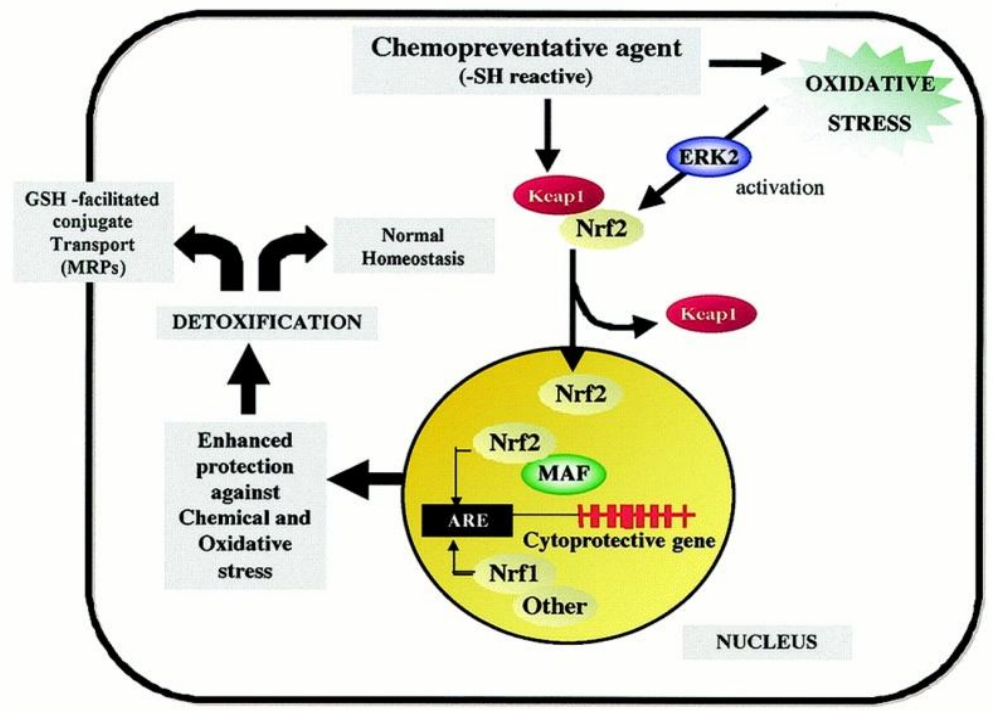


Figure 2-6: Nrf2 pathway (Academy American science, 2011).

2.5.4 Hedgehog pathway

The Hedgehog Hh signalling pathway was first identified in a large *Drosophila* screen for genes that were required for patterning of the early embryo. Subsequent studies showed that three members of this family are present in mammals. These include Sonic (*Shh*), Desert (*Dhh*) and Indian (*Ihh*), all of which encode secreted proteins. Hh ligands undergo posttranslational modifications, which is the fragment that possesses all of the signalling activity (Evangelista *et al*., 2006; Bale, 2002).

Uncontrolled activation of the (Hh) signalling pathway results in distinct cancers of the brain; muscle and skin have received significant attention. The

interest is partly because of the fact that deregulated Hh signalling only seems to cause tumours in a subset of adult cell types potentially a population of adult stem cells that might require Hh signalling for their proliferation and maintenance. Furthermore, specific inhibition of this pathway blocks tumour growth, indicating that active Hh signalling is essential for tumour survive (Laurendeau *et al*. 2010; Briscoe, 2009).

Interestingly, Hh signalling is mediated via a series of inhibitory steps. After secretion, the diffusion of all three Hh ligands is limited by binding to the *Hip1*, Patched1 (*Ptch1*) and Patched2 (*Ptch2*) transmembrane receptors, all of which are expressed on *Hh* responsive cells. Smoothened (*Smo*), and that this inhibition is relieved following ligand binding (Tukachinsky *et al*., 2010).

Three vertebrate *Gli* genes: *Gli1, Gli2* and *Gli3* have been identified. They possess context dependent, distinct repressor and activator functions. *Gli* proteins are posttranslationally modified, and cleavage of the whole proteins results in N-terminal-truncated activator and C-terminal truncated repressor fragments (Varjosalo and taipale, 2009).

In addition, loss of function mutations in negative regulators of the pathway, including *PTCH1* and *SUFU*, have been associated with tumorigenesis, indicating that inhibitors of Hh signalling act as tumour suppressors (Shahi *et al*., 2009; Shi *et al*., 2011).

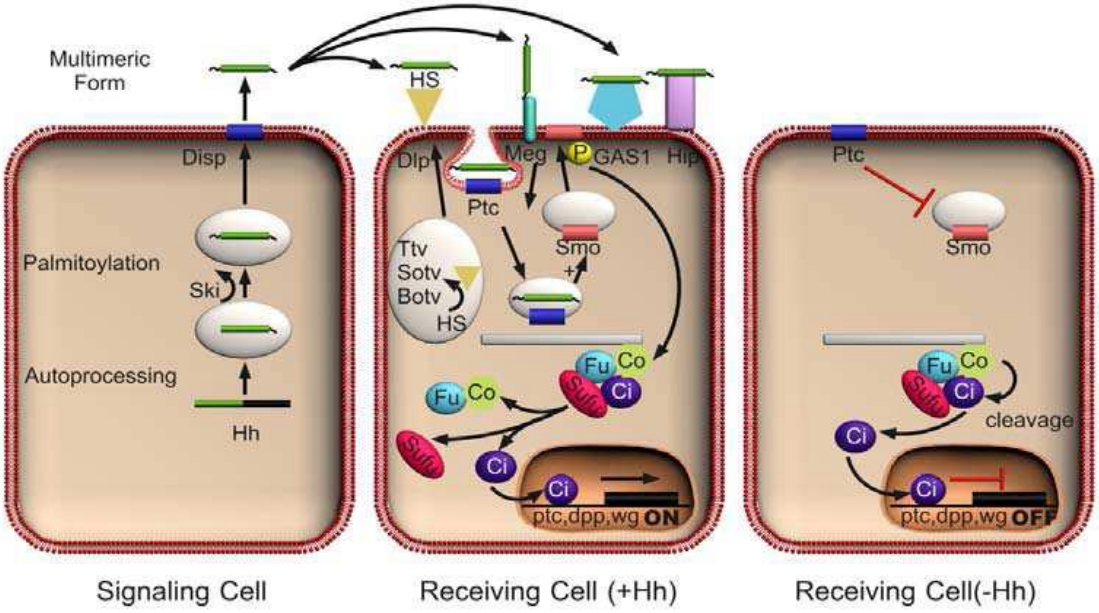


Figure 2-7: A schematic Hh signalling pathway, obtained from combined Drosophila and mammalian data (Villaamil *et al*., 2011).

2.5.5 Inhibition differentiation (ID) pathway

The helix-loop-helix (HLH) family of transcription factors comprises >200 members, which have been identified in organisms from yeast to man. Four main groups of HLH protein can be distinguished on the basis of the presence or absence of additional functional domains *bHLH* leucine zipper, *bHLH-PAS*, *bHLH* and *Id HLH* (Silvia *et al*., 2010; Desprez *et al*., 1998).

The *HLH* domain primarily mediates homo or hetero dimerisation, which is essential for DNA binding and transcriptional regulation (Min *et al*., 2009). Such *ID bHLH* heterodimers are unable to bind to DNA, and hence ID proteins act as dominant negative regulators of bHLH proteins (Norton, 2000). Since most bHLH proteins positively regulate sets of genes during cell fate determination and cell differentiation, the term '*ID*' conveniently alludes to the ability of these proteins to inhibit both DNA binding and differentiation (Wei-Hsuan *et al*., 2012).

Studies had revealed much wider biological roles for this family of regulatory proteins, which impinge on the fields of developmental biology, cell cycle research and tumour biology, as well as, act as 'global' regulators of lineage commitment; and cell fate determination (Rivera *et al*., 2000; Jaleco *et al*., 1999); Required for embryogenesis/organogenesis (Garrell Yokota *et al*., 1999; Lyden *et al*., 1999); Promote cell growth; arrest cell differentiation (Sikder *et al*., 2003); Required for cell cycle progression (Rothschild *et al*., 2006); Induce apoptosis (Kim *et al*., 1999; Norton and Atherton, 1998); Immortalise primary cells (Alani *et al*., 1999; Norton and Atherton, 1998); Function as oncoproteins *in vivo* (Kim *et al*., 1999; Morrow *et al*., 1999); Required for angiogenesis in vivo and promote (Lin *et al*., 2000), and tumour invasiveness (Yuen *et al*., 2007).

However, expression of Id proteins is reactivated in many human cancers.

For example, *Id* expression has been documented in prostate, breast, bladder, colon, and pancreatic cancer; high-grade astrocytoma; and T-cell lymphomas (Gupta *et al*., 2006; Ding *et al*., 2006; Coppe *et al*., 2004).

The *Id* expression profile mirrors that of normal haemopoietic progenitor cells. Given the known 'oncogenic' properties of *ID* proteins and the observation that loss of *Id* expression in tumour cell lines leads to suppression of cell growth, it seems likely that *ID* proteins perform a causal role in tumorigenesis mechanisms. Presumably, the tumour cell associated deregulation of *Id* gene expression arises through perturbations in upstream signalling pathways (Perk *et al*., 2005; Lasorella *et al*., 2001).

Since growth and angiogenesis of tumour cells appear to be strictly dependent on Id function and can be modulated by small changes in intracellular levels of Id protein, it has been suggested that small molecule based inhibitors of Id proteins might provide useful drugs in the treatment of human cancers (Fong *et al*., 2003).

2.6 Cancer cell lines

2.6.1 Prostate cancer cells

Prostate cancer is the most common malignancy among men. While the clinical course of prostate cancer is variable, radical prostatectomy is currently still the standard treatment for organ confined disease. During a prolonged period metastasized prostate cancer was treated with either pharmacological or surgical deprivation of androgens resulting in tumor regression. Most patients eventually experience tumor progression in spite of low androgen levels (Gu *et al*., 2010; Mostaghel *et al*., 2007).

Although docetaxel (Taxotere®) currently represents the most active chemotherapeutic agent it only gives a modest survival advantage with most patients eventually progressing because of inherent or acquired drug resistance (Scher *et al*., 2008).

A number of mechanisms have been proposed to contribute to this

resistance. Firstly, the majority of prostate tumour is slow growing even in metastatic disease and thus are unlikely to respond to drugs that are S phase dependent (Yu *et al*., 2013). Secondly, failure of chemotherapy may be caused by reduced intracellular concentrations of a drug through either increased efflux or decreased intake secondary to alterations in drug transporters, particularly P-glycoprotein (*P-gp*). Increased expression of the Pgp or increased cellular metabolism of drug has been shown to protect the cancer cells against cytotoxic drugs (Van Brussel and Mickisch, 2003). Thirdly, alterations in β-tublin isotypes with different kinetics of microtubule formation have been shown to contribute to resistance with an increase in isotypes III and IV correlating with docetaxel resistance *in vitro* (Makarovskiy *et al*., 2002). Fourthly, mutations in tumour suppressor proteins contributed to resistance (Shen and Abate-Shen, 2007). Finally, as the prostate cancer phenotype progresses expression of survival factors that inhibits the apoptotic cell death pathway (Frigerio *et al*., 2013; Feldman and Feldman, 2001).

2.6.2 Leukemic cancer

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder, which is characterized by a chromosomal translocation, resulting in constitutively activated Bcr-Abl tyrosine kinase (Kosztyu *et al*., 2013).

The chronic leukemic cancer cells line (K562) derived from a human female with chronic myeloid leukemia in blast crisis (Malgorzata *et al*., 2005), and represents a widely used in vitro model system for CML, was also shown to be refractory to apoptosis induced by DNA damaging drugs (Kosztyu *et al*., 2013). Concerning the balance between proliferation and differentiation, the lack of commitment of K562 cells to differentiation can be abrogated with a variety of compounds, both physiological and non-physiological ones, such as anticancer drugs (Malgorzata *et al*., 2005).

The anthracycline antibiotics doxorubicin (DOX) and daunorubicin (DNR) have been used for almost 40 years in treatment of solid tumors and

leukemia. Moreover, K562Dox cell line (resistance to daunurobicin anticancer drug) which is associated with presence of an acquired genetic abnormality, the philidelphia chromosome, which result from translocation between chromosome 9 and 22. Philidelphia chromosome has fusion gene whose code for chimeric protein which is responsible for deregulated tyrosine kinase activity resulting in activation of several transduction pathways in the cells, are responsible for CML phenotypes, Beside, K562Dox has been shown overexpression of P-glycoprotein (Pgp) and allow to resistance of these cells to this drug (Norgaard *et al*., 2004).

2.6.3 Breast cancer

Human breast cancer (MCF7) established in 1973 at the Michigan Cancer Foundation (Holliday and Speirs, 2011). The popularity of MCF-7 is largely due to its exquisite hormone sensitivity through expression of estrogen receptor (ER), making it an ideal model to study hormone response (Mosoyan *et al*., 2013).

3. Materials and Methods

3-1 Materials

3.1.1 Equipment and Apparatuses

The following equipment and apparatuses were used throughout the study:

NO.	**Equipment**	Manufacturers & Origin
1	Autoclave	Gallenkamp, England

2	Caliper	USA
3	Cell Counter	BioRad, Singapore
4	Centrifuge	Thermo-scientific, Germany
5	Centrifuge 5430R	Eppendrof, Germany
6	Deep freez	Kendro-Lab, USA
7	Distillation unit	Millipore, USA
8	Fume hood	Bio-optica, USA
9	Gene Amplification	Applied Biosystems, USA
10	Hood for culture cell	NUAIRE, USA
11	HPLC	Hp Hew Lett Packard, Japan
12	Ice maker	Breama, Japan
13	Image system	Bio-Rad Laboratories, USA
14	Incubator	Vell-Lab, USA
15	ISO- temperature	Fisher Scientific, USA
16	Laminar air flow	Scientific industrial, USA
17	Light microscope	Niko, Japan
18	Lyophilizer	Scientific, USA
19	Magnatic stirrer	Nuova, USA
20	Micropipettes	Eppendrof, Germany
21	Nano Vue	Applied Biosystem, USA
22	Nanopure distal water	Branstead international, USA
23	PH-meter	Crison, Spain
24	Real time-Thermocycl**e**	Applied Biosystem, USA
25	Rotary evaporator	BUCHI, USA
26	Sensitive balance	OHAUS, Switzerland
27	Sonicator	Life scientific, USA
28	Spectrophotometer	Bio-TEK, USA
29	Vortex	Scientific industries, USA
30	Water path	Sheldon manufacturing, USA

3.1.2 General Instruments

NO.	Instruments	Manufacturers & Origin
1	Plastic ware	Disposable sterile plastic tissue culture flasks with different surface areas, multi-well plates (6-well, 96-well; BD Falcon, USA), sterile polypropylene conical tubes (15ml and 50ml), polystyrene conical tubes 15ml and polystyrene round-bottom tubes 5ml (BD Falcon, USA), disposable sterile pipettes (Sterilin, UK), syringes (1, 10 and 20ml;BD, USA), syringe driven filters (0.22 and 0.45μm; Millipore, USA) and Eppendorf tubes (Eppendorf, USA).
2	Glass ware	Beakers (50, 100, 200, 500 and 1000ml), Pasture pipettes, funnels, cylinders, volumetric flasks (50, 250 and 1000ml) and bottles (75, 200 and 1000ml) were purchased from VWR, USA. All glasswares were sterilized either by autoclave (121°C, 1.5 pounds/ $inch^2$ for 30 minutes) or oven (160°C for 3 hours).

3.2 Plant Sample Collection

Ficus religiosa leaves were collected from garden in Baghdad University, Iraq, during the period April-June 2012.

They were authenticated by Professor Ali Al-Mosawy, Ph.D. in Plant

Taxonomy, Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.

The fresh leaves of *F. religiosa* were collected washed thoroughly under tap water to remove traces and dust then let's to dry by air dry. They were grained to be powder. After that, the plant powder leaves transported to the Western University of Health Science, College of Pharmacy, Center for Advancement of Drug Research and Evaluation (CADRE), California, USA at which the extraction, chemical analysis and the other Laboratory investigations were carried out.

3.3 Preparation of Plant Extract

❖ **Solutions**

i. Absolute methanol (EMD science, USA).

ii. Chloroform (EMD chemicals, USA).

iii. Butyl alcohol (EMD chemicals, USA).

❖ **Methods**

The extract was prepared according to method presented by (Taskeen *et al*., 2009) with modification as illustrated in Figure 3-1.

Fifty grams powder was soaked in (1L) 80% methanol, for one hour in sonication, then 24 hours of stirring the mixture, was then filtered using a Buchner funnel under vacuum pressure repeated two times. The filtered solution was dried using a rotary evaporator under vacuum, then dry freezing weighted and the dark brown sticky residue. Methanol extract plant powder was extracted by using chloroform, butyl alcohol and then absolute methanol. Finally, three different extracts were obtained. While crud water extract (50g)

of the ground plant was weighted and extracted with (1L) of distal water as extraction solvent. The sample was ultrasonic bath at room temperature for 60 min. Then the mixture was stirred, filtered through (whatman filter paper No. 0.4) and dry freezing to get powder dark brown extract. Four extracts were stored under sterile conditions, protected from light in a dry and cool place at -20°C until use.

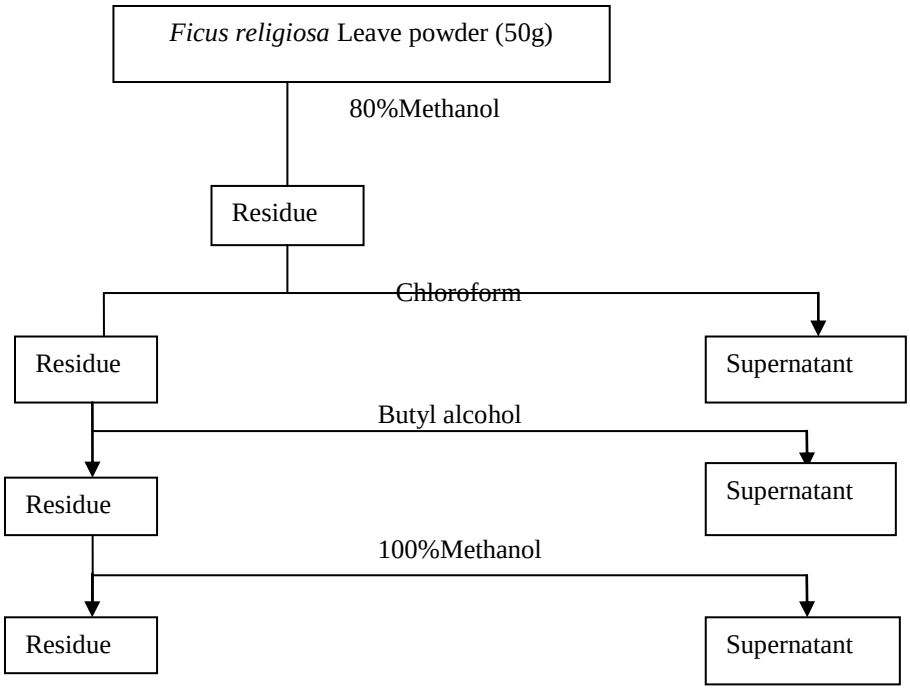


Figure 3-1: Scheme for *Ficus religiosa* extract preparation to obtain chloroform, Butyl alcohol and Absolute methanol extracts.

3.4 Laboratory Investigations

Different laboratory investigations were carried out to achieve the aims of

the present study. They are out lined in the following:

3.4.1 *In Vitro* Investigations

3.4.1.1 Cell Lines and Cell Cultures

❖ **Materials and Solutions**

The solutions were prepared according to methods given by Eeba *et al*., 2001 and Tavecchio *et al.*, 2008.

i. *Ficus religiosa* extracts preparation: They were prepared by weighted 200 mg from leaf powder and dissolved in 1 ml of DMSO (Sigma, USA) as a stock, which kept at -20°C until used.

ii. Roswell Park Memorial Institute medium (RPMI-1640) medium: Ready used solution with glutamine (Cellgro, USA), but in the establishment of cultures, it was supplemented with 10% heat-inactivated (water bath 30 minutes at 56°C) fetal bovine serum (FBS) and 1% antibiotic (100 IU/ml Penicillin and 100 IU/ml streptomycin).

iii. Dulbecco's modified eagle medium (DMEM): Ready used solution (Cellgro, USA), but in the establishment of cultures, it was supplemented with 10% FBS and 1% antibiotic (100 IU/ml penicillin and 100 IU/ml Streptomycin).

iv. Phosphate Buffer Saline (PBS): Ready used solution (Gibco by life technologies, USA).

v. Trypsin-EDTA (trypsin/Ethylene-diamine-tetra-acetic-acid): Ready used solution (Invitrogen, USA).

❖ **Cell Lines**

Human prostate cancer sensitive to docetaxel drug (PC3) and acquired resistance to docetaxel (PC3-TxR), passages range were (22-40), were kindly authorized and provided by Department of Medicine, University of Pittsburgh and Partners Healthcare, CA, USA. Human leukemic cancer sensitive sensitive

to daunorubicin (K562) and acquired resistance to daunorubicin (K562Dox) were obtained from Western University, College of Pharmacy Laboratory in Pomona, CA, USA. And sensitive human breast cancer (MCF7) cell line was purchased from ATCC (ATCC, Manassas, VA, USA). Cells were recovered from the cell bank by rapid thawing at 37°C in a water bath, then vortex for 10 seconds at room temperature, resuspended in 5 ml culture medium and transferred to tissue culture flask, which was incubated at 37°C for 24 hours, then the medium was removed and a new fresh medium was added.

3.4.1.2 Cell Culture

Four human cancer cell lines PC3, PC3TxR, K562 and K562Dox, were suspended in RPMI-1640 medium which grown and propagated in culture flasks in a humidified atmosphere supplemented with 5% CO_2 and 95% at 37°C. While MCF7 were suspended in DMEM medium and maintained under the same condition. The grown cells were detached from the surface of flasks and collected by trypsin-EDTA solution. To maintain the cell lines in an expontial growth, the culture were sub-cultured every three days. The cells were rinsed with PBS, counted and seeded at the required density (Pitchakar *et al*., 2012).

3.4.1.3 Cell growth and Cytotoxicity assays

For cell growth and cytotoxicity assays, PC3, PC3TxR, K562, K562Dox and MCF7 cancer cells were seeded in 96 well flat bottom tissue culture plates at densities 3×10^3 cell.well^{-1} for PC3, PC3-TxR and MCF7 while K562 and K562/Dox seeding at densities 10×10^3 cell.well^{-1} (in duplicate). The plates were incubated in humidified atmosphere supplemented with 5% CO_2 and 95% air at 37˚C overnight to allow attachment onto the wells, then 100 µL of different concentrations of plant extracts ranged (4

mg.ml^{-1}-0.4µg.ml^{-1}) were added (Lee *et al*., 2000), following incubation for 48 hours under the same conditions, as shown in Figure 3-2. Then cytotoxicity was assessed using Sulforhodamine-B (SRB) assay to calculate inhibitor concentration (IC_{50}).

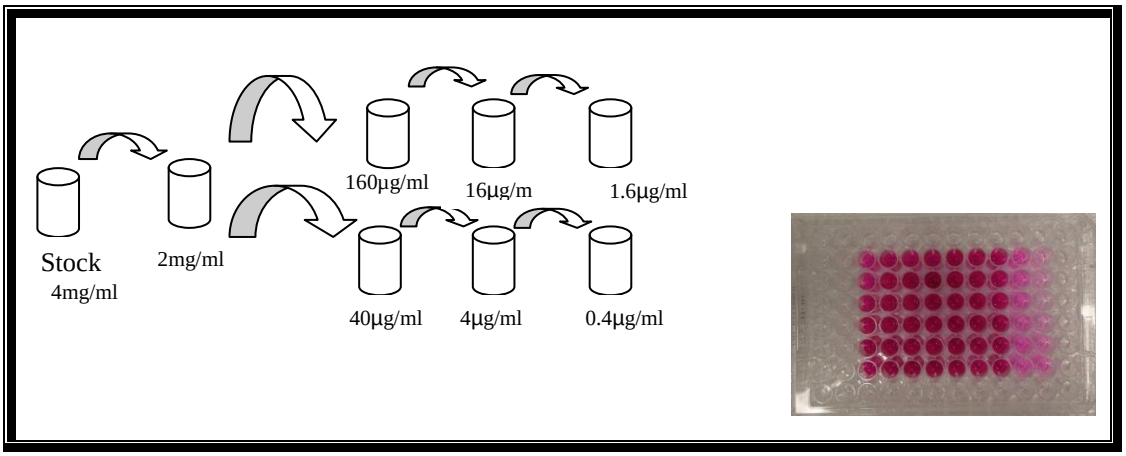


Figure 3-2: Scheme of prepare different concentrations for cytotoxic assay.

3.4.1.4 The Chemosensitizing Effect (CE)

Prostate cancer cells PC3 and PC3-TxR were seeded to a 96-well plate at cell density of 3×10^3 cell.ml^{-1} and Leukemic cancer cells K562 and K562Dox were seeded at cells density 10×10^3 cell.ml^{-1}, and incubated for 24 hours at 37˚C in an atmosphere of 5% CO_2 and 95% air. The four *F. religiosa* extracts with concentrations of 0.062 and 0.125 mg.ml^{-1}, were added to PC3 and PC3TxR cells, respectively and 0.25 mg.ml^{-1} was added to K562 and K562Dox cells. After one hour of incubation, docetaxel at serial concentrations ranged from of 0.1 to 100 nM for docetaxel or 0.04 to 10 mM for daunorubicin were added. After incubation for another 72 hours, the cell viability was determined using the SRB assay to calculate Inhibitor concentration (IC_{50}), (Kogias *et al*., 2012) of treatment groups then determinate chemosensitizing effect (CE) The chemosensitizing was defined as the IC_{50} value in the presence of *F. religiosa* extract minus the IC_{50} value in the absence of *F. religiosa* extract, as the following equation (Wang *et al*., 2012):

CE = IC_{50} (Combination *F. religiosa* +Docetaxel) – IC_{50} (Docetaxel

alone)

3.4.1.5 Sulforhodamine-B (SRB) assay

❖ **Materials and Solutions**

i. Sulforhodamine-B-Sodium (SRB dye): Weighted 0.4g of SRB (Sigma, USA) salt dissolved in 100 ml of 1% acetic acid.

ii. Trichloroacetic acid (80% TCA): Weighted 80g of TCA salt (Sigma, USA) and dissolved in 100 ml of distal water filtered with 0.22µm and kept at 4°C.

iii. Trichloroacetic acid (10% TCA): Weighted (10g) of TCA salt and dissolved in 100 ml of distal water, filtered with 0.22µm and kept at room temperature.

iv. Trishydroxymethyl-aminomethane (Tris-base): Electrophorsis purify reagent (Bio-Rad, USA) weighted 121.14g and dissolved in 100 ml distal water and adjust pH=10 which was kept at room temperature.

❖ **Method**

The Sulforhodamine-B (SRB) assay is used for cell density determination, based on the measurement of cellular protein content. The method described here has been optimized for the toxicity screening of compounds to adherent cells in a 96-well format. After an incubation period (72 hrs), cell mono-layers were fixed with 10% TCA solution for prostate cancer cell lines and breast cancer cell line while leukemic cancer cell lines were fixed with 80% TCA (cold). All cell lines were incubated for one hour at 4˚C, washed 3-4 times with tap water, and dried in the air. Cells were stained with 0.4% SRB, and washed with 1% acetic acid solution. The protein-bound dye was dissolved in 10 mM Tris solution (pH 10.0) for OD determination at wave length 565nm for prostate cancer and breast cancer cell lines and 515nm for leukemic cancer cell lines, using a microplate reader (Vichai and Kirtikara, 2006).

3.4.2 Qualitative and Quantitative Analysis of *F. religiosa* Chloroform Extract using HPLC

❖ **Materials and Solutions**

i. *Ficus religiosa* extract and Standards sample preparation:

The *F. religiosa* samples and standards compounds were dissolved at a concentration of 1 $mg.ml^{-1}$ in absolute methanol such preparations were based on a series of experiments that determined the optimal solubility, and they were considered as stock solutions.

ii. Solvent A: It was prepared by diluting 200µl of phosphoric acid (Sigma, USA) in 500ml distal water.

iii. Solvent B: It was ready used acetonitrile solution (CH_3CN, Sigma, USA).

❖ **Methods**

The forthcoming *F. religiosa* plant leaves extract was diluted 1:200 (v/v) with 80% methanol. Qualitative and quantitative analyses of *F. religiosa* extract were done by HPLC system which consisted of a 1050 series capillary with LC pump equipped with autosampler, multiple wavelength detector and diode array detector.

According to Gao and his colleges (2008), the HPLC separation was obtained with a zorbax SB-C 18 column (100×0.5 mm; 5µm particle size), using an elution mixture composed of solvent A and solvent B. The injection volume was 1-2µl and the flow rate was 10µl minute. The elution gradient of solvent B was from 20 to 100% in 30 minutes (hold at 100% for 6 minutes) at a total flow rate of 10 µl min^{-1}. The separation was performed at room temperature (20-25°C). Samples (1µl) were automatically injected into the HPLC column (triplicate), which was directly coupled to the ion source spray capillary by a liquid junction. Data processing and calculation were done by using the LTQ-Orbitrap Xcalibur 1.4 software.

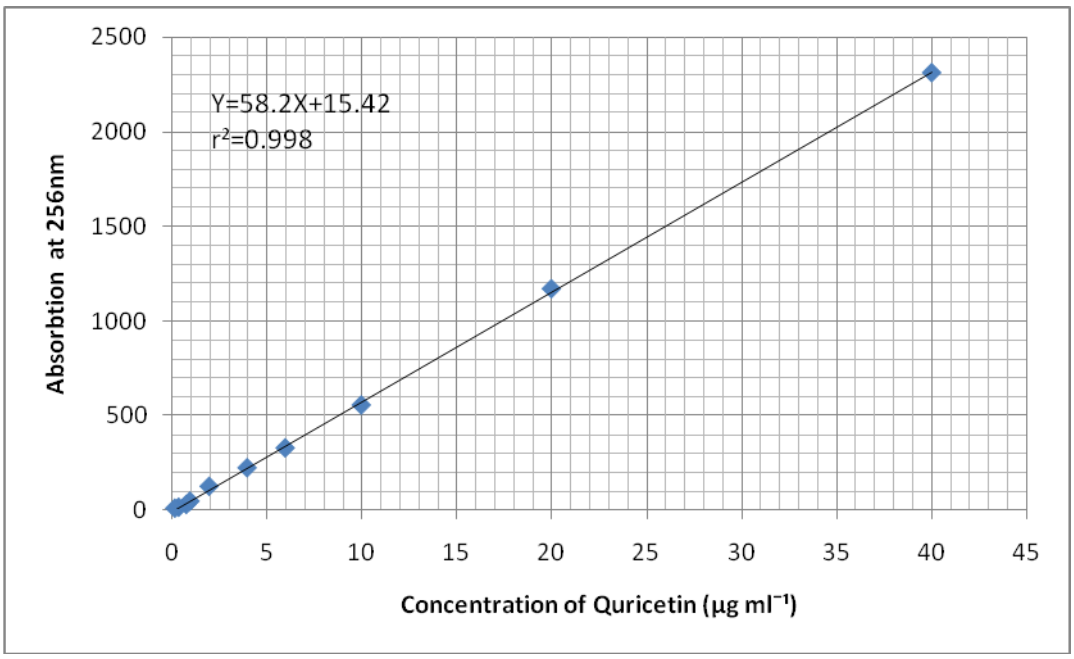


Figure 3-3: Standard curve of quricetin concentrations.

3.4.3 Determination Gene Expression

According to Murphy and his colleges (2009), this method was done:

❖ **Materials and solutions**

i. Specific primer sets as table 3-1 (AppliedBiosystem, USA).

Table 3-1: Gene's primer of (*patch1, Gli2, Id1, Id2, Id3, EGFR, Nrf2* and *P-gp*) used to detect gene expression by RT-PCR.

Genes	Forward	Reverse
GAPDH	agccacatcgctcagacac	gcccaatacgaccaaatcc
Patch1	cttcgctctggagcagattt	acccagtttaaataagagtct
Gli2	cacgctctccatgatctctg	ccccctctccttaggtgctc
ID1	ttggagctgaactcggaatc	gagacccacagagcacgta
ID2	gctatacaacatgaacgactgct	aatagtgggatgcgagtccag
ID3	catcgactacattctcgacctg	tccttttgtcgttggagatgac
EGFR	cttcttgcagcgatacagctc	atgctccaataaattcactgc

PGP	Tgaggctgtaacaagggc	cagcaaaggaggccaacat
NRF2	Cagtcttcgcccctgtctac	tgttggggaaggtgaagaag

ii. Trizol reagent: Ready used (Invetrogen, USA).

iii. Chloroform for RNA only: Ready used (Fisher scientific, USA).

iv. Isopropyl alcohol For RNA only: ready used (Fisher scientific, USA).

v. Absolute ethanol for RNA only: Ready used (Sigma, USA).

vi. Ribonuclease (Rnase) free water: Ready used (fisher scientific, USA).

vii. Master Mix PCR (RT2-SYBR-Green/Rox): Ready used (AppliedBiosystem, USA).

viii. DeoxyribonucleaseI (DNaseI) treatment Kit: (Invetrogene, USA) contain: (Taq Man RT buffer, dNTP mix, RT Random primers, Multiascribe Reverse transcriptase and DnaseI, Amp Grade)

❖ **Method**

According to Murphy (2009), PC3TxR cells were seeded into 6 well plates 5×10^5 cells.well^{-1} and incubated for 24 hours afterwards, the cells were treated by *F. religiosa* chloroform extract at two concentrations of 0.125 and 0.25 mg.ml^{-1}, docetaxel 20 nM, and the combination *F. religiosa* (0.125 mg.ml^{-1}) and docetaxel (10mM) for another 24 hours. The total RNA was extracted from monolayer cultures with 2 ml Trizol reagent according to the manufacturer's instruction.

For phase separation

1- Incubated the homogenized samples for 15 minutes at 15-30°C.

2- 0.2 ml of chloroform per 1 ml of Trizol reagent was added.

3- Cap sample tubes securely. Shacked tubes vigorously by hand for 15 seconds.

4- Tubes at 15-30°C for 2-3 minutes were incubated.

5- Centrifuged samples at 4°C at 12.000 rpm for 15 minutes.

6- Phases after centrifugation lower red phenol-chloroform interphase and colorless upper aqueous.

For RNA transcipitation

7- Transferred the aqueous phase to clean dolphin tubes.

8- Mixed with 0.5 ml of isopropyl alcohol.

9- Caped tubes securely and hand mix by inverting 15 times.

10- Incubated samples at 15-30°C for 10 minutes and centrifuge at 12000 rpm for 10 minutes at 4°C. Pellet should be visible after centrifuged.

For RNA wash

11- Slowly pour out supernatant without disturbing pellet. RNA pellet was washed once with 1 ml of 75% ethanol.

12- Centrifuged samples at 7400 rpm for 5 minutes at 4°C.

For redissolving the RNA

13- After centrifugation, slowly poured out supernatant.

14- Briefly dried the RNA pellet with caps open and tubes standing up-side-down on a piece of clean paper (5minutes) at room temperature.

15- Dissolved RNA pellet in 55°C RNase free water (10µl total for each sample) by pipette mixed until no pellet more. Allowed

the samples to site on ice for 15 minutes to sure everything dissolved.

16- RNA concentration and purity were measured by spectrophotometer at 260 and 280 nm.

To prepare RNA used Dnase I treatment kit

1- Added master mix 2µl of (8µl of 10x Dnase I reaction buffer and 1 µl of DnaseI, Amp Grade) to RNA Samples (8 µl per sample).

2- Tubes for 15 minutes at room temperature were incubated.

3- Inactivated DNaseI by adding 1 µl of 25 mM EDTA to each reaction mixture then heated for 10 minutes at 65°C. RNA sample is ready to use.

4- For real time PCR; total RNA was subjected to cDNA synthesis by mixing 1 µl of diluted RNA with 10 µL of master mix per reaction (2 µl of 10x RT buffer, 0.8µL of dNTP mix (100mM), 2µL RT Random primers, 4µl of nuclease free water and 1µL Multiscribe Reverse transcriptase).

5- The reverse transcription reaction was performed sequentially for 10min at 25˚C, for 120 min at 37˚C and 5 min at 85˚C.

Quantitive real time reverses transcription PCR (RT-PCR);

Quantitive RT-PCR assays were carried out by using ABI PRISM 7300 with SYBR-green fluorescence. Real Time PCR amplification was performed in 24µL of reaction mixture containing 10.5 µL of RNase free water, 12.50µL of RT2-SYBR Green/Rox PCR master mix and specific primer sets which are shown in Table 1.

Real time-PCR was carried out starting with a 15min hot start at 95˚C followed by a denaturation step at 94˚C for 15 sec, an

annealing step at 60˚C for 30s, and an extension step at 72˚C for 1min. Data were analyzed by using sequence detector system version 1.4 software.

3.4.4 Western blot Assay

According to Mahmood and Yang (2012), this method was done as follows:

- **Materials and solution**

i. Lysis buffer: Prepared immediately and put in ice (Santa cruzbiotechnology, USA)**:**

   a. Ripa buffer (1µl).

   b. Sodium-orthovanadate 100mM (Na_3VO_4) (10µl).

   c. Phenylmethanesulfonylfluoride (PMSF) 200mM (10µl).

   d. Protease inhibitor cocktail (10µl).

ii. BCA™ protein assay kit (Thermo Scientific, USA)**:**

   a. Reagent A (BCA™ protein) assay**.**

   b. Reagent B (BCA™ protein) assay**.**

   c. Albumin standards ampoules.

iii. Bench mark prestained protein ladder (Invitrogen, USA).

iv. Skim milk 5%: (5g) of Blotting Grade blocker non-fat dry milk (Bio-Rad Laboratory, USA) dissolved in 100 ml TBST.

v. Gel (Mini-protean TGX) long shelf life precast gels for separation of a wide range of proteins ready used (Bio-Rad Laboratories, USA).

vi. Monoclonal anti-P-glycoprotein (MDR) antibody produced in mouse which prepared 1:500 of 5% milk (Sigma, USA).

vii. Secondary antibody goat anti-mouse which prepared 1:500 of 5% milk (Sigma, USA).

viii. Luminol reagent kit: Western blot reagent contains reagent A and reagent B which prepared 1:1 (Sigma, USA).

ix. Primary antibody MsX actin (Millipore, USA).

x. Transfer buffer stock 10X (TBS): Dissolved 30.3g Tris base and 144.1g glycine (Bio-Rad Laboratories, USA), in ddH_2O 1 Liter and store at 4°C.

xi. Transfer buffer 1X (TB): 100ml of 10X TBS, 500ml ddH_2O, 200ml methanol and store at 4°C.

xii. Tank buffer 10X (Running buffer): Dissolved 30.3g Tris base, 144g Glycine, 10g SDS in 1L ddH_2O, adjust pH=8.3 and store at room temperature.

xiii. Tween20 buffer + TBS 10X: Dissolved 88g sodium chloride (Bio-Rad laboratory, USA), 12g Tris base, 5ml Tween20 (Bio-Rad laboratory, USA), in 800ml ddH_2O final volume 1L and adjusted pH=7.4 and stored at room temperature**.**

xiv. Blocking buffer: 1X TBST and 2% Bovin serum albumin or 5% milk.

xv. Stripping buffer: Dissolved 7.5g Glycine, 0.5g SDS, 5ml Tween20 in1L ddH_2O and adjusted pH=2.2 and stored at 4°C.

xvi. Laemmil buffer (Load dye) 4X: 4.4ml Tris base (pH=6.8), 4.4ml Glycerol, 2.2ml 20% SDS, 0.5ml 1% Bromophenol blue (BioRad laboratories, USA), 0.5ml β-ME and store at -20°C.

❖ **Method**

1- Prostate cancer cells (PC_3TxR) were cultured at density 5×10^5 cell.well^{-1} using 6 well plate after 24 hours of incubation, the cells treated with different concentrations of *F. religiosa* (0.25 and 0.125 mg.ml^{-1}) alone, Docetaxel (20mM) and combination *F. religiosa* (0125mg.ml^{-1}) +Dcetaxel (10mM).

2- After 24 hours incubation, Aspirated medium from wells and washed cells 2X with cold PBS.

3- Added 100µl lysis buffer to each well one at a time. Scraped cells and put in tubes (pre-chilled). Vortex tubes and put in ice for 30 minutes. Spanned at 14000 rpm for 10 minutes at 4°C. Transferred supernatant to anther tubes.

4- For measure protein concentration;

a- Standards can be made a head of a time and kept in 4°C. 200µl (50µl reagent A: 1µ reagent B) solution per well + 10 µl samples or standards or blank.

b- Incubated 37°C in the dark for 30 minutes. Read plate using BTA/BCA protocol at 562nm.

5- After calculating protein concentrations, prepared proteins for denature; 30µg for loading: 3X protein and 1X loading dye per sample. Denatured protein 95°C for 5minutes.

6- For loading samples and run gel;

a- Pipette same tank buffer in the wells before loading samples.

b- Full reservoir with tank buffer 1X all the way to the top. Full gel box with tank buffer.

c- 4µl ladder and 15µl of samples loaded for run the gel at 100 volts to black line.

7- After 1hour transfer gel; into bucket full of ice and put gel apparatus inside, prepared in transfer buffer from bottom up:

a- Fiber pad (the black side of cassette, down on transfer buffer),

b- Filter paper,

c- Gel, Membrane,

d- Filter paper ,

e- Filter pad. Then black side of cassette face black case.

f- Rolled out bubbles with roller before clamping shut to load onto apparatus transfer buffer must be cold and cut right corner of membrane to indicate that it is the front side.

8- For blocking with 5% milk for 1hour at room temperature on 30 rpm,

9- Blotted with primary antibody at least overnight at 4°C on 30 rpm.

10- Washed 3 times with 1X TBST for 15 minutes per wash, at room temperature to remove all antibodies.

11- Then blotted with secondary antibody; prepared total volume 6ml of whatever block with.

12- Washed 4-5 times with 1X TBST for 5minutes each wash, 30 rpm at room temperature.

13- Added 2ml luminal reagent by washed membrane with solution mixture for 2 minutes away from light.

14- Exposed for 1minute, luminescence, one exposure without filter then turned light dial.

15- For ladder, changed values to 0.005 second exposure one exposure with white light without filter and adjust to lower amount of light to read the image system.

16- Washed membrane with 1X TBST 4-5 times for 5minutes per wash before storing in 4°C.

a- Membrane need to be stripped before blotting with actin antibody, for 45 minutes in stripping buffer at room temperature on 30 rpm.

b- Washed 4 times in 1X TBST for 15 minutes per wash at room temperature.

c- Blocked with 5% milk in 4°C at room temperature for 1hour then with primary antibody for one hour.

d- Washed 3 times with 1X TBST for 15 minutes per wash, at room temperature to remove all antibodies.

e- Then blotted with secondary antibody; prepared total volume 6 ml.

f- Washed 4-5 times with 1X TBST for 5 minutes each wash, 30 rpm at room temperature.

g- Added 2ml luminal reagent by wash membrane with solution mixture for 2 minutes away from light.

h- Exposed for 1 minute, luminescence, one exposure without filter then turned light dial.

i- For ladder, changed values to 0.005 second exposure one exposure with white light without filter and adjusted to lower amount of light to read the image system.

3.5 *In vivo* Investigation

All animals experiments were approved by the institutional animals care used committee (IACUC) at Western University of Health Sciences. Animals use and care was in strict compliance with institutional guidelines and all experiments conformed to the relevant regulatory standards established by Western University.

3.5.1 Determination Maximum Tolerance Dose (MTD)

The study was carried out in 2 parts according to Ginsburg and his colleges (2008): determine the single dose maximum tolerated dose (sdMTD) and multiple dose MTD (mdMTD), as in Figure 3-8. The sdMTD is defined as the highest dose level with <10% mortality, while mdMTD is defined as the dose that leads to no deaths of any animals, no more than 10% or greater retardation of body weight gain as compared with control animals, and no overt organ dysfunction or side effects (assessed by the use of tissues, organs and blood collected at necropsy).

Maximum tolerance dose (MTD) studies were performed on normal CD-1 mice, 6~10 weeks old, both genders (cohorted with same gender). Animal will be monitored closely at least 3 times per day. If a sign of distress was recognized, such as loss more than 10% of body weight, dehydration, wasting of muscles on back, unresponsive, hunched posture and ataxia, animal was euthanized to prevent irrelievable severe pain or distress.

Part 1: To determine the toxicity of single-dose MTD (sdMTD), 12 mice (half male and half female) were divided into 2 groups (6 mice each with equal number of gender). According to our previous in vitro data, the chloroform

extract appeared to be more active. *F. religiosa* chloroform extract was tested at two doses are 11.3 $g.kg^{-1}$ and 7.5 $g.kg^{-1}$. All animals were monitored for a period of 4 hours immediately for acute toxicity after administration of the extract and was continued to be monitored at least 3 times daily for period of 1 week to observe for any signs of delayed toxicity. If any mouse was exhibited any adverse effects such as death or loss more than 10% body weight, it would be euthanized. Nonspecific deaths from each group was counted and the doses that resulted in 50% mortality (LD_{50}), the sdMTD was defined as the highest dose level with <10% mortality.

Animal number calculation:

Species & strain: CD-1 mice

3 mice × 2 doses × 2 genders = 12 mice

Part 2: To determine the toxicity of multiple-dose MTD (mdMTD), 6 mice were administered a dose based on the sdMTD determined from step 1, once daily for 12 consecutive days. All animals were monitored for a period of 4 hours immediately for acute toxicity after administration of the drugs and was continued to be monitored at least 3 times daily for a period of 12 days to observe for any signs of delayed toxicity. The mice were also be weighed 24 hours post treatment and every other day during the 12 days. If any mouse exhibited any adverse effects such as death or loss more than 10% body weight, the mouse will be euthanized. The mMTD is defined as the dose that leads to no death of any animals, no more than 10% or greater retardation of body weight gain as compared with control animals.

3mice × 1 dose × 2 genders = 6 mice

3.5.2 Determine the Cytotoxic Effect of *F. religiosa* on docetaxel using a Xenograft Cancer model

3.5.2.1 Laboratory animals

Severe combined immunodeficiency (SCID) is a sever immunodeficiency genetic disorder that is characterized by the complete

inability of the adaptive immune system to mount, coordinate, and sustain an appropriate immune response, usually due to absence of or a typical T and B lymphocytes.

The age of male SCID mice (6-8 weeks) and their weights range (18-25 g). They were maintained in a controlled animal house at 25°C, 60-70% humidity, 12 hours of artificial light (8:00am to 20:00pm) and under specific pathogen free (SPF) environment in cages with a filter paper cover and supplied with sterilized food and water *ad libitum*. Before carrying out experiments, the animals were kept for at least 3 days prior to dosing in the animal room at the animal house of western university to allow for their acclimatization for the laboratory conditions.

6 mice × 6 dose × 1 gender (Male) = 36 mice

3.5.2.2 Tumour Transplantation

❖ **Materials and Solution**

i. Roswell Park Memorial Institute RPMI-1640 medium free serum: Ready use, Trypsin-EDTA and PBS were prepared as in section 3.3.2.1.

ii. Matrigel: Ready used (BD Bioscience, USA).

❖ **Method**

PC3-TxR cells were seeded at density 5×10^5 cell.ml^{-1} in culture flasks and incubated for 7 days in a humidified atmosphere supplemented with 5% CO_2 and 95% air at 37°C. Sterile hood and warm up buffer, trypsin and complete medium (except serum-free medium) were prepared. Animals were shaved by using an electric razor. Pipette tips, tubes, syringes and needles were pre-chilled. If a new bottle of Matrigel were recieved, once it has liquified and refreeze unused vials in -20˚C would be a liquoted. Cells (i.e. 5 ml of trypsin-EDTA were added to each flask) were trypsinized and collected in a 50 ml conical tube. Cells at 1500 rpm for 5 minutes at room temperature were Spend down. Cells were washed twice with DPBS. Each time spend down cells at 1500 rpm for 5 minutes at room temperature. Cells in cold serum-free medium were resuspended. Cells to Matrigel should be a 1:1 ratio

(i.e. If injected 3 x 10^6 cells per injection and a total of 0.3 ml per injection, then 3 x 10^6 cells/ 0.15 ml = 2 x 10^7 cell/ml (prepared this concentration with cold serum-free medium), 0.15 ml of cell suspension (in cold serum-free medium) + 0.15 ml of matrigel = 0.3 ml total per injection).

The desired concentrations of cells were prepared in cold serum-free medium and place on ice. 750 µL of cells were aliquoted into sterile pre-chilled 5.0 ml tubes on ice. Prepared 1 tube per 4 injections with 25% excess = 1.5 ml total volume in each tube. 750 µL of Matrigel were added into each tube of cells on ice. Mixed well with pipette and placed on ice. Without vortex. Performed subcutaneous injections in mice using 23G needle in the right flank with 0.2 ml of cell suspension containing 10^7 cells.ml^{-1} of PC3-TxR (resistant cell line) in serum-free medium. After injection, syringe was holed in place for 10-20 seconds to allow Matrigel to being to polymerize. Syringe was removed slowly with rotation to seal the hole. Some leaking is acceptable (ReÁme *et al*., 2001).

3.5.2.3 Dosage and Adminstration of *F. religiosa* extract

- **Materials and Solutions**

i. Saline: 0.9% saline was used.

ii. Docetaxel drug: 20 mg.Kg^{-1} was prepared.

iii. *Ficus religiosa* extract preparation: Only chloroform extract was used, and the doses were 7.5g.$Kg^{-1}$and 3.8 g.Kg^{-1}. The FR extract was prepared immediately before use by dissolving with saline to get the required concentration.

- **Method**

A single dose of docetaxel (Doc) in saline will be administered intravenously into the tail vein at dose of 20 mg.kg^{-1}. For each group, the *F. religiosa* extract was administered by gavage daily (single dose per day; 0.3ml) for 12 days and scarified in day 13^{th}. The saline was used as control and injected at the equivalent volume (Figure 3-4). Mice were weighed twice per week (to assess the toxicity of each formulation). Tumor size was measured

twice per week for the duration of the study, including the day treatment was initiated (day 0) and the day mice were terminated from the study. The mice were euthanized when total tumor burden reached 1.5 cm^3.

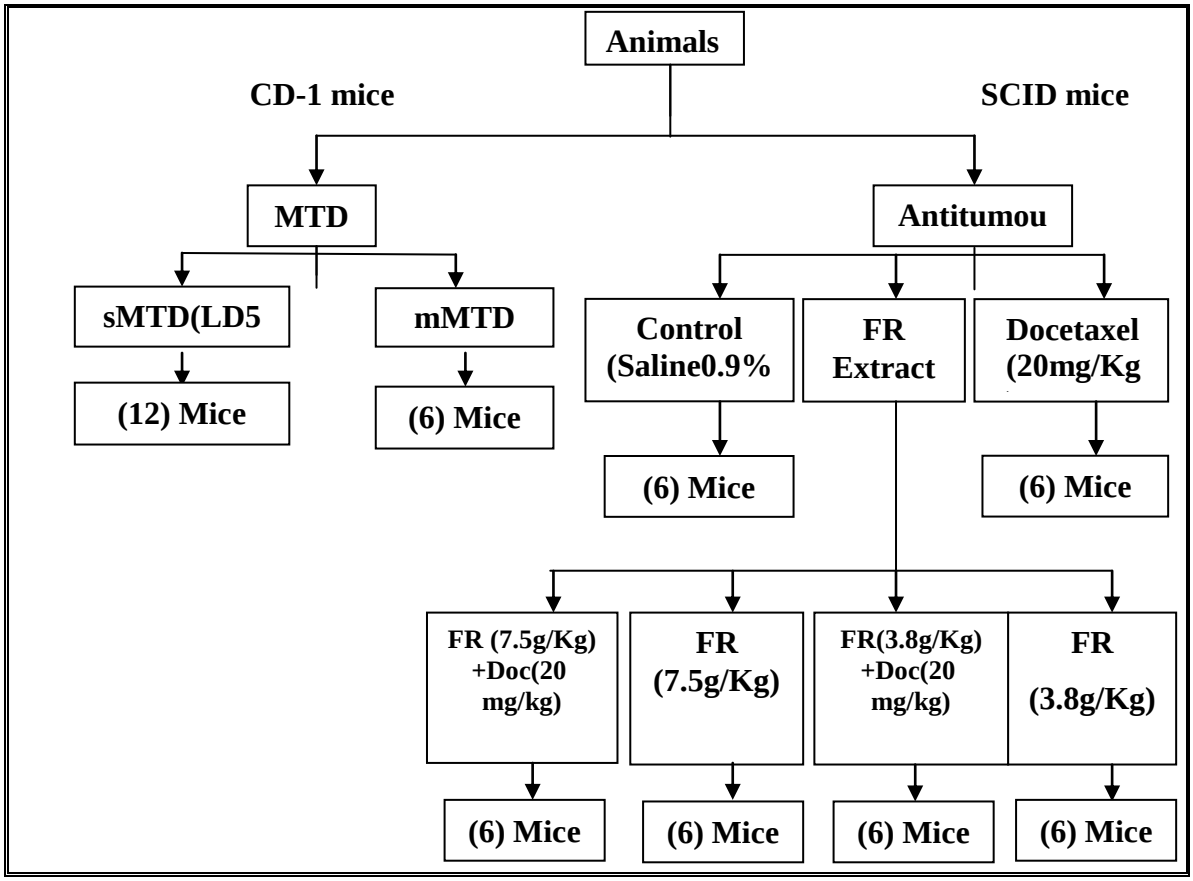


Figure 3-4: Scheme animals experiment design.

3.5.2.4 Assessment of Tumour growth

The tumour volume was assessed for 12 days, with tumor volumes being measured twice per week by using caliper measurement of two dimensions using following equation: (calculated tumor volume [(W^2 x L)/2], where W is the tumor measurement at the widest point, and L is the tumor dimension at the longest point) (Orsolic *et al*., 2006). When the tumors reach 30-40 mm^3, treatment will start, and this day will be designated day 0. On day 0, for each cell line injection.

3.5.3 Immunohistochemistry

3.5.3.1 Histopathology

❖ **Materials and solutions**

i. Buffered formalin: 10% buffered formalin was purchased from BD Pharmingen, USA.

ii. Paraffin wax: Paraffin wax with a melting point ranged 56-58°C (BD scientified, USA).

iii. Ethanol: 70%, 80%, 95% and 100% concentrations of ethanol were used (EM science, USA).

iv. Xylene: It was purchased from BD scientified, USA.

v. Haematoxylin: Mayer's haematoxylin solution was purchased from Fluka, Switzerland.

vi. Eosin: It was prepared by dissolving 5 grams of eosin Y (Merck, Germany) in 100 ml of distilled water.

❖ **Methods**

The cancer growth was removed completely and immersed in (10%) neutral buffered formalin solution. Specimens were processed by using standard histological procedures for preparation of 5 micrometers thick

sections which were collected on glass slides. Sections were stained with hematoxylin and eosin (H&E) and studied using Nikon E 400 light microscope.

3.5.3.2 Immunocytochemistry

According to Sartippour and his colleges (2008), this work was performed as follows:

❖ **Materials and Solutions**

i. Ethanol, xylene and haematoxylin were prepared as in section 3.4.3.1.

ii. Triton**:** it was purchased from Millipore, USA.

iii. Normal Horse Serum (NHS) (Horse serum donor herd sterile, non-hemolyzed delipidized, heat inactivated, Pel-Freez biological , AK code 39327-5)

iv. Mouse anti P-glycoprotein (P-gP): (Calbiochem, USA).

v. Goat anti mouse secondary antibodies (Jackson Immunosearch, West grove PA, USA).

vi. Dye-conjugated 488 tyramide solutions (Life technologies, Grand island NY)**.**

vii. Malinol (Vectashield® hard+set) mounting medium with DAPI: It was purchased from (Daido sangyo, Japan).

❖ **Methods**

Immunocytochemistry was performed on 5 micron thick paraffin sections mounted on glass slides. After deparaffinization in xylene and rehydration through downgraded series of ethanol, the sections were placed flat on a plastic grid, preblocked in 1X 0.1M phosphate buffer (PB) solution with 1 % triton, containing 5% normal horse serum (NHS) and 3% peroxide

for 20 minutes at room temperature. The sections were then incubated overnight (18 hours) at 4 ^{O}C with mouse anti P-glycoprotein (P-gp) antibody at 1:200 in 0.1M PB and 5% NHS. The sections were then rinsed 3X with PB followed by incubation in HRP conjugated goat anti mouse secondary antibodiesat 1:1000 in 0.1M PB and 5% NHS for 90 minutes at room temperature then washed 3X with PB. The sections were then incubated in fluorescent dye- conjugated 488 tyramide solution, at 1:300 and 3% peroxide at 1:500 for 20 minutes. Sections were washed 3X with PBS, mounted with Malinol (Vectashield® hard+set) and mounted medium with DAPI. Optical sections were imaged with Nikon E400. Eclipse light and fluorescent microscope was used appropriate filter to visualize immunoreactive materials on the surface of cancer cells and other tissues.

Pre-block: In 1X PBT (0.1M PB + 1 % Triton), Add 5 % NHS (Horse serum donor herd sterile, non-hemolyzed delipidized, heat inactivated, Pel-Freez biological , AK code 39327-5) and 3% Peroxide. So 20 ml PBT + 1 ml NHS + 0.6 ml Peroxide (30 %). Cover the slides and incubate for 20 minutes at room temp. 3X washes with PBS. Primary antibodies: Anti P-glycoprotein Mouse (Calbiochem) at 1:200. So 20 ml PBT + 1 ml serum + 100 µl antibody. Cover the slides and incubate at 4°C O/N. 3X washes with PBS. Secondary antibodies: HRP Goat anti Mouse at 1: 1000. 20 ml PBT + 1 ml Serum + 20 µl antibody. Incubate for 1.5 hours. 3X washes with PBS. 488 tyramide at 1: 300, 3 % peroxide at 1: 500. 20 ml PB + 40 µl 3% peroxide + 66.6 µl 488 tyramide. 3X washes with PBS. Dehydrate and cover slips.

3.6 Statistical Analysis

Data were either presented as mean± standard deviation (S.D.) for the results *in vitro* studies, while mean± standard error (S.E.) was determined for *in vivo* studies. To get such data, the individual values were tabulated in a sheet of the statistical programme GraphPad Prism version 5.01 (GrapgPad software,

Inc., La Jolla, CA, USA), and Statistical analysis system- SPSS version 14 were used to effect different actors in study parameters. LSD test and the difference between means were assessed by Duncan's test, in which $P \leq 0.05$ was considered significant.

4. Results

4.1 In vitro Investigations

4.1.1 Cytotoxic Effect of *F. religiosa* Extracts on Cancer Cell Lines

The *in vitro* cytotoxic effects were assessed for four *F. religiosa* extracts against five tumour cell lines (MCF7, PC3, PC3-TxR, K562 and K562Dox) after incubation periods (72 hours).

The results demonstrated that a treatment of cell cultures with chloroform extract and Butyl alcohol extract of *F. religiosa* plant decreased the growth of cancer cell lines significantly ($P \leq 0.05$), In contrast, there was a less cytotoxicity after a treatment with absolute methanol extract ($P \leq 0.05$), and crud aqueous extract displayed more less effect than other extracts. The inhibitory concentration (IC_{50}) was different with the investigated extracts and different type of cancer cell lines in which IC_{50} was based.

Inhibitory effects of chloroform extract on the concentration inhibition IC_{50} for (MCF7, PC3, PC3-TxR, K562 and K562Dox) cells *in vitro* were (0.38±0.028, 0.25±0.07, 0.3±0.028, 0.3±0.056 and 0.4±0.07) $mg.ml^{-1}$ respectively, after 72 hours was more effective than butyl alcohol extract

which has IC_{50} (1.025±0.02, 0.71±0.042, 0.8±0.25, 0.6±0.15 and 0.6±0.212) $mg.ml^{-1}$, and absolute methanol extract which has IC_{50} (1.2±0.353, 1.2±0.26, 0.84±0.014, 1.17±0.098 and 1±0.22) $mg.ml^{-1}$ respectively, while crud aqueous extract has IC_{50} (1.6±0.212, 1.18±0.08, 1.7±0.09, 1.62±0.02 and 1.06±0.08) $mg.ml^{-1}$ on the same cancer cell lines, respectively. However, depending on these results, chloroform extract of *F. religiosa* is high in cytotoxicity than other extracts on different cancer cell lines (Figure 4-1).

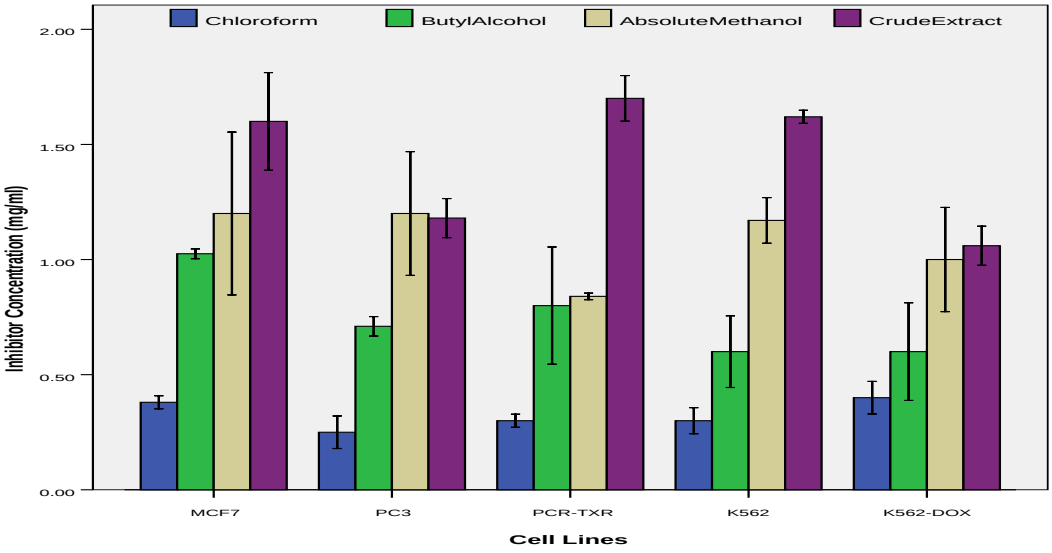
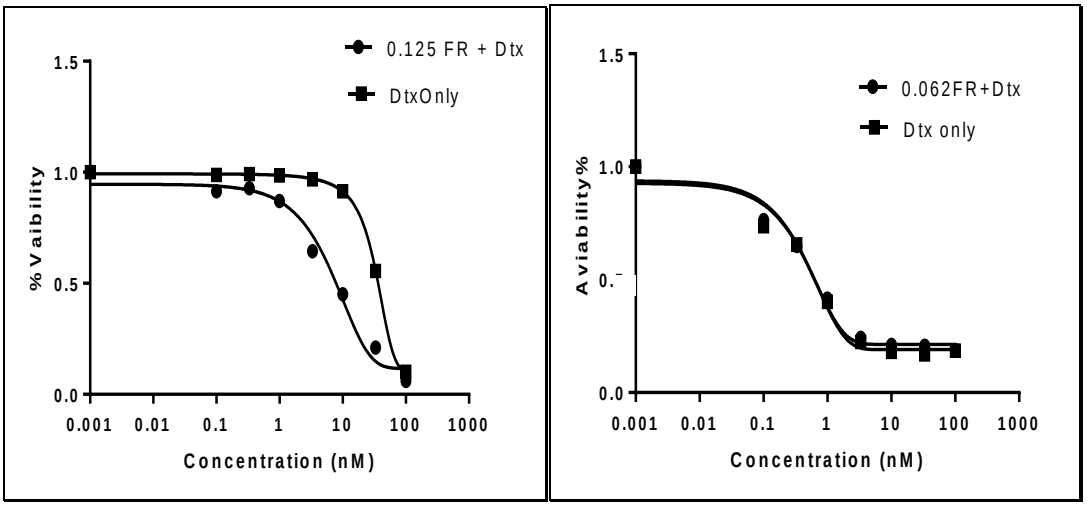


Figure 4-1: Cytotoxic effect of four *F. religiosa* extracts (Chloroform, Butyl alcohol, Absolute Methanol and crud aqueous) extracts on (MCF7, PC3, PC3-TxR, K562 and K562Dox) cancer cell lines.

4.1.2 Chemosensitizing Effect of *F. religiosa* Chloroform Extract

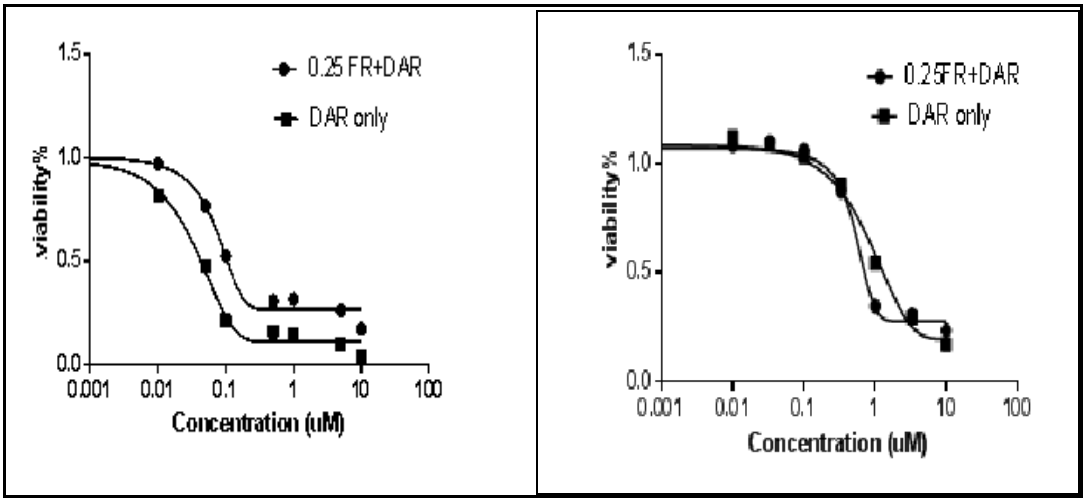
Chloroform extract at concentration ($0.125mg.ml^{-1}$) caused high significant decrease on chemoresistance (increase in chemosensitive CE) effect on PC3-TxR (-16.20±0.28) nM, ($P \leq 0.05$) than PC3 has (0.05±0.007) nM, depending on dose manner, as (figure 4-2).

In addition, the same extract at concentration ($0.25\ mg.ml^{-1}$) showed significant decrease in chemoresistance effect (increase in chemosensitive CE) on K562Dox has (-0.4±0.02) nM, ($P \leq 0.05$) than leukemic cancer cell sensitive to daunorubicin (0.61±0.01) nM, this effect was in a dose dependent manner , as shown in (Figure 4-3).



(a) **(b)**

Figure 4-2: Synergestic effects of chloroform extract of *F. religiosa* (0.125 mg.ml^{-1}) and docetaxel on viability of (a) PC3 resistance and (b) sensitive cancer cell line.



(a) (b)

Figure 4-3: Synergestic effects of chloroform extract of *F. religiosa* (0. 25 mg.ml^{-1}) and daunorubicin on viability of (a) K562 sensitive and (b) resistance cancer cell line.

4.2 Qualitative and Quantitative Analyses by HPLC of *F. religiosa* Chloroform Extract

The chemical composition of *F. religiosa* chloroform extract was identified by HPLC-DAD analysis.

Lower Limit of Quantification LLOQ, and Quality Control QC (Low, Medium and High) were detected for each standards compounds (quercetin

(QE), serotonin (SER), gallic acid (GA), tannic acid (TA) and myricetin (MYR)), and accuracy (test results obtained by the method to the true value (concentration) of the analyte, the mean value should be within 15% of the coefficient of variation except LLOQ where it should not deviate by more than 80-120%), precision (determined at each concentration level should not exceed 20% of CV except LLOQ where it should not deviate by more than 20%).

Table 4-1indicates that HPLC-DAD method is quite sensitive with the LLOQs for accuracy and precision of QE (113.9% and 2.4%), SER (11.9% and 8.0%), GA (119.9% and 18.8%), TA (83.00% and 9.4%) and MYR (83.00% and 3.10%) within the range of true value of LLOQ.

Three concentrations representing the entire range of the standard curve were studied for each standard compounds, one within low QC sample, one near the center (middle QC), and one near the upper boundary of the standard curve (high QC) which gave different values were used to evaluate the assay sensitivity, the accuracy for all the QC samples was between 82.9 to 119.9%, while the precision were less than 13.4%, as it is shown in the Table 4-1.

In the Figure 4-4, illustrates the ion chromatograms of standard compounds (QE, SER, GA, TA and MYR), depending on peak area and retention time (RT min) for each compound evident that *F. religiosa*

Table 4-1: LLOQ and QC (Low, Medium and High) of quricetin (QE), serotonin (SER), gallic acid (QA), tannic acid (TA) and myrecitin (MYR) determined by HPLC-DAD method.

Compounds	Test	LLOQ	LQC	MQC	HQC
Quercetin (QE)	Norm. Conc.	0.2	0.4	4	40
	Cal. Conc.	0.23	0.36	4.06	42.11
	Accuracy	113.9%	90.0%	101.5%	105.3%
	Prescion	2.4%	11.8%	6.0%	1.4%
Serotonin (SER)	Norm. Conc.	1	2	20	200
	Cal. Conc.	1.12	1.66	20.46	189.48
	Accuracy	111.9%	82.9%	102.3%	94.7%
	Prescion	8.0%	8.2%	8.9%	10.8%
Gallic acid (QA)	Norm. Conc.	0.25	0.5	2.5	10
	Cal. Conc.	0.3	0.56	2.34	9.84
	Accuracy	119.90%	112.90%	93.50%	98.40%
	Prescion	18.80%	2.10%	2.60%	2.90%
Tannic acid (TA)	Norm. Conc.	2	4	40	400
	Cal. Conc.	1.66	4.74	38.36	366.95
	Accuracy	83.00%	118.60%	95.90%	91.70%
	Prescion	9.40%	1.70%	14.80%	15.40%
Myrecitin (MYR)	Norm. Conc.	0.4	1	3	20
	Cal. Conc.	0.33	1.04	2.71	21.37
	Accuracy	83.00%	104.30%	90.40%	106.80%
	Prescion	3.10%	2.80%	4.50%	13.40%

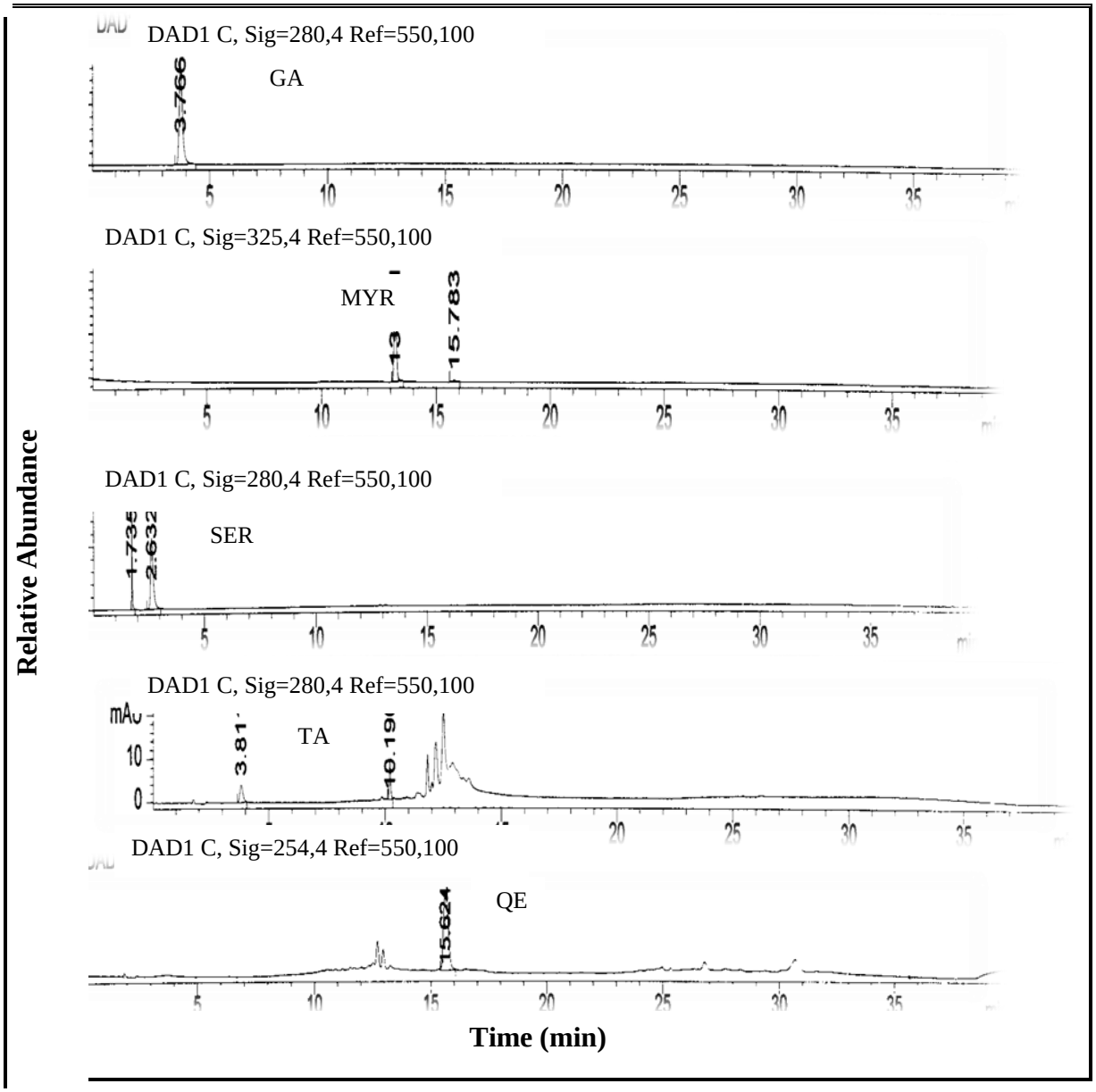


Figure 4-4: Extracted ion chromatogram of calibration standard obtained by HPLC-DAD scanning. The concentration of analytes in standard is 1 µg/ml for gallic acid (GA), Serotonin (SER), Myreitin (MYR), tannic acid (TA) and qurecetin (QE)

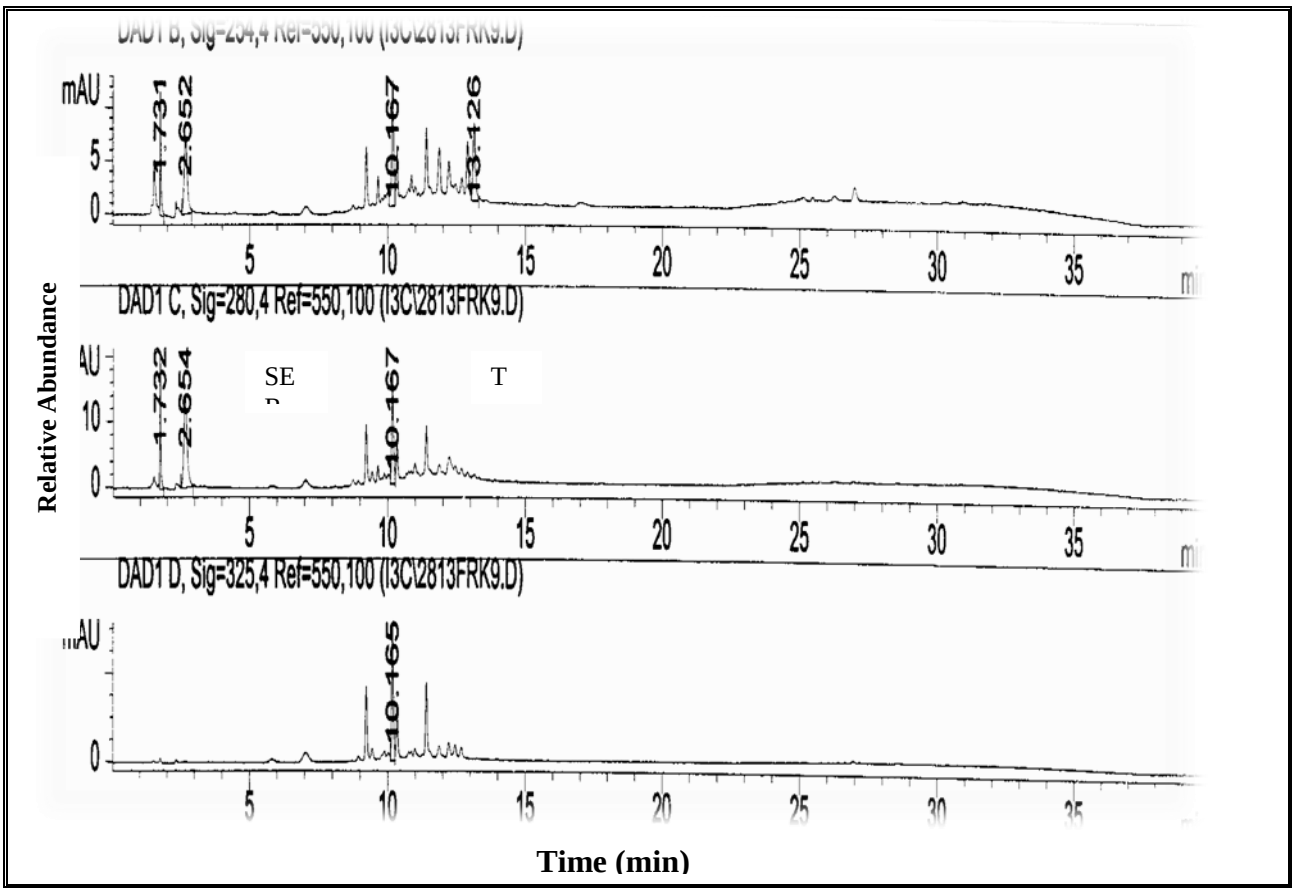


Figure 4-5: Extracted ion chromatogram of *Ficus religiosa* chloroform extract at the concentration (15 µg/ ml) obtained by HPLC-DAD scanning.

The data presented in Table 4-2 were based on information obtained from figures 4-4 and 4-5. The calibration curves for the standard compounds display good linear 0.2-40, 1-200, 0.25-10, 2-400 and 0.4-20 µg/ml for quercetin, serotonin, gallic acid, tannic acid, and myrecitin, respectively, ($r^2 \geq 0.98$) relationships under the chromatographic conditions. The content of standard compounds were detected in *F. religiosa* extract showed low concentration of serotonin (26.192±7.191) and high concentration of tannic acid (47.208±8.582), while gallic acid, quercetin and myricetin were not detected.

Table 4-2: Linearity of test compounds (quricetin, serotonin, qallic acid, tannic acid and myrcitin) and content of detect compounds in *Ficus religiosa* chloroform extract.

Test Compounds	r^2	µg/mg of raw extract (r
Qurecetin (QE)	0.998931	ND
Serotonin (SER)	0.99796	26.19235±7.19160
Qallic acid (CA)	0.999179	ND
Tannic acid (TA)	0.99797	47.2080±8.582978
Myreitin (MYR)	0.990042	ND

4.3 Effect of *F. religiosa* Chloroform Extract on Genes Expression

To evaluate the effect of chloroform extract and docetaxel drug on PC3-TxR cancer cells line, expression of eight genes had been detected using RT-PCR (P-glycoprotein (*p-gp*), Epidermal Growth Factor Receptor (*EGFR*), Nuclear related factor (Nrf_2), Patch (*Ptch1*), *Gli2* and Inhibition differentiation (*Id1*, *Id2* and *Id3*) genes which were related to apoptosis and the results were shown as follows:

The expression fold change of target gene (*P-gp*) (2^{-ddct}) was significantly down regulated when treated with *F. religiosa* chloroform extract in a dose dependent manner and when treated with *F. religiosa* extract combined with docetaxel (10 nM) ($P \leq 0.05$), In Figure 4-6, *F. religiosa* (0.125 $mg.ml^{-1}$) and *F. religiosa* (0.25 $mg.ml^{-1}$) caused down regulation of expression fold change of *ABCB* gene (0.68±0.12) and (0.388±0.08) respectively, and when combination *F. religiosa* (0.125 $mg.ml^{-1}$) with docetaxel (10 nM) significantly down regulation of the expression fold change of the same gene (0.38±0.07) was

observed but docetaxel at concentration (20 nM) alone was significantly up regulation of *ABCB* expression gene (0.96±0.087) on PC3-TxR cancer cell lines.

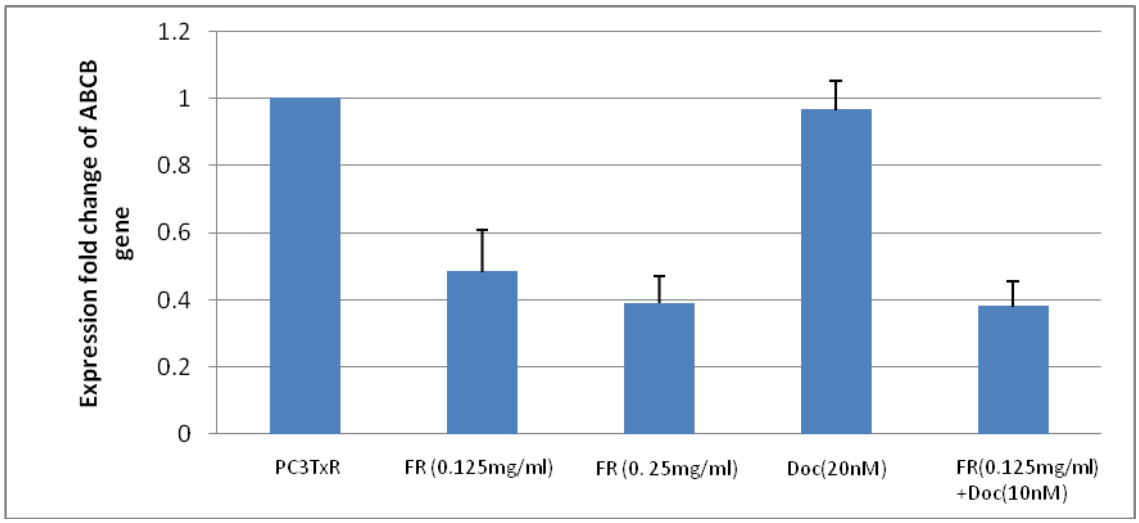


Figure 4-6: Effect of *F. religiosa* extract and docetaxel on *ABCB* gene expression in P-gp signaling pathway on PC3-TxR cancer cell lines.

Figure 4-7, showed that the expression fold change of *EGFR* gene was non-significantly affected when treated with *F. religiosa* extract (P≤0.05) at any concentration used, as well as, non-significant effect was found on the same genes when treated with docetaxel (P≤0.05) compared with control (non treated group). However chloroform extract at concentrations (0.125 $mg.ml^{-1}$) and (0.25 $mg.ml^{-1}$) have (0.79±0.13) and (0.65±0.18) respectively. When combined *F. religiosa* (0.125 $mg.ml^{-1}$) with docetaxel (10 nM) was displayed (0.71±0.19), whereas docetaxel alone showed (20 nM) (0.73±0.41).

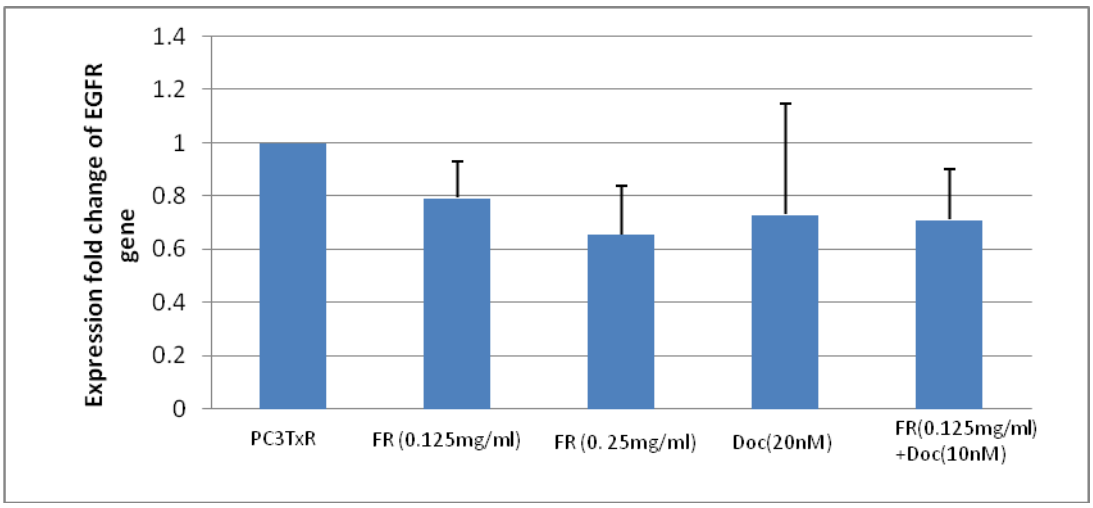


Figure 4-7: Effect of *F. religiosa* extract and docetaxel on *EGFR* gene expression on PC3-TxR cancer cell lines.

Figure 4-8 shows that expression fold change of *Nrf2* gene (2^{-ddct}) was not significantly affected when treated with *F. religiosa* extract (0.125 and 0.25 $mg.ml^{-1}$) ($P \leq 0.05$), which were (0.8±0.29 and 0.814±0.25) respectively. But PC_3TxR cells were treated with docetaxel alone (20 nM), it showed up regulation of gene expression (0.533±0.436). Finally non-significant effect was observed when combination of *F. religiosa* (0.125$mg.ml^{-1}$) with docextaxel (10 nM) (0.880±0.088) on the same gene when compared with control group.

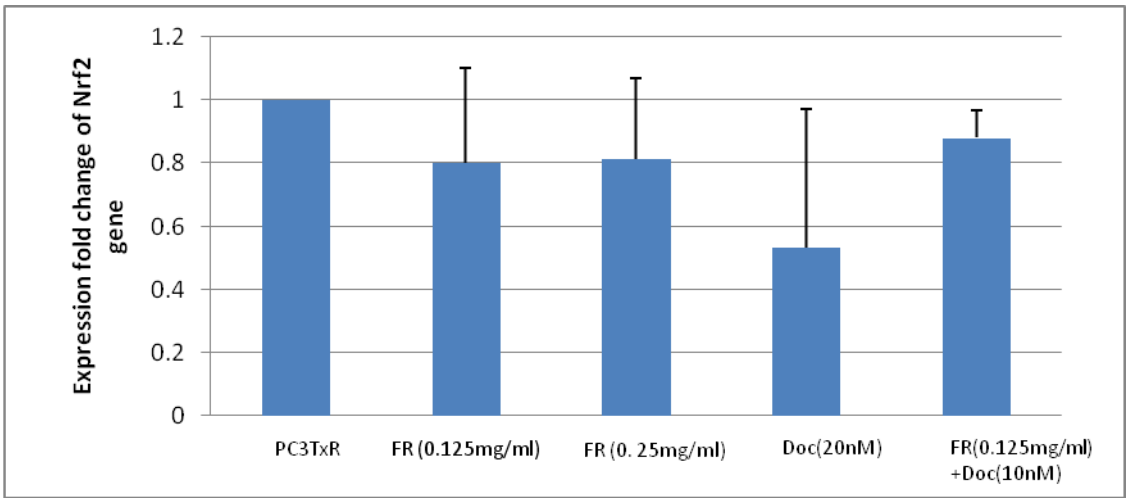


Figure4-8: Effect of *F. religiosa* chloroform extract and docetaxel on *Nrf2* gene expression on PC3-TxR cancer cell lines.

The *Patch1* and *Gli2* genes expression were not significantly affected when treated with chloroform extract and docetaxel (P≤0.05). Figure 4-9, *F. religiosa* extract at concentration (0.125 and 0.25) mg.ml^{-1} have up regulation of the expression fold change of *Gli2* gene (1.001±0.33 and 0.84±0.177) respectively, and expression fold change when treated with docetaxel alone (20 nM) has (1.373±0.36) or when recombination *F. religiosa* (0.125 mg.ml^{-1}) has (1.267±1.08) + doctaxel were up regulation expression of *Gli2* gene when compared with control.

Figure 4-10 shows that, chloroform extract induced up regulation of *Patch1* gene expression fold change (1.414±0.742) at the concentration (0.125 mg.ml^{-1}) and at the concentration (0.25 mg.ml^{-1}) was (1.673±0.10), as well as docetaxel (20 nM) displayed non- significantly affect on the *Patch1* gene (0.982±0.915) and when *F. religiosa* extract combined with docetaxel has (1.536±1.282) as compared with control.

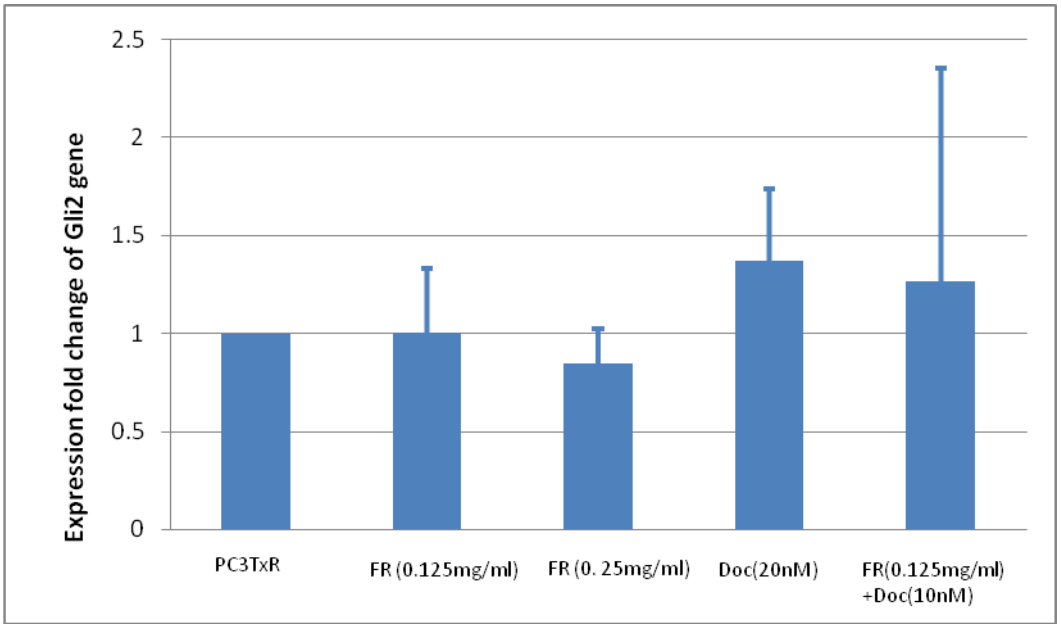


Figure 4-9: Effect of *F. religiosa* extract and docetaxel on *Gli2* genes expression in hedgehog pathway on PC3-TxR cancer cell lines.

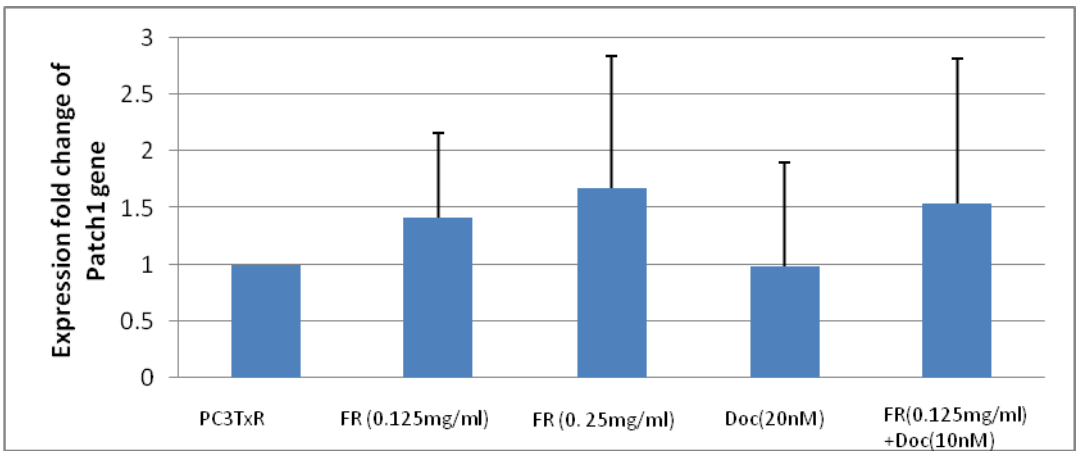


Figure 4-10: Effect of *F. religiosa* extract and docetaxel on *Patch1* genes expression in hedgehog pathway on PC3-TxR cancer cell lines.

Inhibitor DNA or Inhibitor differentiation (*Id*) genes expression have been found in many types of human cancers and its expression level has been indicated as a marker for malignant progression in a number of human cancers including the prostate cancer.

In both concentrations of chloroform extract of *F. religiosa* employed no significant affect on regulation of *Id1* gene expression fold change (2^{-ddct}) (0.695±0.12 and 0.824±0.107) respectively, were detected and the combination of *F. religiosa* (0.125 $mg.ml^{-1}$) with docetaxel (10 nM) has (0.645±0.448), or docetaxel (20 nM) alone display non significant affect gene expression (0.68±0.03), as seen in Figure 4-11.

Moreover, The results presented in figure 4-12 showed that *Id2* gene expression fold change significantly down regulation (0.763±0.182 and 0.852±0.108) when treated with *F. religiosa* extract at concentrations (0.125 and 0.25) $mg.ml^{-1}$ respectively, a dose dependent manner, and the combination between docetaxel (10 nM) with *F. religiosa* (0.125$mg.ml^{-1}$) was caused significantly down regulation *Id2* gene expression (0.67±0.13) ($P \leq 0.05$), but docetaxel (20 nM) alone showed no sense effect on PC3-TxR cells (0.93±0.23).

PC3-TxR cancer cells were treated with docetaxel (20 nM) alone caused significantly down regulation of Id3 gene expression fold change (0.632±0.12)

and secondly affect displayed significant down regulation of *Id3* gene expression when combination of *F. religiosa* (0.125 mg ml^{-1}) with docetaxel (10 nM) (0.734±0.16), while *F. religiosa* extract caused less effect on the same gene, a dose dependent manner ($P \leq 0.05$). The results are presented in Figure 4-13.

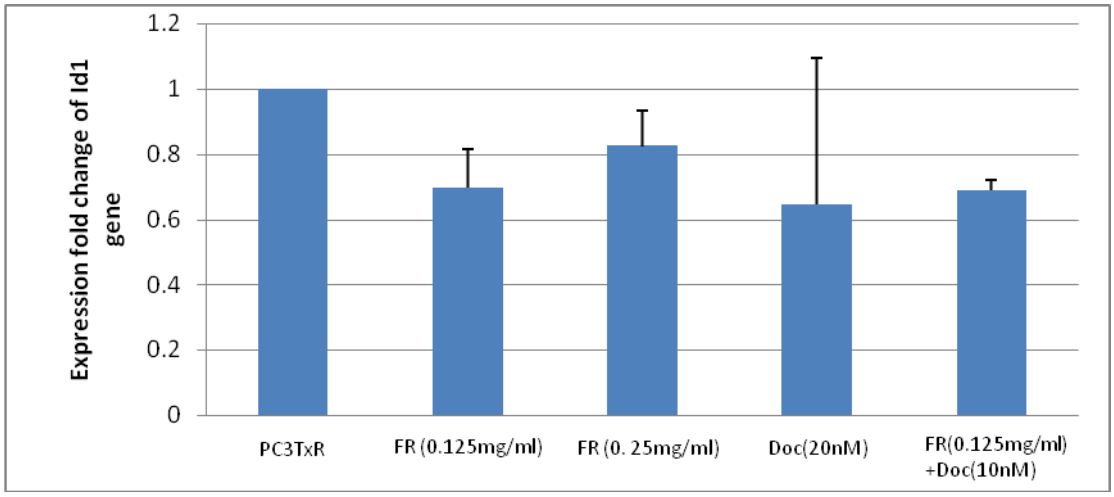


Figure 4-11: Effect of *F. religiosa* extract and docetaxel on *Id1* genes expression in Inhibition differentiation pathway on PC3-TxR cancer cell lines.

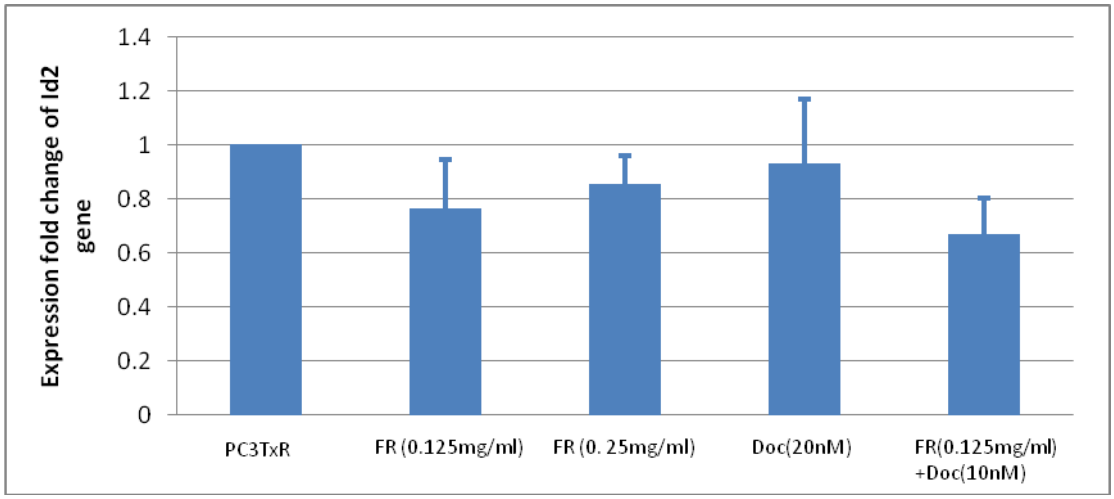


Figure 4-12: Effect of *F. religiosa* extract and docetaxel on *Id2* gene expression in Inhibition differentiation pathway on PC3-TxR cancer cell lines.

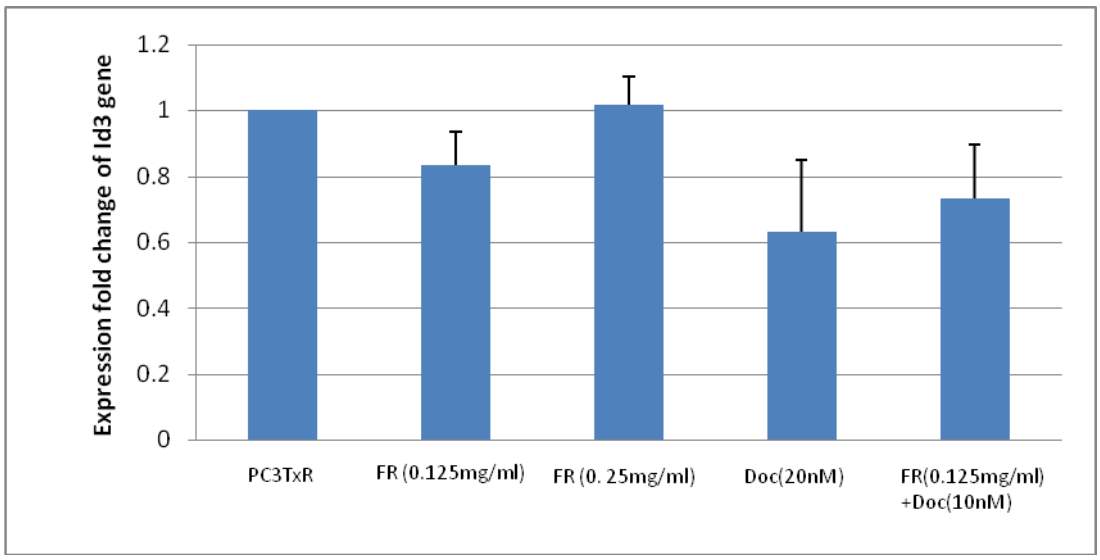


Figure 4-13: Effect of *F. religiosa* extract and docetaxel on *Id3* gene expression in Inhibition differentiation pathway on PC3-TxR cancer cell lines.

4. 4 Western blot Assay

According to the obtained results in section 4.3.1, chloroform extract caused down-regulation of *ABCB* gene. Furthermore, an investigation was performed to prove the inhibition of P-gp protein by *F. religiosa* chloroform extract as represent in Figure 4-14. It showed that *F. religiosa* extract at different concentrations employed inhibition of P-gp protein synthesis, in dose dependant manner and when using combination of *F. religiosa* (0.125 $mg.ml^{-1}$) with docetaxel (10 nM) caused a decrease in protein synthesis, but P-gp protein inhibition was not observed in PC3-TxR cells when treated with docetaxel (20 nM) in comparison with control and β-Lactin protein.

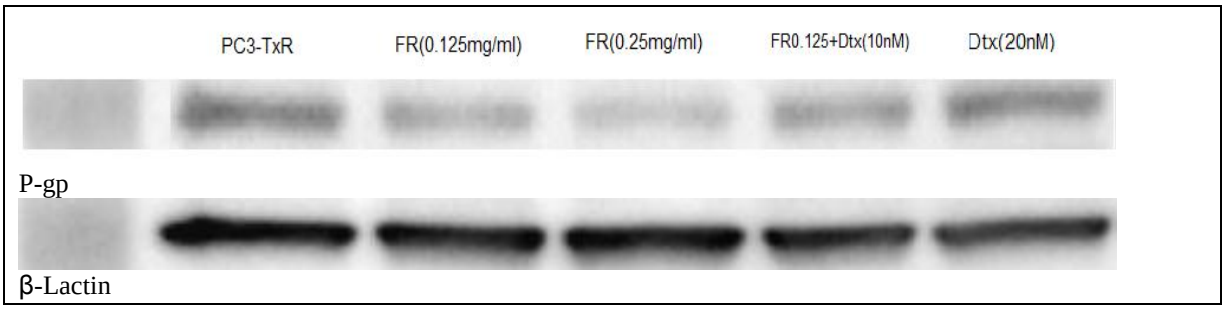


Figure 4-14: Westren blot showed the effect of *F. religiosa* extract (0.125 and 0.25) $mg.ml^{-1}$, docetaxel (20 nM) alone and combination *F. religiosa* (0.125 $mg.ml^{-1}$) + docetaxel (10 nM) on inhibition P-gp protein synthesis in PC3-TxR cancer cell lines.

4.5 *In Vivo* Investigations

4.5.1 Maximum Tolerance Dose (MTD)

To assess a safely dose of *F. religiosa* chloroform extract that used as anti-tumour potential LD_{50} and the maximum tolerance dose for the extract in CD1- mice should be determined

4.5.1.1 Single Maximum Tolerance Dose (sMTD)

Six mice (3males+ 3females) were gavaged (11.3g.Kg^{-1}). Depending on observation, which half of animals died after 24 hour. It was detected as LD_{50}. While other six mice were gavaged (7.5g.Kg^{-1}) did not show any symptoms.

4.5.1.2 multiple Maximum Tolerance Dose (mMTD)

Six mice (3males+ 3females) which were gavaged with chloroform extract (7.5g.Kg^{-1}) for 12 day. Depending on observation, no symptoms were displayed as well as no significant decrease in their weights (>10%), as shown in Figure 4-15.

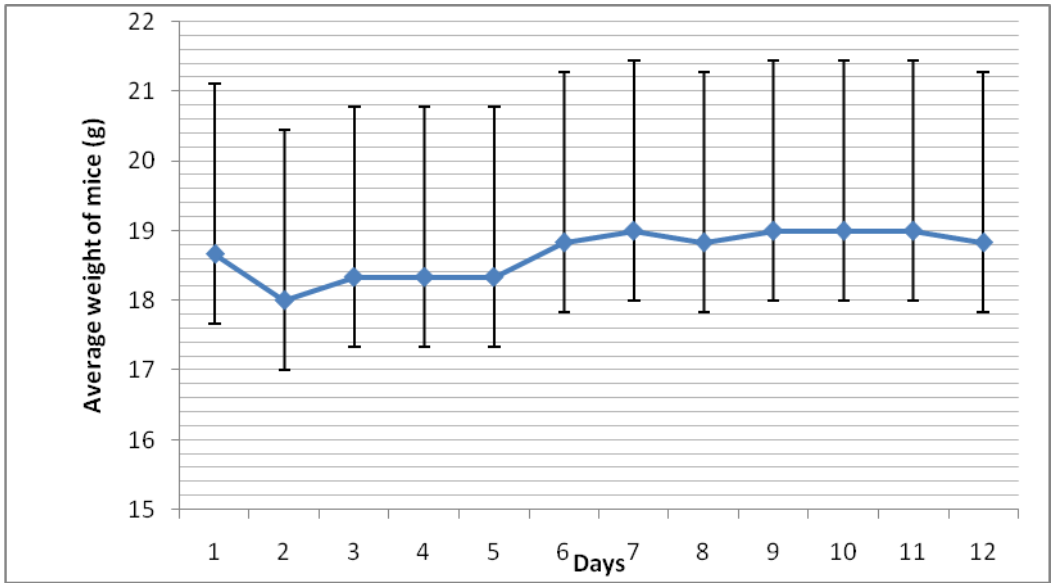


Figure 4-15: Multiple Maximum Tolerance Dose (mMTD), weights of CD-1 mice when gavage with 7.5 g.kg^{-1} *F. religiosa* chloroform extract for 12 day.

4.5.2 Assessment of Anti-tumour Effect

To evaluate the anti-tumour potential of chloroform extract, thirty six SCID mice were xenografted with PC3-TxR tumour cells. When the tumours

reached more than 100 mm^3 on day 14, the animals were divided into six groups as mentioned in section 3.5.2.3. In treated animals, the tumour size was measured on days 15, 19, 23 and 27. However, mouse-bearing tumours are given in Figure 4-16.

In the negative control (saline 0.9%) a tumour size 188.67 mm^3 was noticed on day zero of treatment (i.e. day 15 post-tumour cell implantation), and then the size of tumour increased gradually during the investigated period and reached 310.55, 515.06 and 563.21 mm^3 on days 4, 8 and 12, respectively (i.e. days 19, 23 and 27 post-tumour cell implantation). Such differences were significant ($P \leq 0.05$), with the exception of days 8 and 12, in which no significant difference between tumour size was observed. Furthermore, positive control group (docetaxel 20 $mg.Kg^{-1}$) significantly increased tumour growth 267.62, 537.86mm^3 and 754.46 mm^3, respectively, and significance difference on day 12 as compared with control (saline 0.9%). Treating tumour-bearing mice with 3.8$g.kg^{-1}$ of *F. religiosa* extract was associated with a decreased size of the xenografted tumours, and the corresponding sizes were 263.45, 336.2 and 435.91 mm^3, respectively, but a significant difference was reached on day 12 (422.27 *vs*. 563.21 mm^3) (Table 4-3).

When treated xenograft mice with 3.8$g.Kg^{-1}$ of *F. religiosa* extract daily gavaged for 12 day in combination with docetaxel (20 $mg.Kg^{-1}$) which intravenous once weekly, Chemosensitizing result was noticed significantly decreased in tumour growth 357.66, 493.93 and 517.0 mm^3,with exception day 8 and 12 which showed no significant difference when compared with negative control. This reduction in tumour growth was significant when compared with negative and positive control at day12. When treating mice bearing tumour with 7.5$g.Kg^{-1}$, no significant difference on tumour size were shown 175.35, 193.21 and 272.97 mm^3, respectively. But there were further reductions of tumour size as compared to the corresponding size in mice treated with saline 0.9% or docetaxel 20 $mg.Kg^{-1}$. In addition, chemosensitizing result of *F. religiosa* chloroform extract at the dose 7.5$g.kg^{-1}$ with docetaxel (20 $mg.Kg^{-1}$)

was used. No significant reductions of tumour size were observed as showed in 129.6, 198.85 and 233.56 mm^3, respectively. Such reductions were significant as compared to the corresponding sizes in negative and positive control, as Figure 4-17.

To evaluate the deleterious effects of tumour implantation and *F. religiosa* extract treatments on mice throughout the investigated period, the body weight was considered a parameter in this regard. Untreated group (Saline 0.9%) has further significant reduction in weight 27.43, 21.33 g and 18.76 g at days 5, 9 and 12 (post- tumour cells implantation), although the animals treated with docetaxel (20 $mg.Kg^{-1}$), they showed a significant weight reduction more than (10%), 24.93, 21.53 and 18 g, respectively (Table 4-3). The animals treated with low dose of *F. religiosa* extract (3.8$g.Kg^{-1}$) displayed a significant reduction in weights which were 27.93, 22.46 and 18.66 g (more than 10%), and when investigated chemosensitizing result of this dose with docetaxel, they displayed a significant reduction in the body weight was 26.63, 20.1 and 19.1 g respectively, but no significant reduction appeared in the body weight when the animals treated with high dose of *F. religiosa* extract (7.5$g.Kg^{-1}$) was 27.16, 26.16 and 23.66 g, respectively, and when gavaged the animals 7.5 $g.Kg^{-1}$ of *F. religiosa* extract for 12 days with intravenous docetaxel 20 $mg.Kg^{-1}$ once weekly showed no significant reduction in weights were appeared 26.76, 26.9 and 23.66 g, respectively, but there are significant variation in the body weight when compared with positive and negative control at day 12, as shown in Figure 4-18.

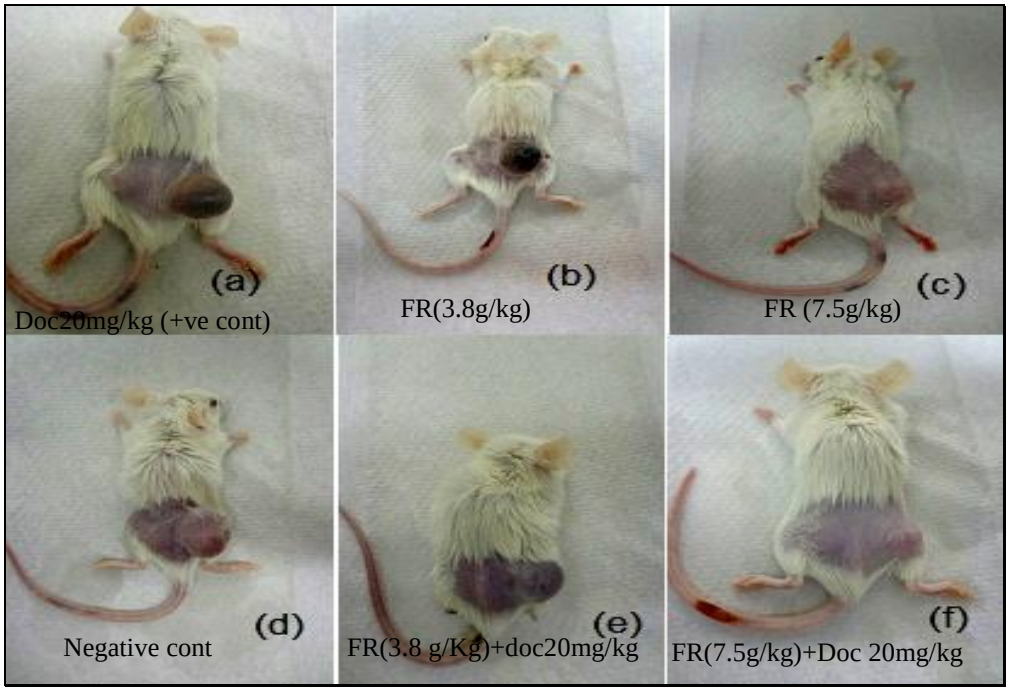


Figure 4-16: Day 12 of SCID nude mice xenografted with PC3-TxR cells showing tumour mass treated with (a) Docetaxel 20 mg.Kg^{-1} (b) *F. religiosa* 3.8 g.Kg^{-1} (c) *F. religiosa* 7.5 mg.Kg^{-1} (d) Saline 0.9% (e) *F. religiosa* 3.8 g.Kg^{-1}+ Docetaxel (20 mg.Kg^{-1}) (f) *F. religiosa* 7.5 g.Kg^{-1}+ Docetaxel (20mg.Kg^{-1}).

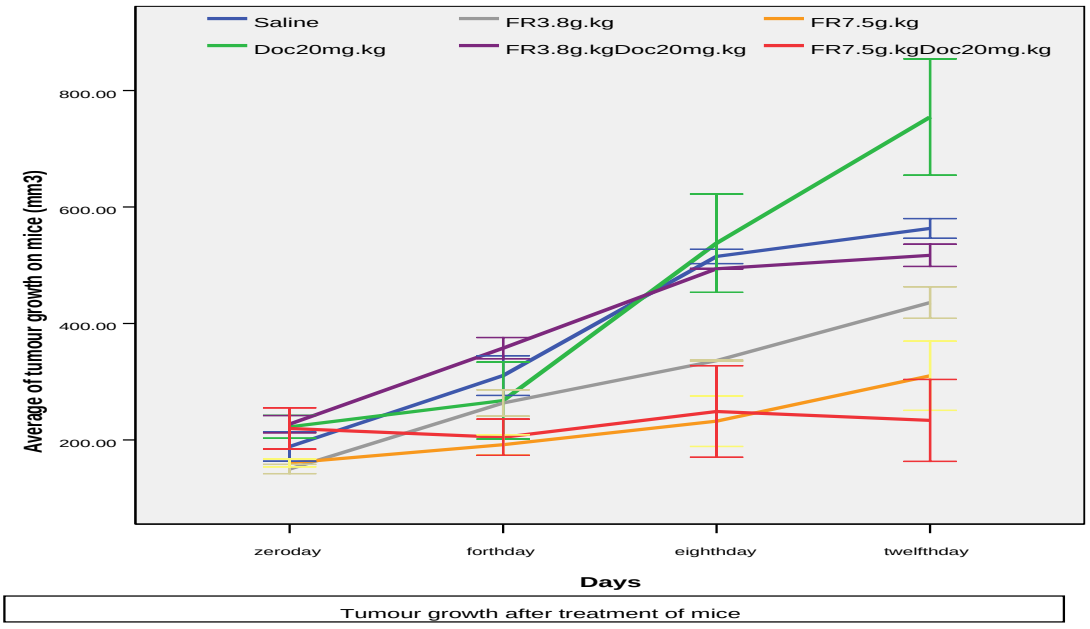


Figure 4-17: Tumour inhibition in tumour-bearing nude mice treated with *F. religiosa* chloroform extract and docetaxel.

Table 4-3: Tumour size and mouse weight in tumour-bearing nude mice treated with *F. religiosa* chloroform extract and docetaxel drug.

Groups Dose		Dose	Day *	Tumour size Mean±S.E. (mm^3)**	Mouse Weight Mean±S.E. (gram)**
Negative Control (Saline)		0.9%	0	$188.67 \pm 25.19^{C\ ab}$	$26.76 \pm 0.3^{A\ a}$
			4	$310.55 \pm 34.06^{B\ a}$	$27.43 \pm 0.7^{A\ a}$
			8	$515.06 \pm 12.23^{A\ a}$	$21.33 \pm 1.26^{BA\ b}$
			12	$563.21 \pm 17.00^{A\ b}$	$8.76 \pm 0.39^{B\ c}$
Positive Control (Docetaxel)		20 $mg.Kg^{-1}$	0	$222.6 \pm 19.58^{B\ ab}$	$26.5 \pm 0.58^{A\ a}$
			4	$267.62 \pm 66.05^{B\ ac}$	$24.93 \pm 1.72^{A\ a}$
			8	$537.86 \pm 84.43^{A\ a}$	$21.53 \pm 2.66^{AB\ b}$
			12	$754.47 \pm 99.89^{A\ a}$	$18.00 \pm 0.75^{B\ b}$
FR Chloroform Extract Treated Mice	*F. religiosa* Low Dose	3.8 $g.Kg^{-1}$	0	$150.15 \pm 7.9^{D\ b}$	$27.36 \pm 0.03^{A\ a}$
			4	$263.45 \pm 22.59^{C\ ac}$	$27.93 \pm 0.29^{A\ a}$
			8	$336.2 \pm 0.93^{B\ ab}$	$22.46 \pm 0.03^{B\ b}$
			12	$453.91 \pm 27.08^{A\ bc}$	$18.66 \pm 0.66^{B\ c}$
	F. religiosa Low dose+ Doc	3.8 $g.Kg^{-1}$+ 20 $mg.Kg^{-1}$	0	$227.2 \pm 14.75^{C\ ab}$	$26.56 \pm 0.23^{A\ a}$
			4	$357.66 \pm 18.21^{B\ a}$	$26.63 \pm 0.53^{A\ a}$
			8	$493.93 \pm 0.69^{A\ b}$	$20.1 \pm 1.3^{A\ b}$
			12	$517.0 \pm 19.05^{A\ b}$	$19.1 \pm 0.1^{AB\ b}$
	F. religiosa High Dose	7.5 $g.Kg^{-1}$	0	$229.75 \pm 70.44^{AB\ ab}$	$26.86 \pm 1.31^{A\ a}$
			4	$175.35 \pm 32.37^{B\ b}$	$27.16 \pm 1.22^{A\ a}$
			8	$193.21 \pm 50.09^{AB\ c}$	$26.16 \pm 1.60^{A\ a}$
			12	$272.98 \pm 66.76^{A\ c}$	$23.66 \pm 1.43^{AB\ a}$
	F. religiosa High Dose+ Doc	7.5 $g.Kg^{-1}$+ 20$mg.Kg^{-1}$	0	$219.72 \pm 35.49^{AB\ ab}$	$27 \pm 1.15^{A\ a}$
			4	$129.6 \pm 22.11^{B\ b}$	$26.76 \pm 1.76^{A\ a}$
			8	$198.85 \pm 94.13^{AB\ c}$	$26.9 \pm 1.75^{A\ a}$
			12	$233.65 \pm 70.38^{A\ c}$	$23.66 \pm 0.66^{AB\ a}$

*Days Post-tumour Cells implantation.

**Different letters: Significant difference ($P \leq 0.05$), small letters between means of the same column and capital letters between groups at the same day of treatment.

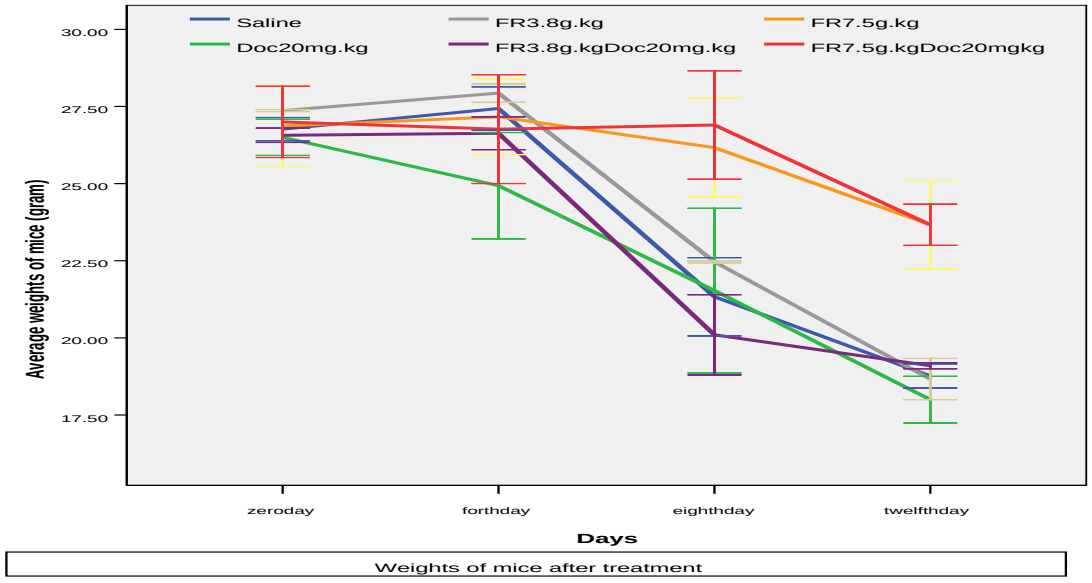


Figure 4-18: Weights of animals treated with *F. religiosa* chloroform extract and docetaxel.

4.5.3 Immunohistochemistry

Only tumour excised from groups (positive control (docetaxel), *F. religiosa* extract (high and low doses) and chemosensitizing of *F. religiosa* extract (combination between *F. religiosa* extract +docetaxel)) on day 13 were subjected to histopathological and immunohistochemical examinations, in Western University, appreciated by Professor Mohammed Al-Tikriti (Ph.D. in College of Osteopathic Medicine of the Pacific and College of Optometry), and appreciated by Professor Wael A. Khamas (Ph.D. in College of Veterinary Medicine) had been carried out.

4.5.3.1 Histopathological analysis

These evaluations were based on a report given by Professor Mohammed Al-Tikriti (Ph.D.), and the findings are presented in the following:

- **Data Analysis**

Tumour sections stained with hematoxylin and eosin were evaluated in terms of mitotic factor (MF) and blood vessels (BVS) These two parameters were used to assess the growth of tumor.

The data were evaluated upon the effect of the extract against other

treatments and report its significant effect on the growth (reflected by mitotic figures) of the cancer cells and the vascularization of the cancerous nodules (reflected by the number of vessels in the area of viewing field (20X)) ($P \leq 0.05$).

The data presented in the Table 4-4, showed mitotic factor (MF) in tumour section from mice treated with low dose of chloroform extract (3.8 $g.Kg^{-1}$) and mice treated with combination of *F. religiosa* (3.8 $g.Kg^{-1}$)+ Doc (20 $mg.Kg^{-1}$) significant reduction in MF was observed (2.76±0.30 and 3.13±0.49) respectively, when compared with positive control (docetaxel 20 $mg.Kg^{-1}$) was (6.50±1.04), while no significantly difference was appeared between *F. religiosa* extract at high dose (7.5$g.Kg^{-1}$) and combination of *F. religiosa* extract (7.5$g.Kg^{-1}$) + docetaxel (20 $mg.Kg^{-1}$) were (7.26±0.87 and 8.00±0.43), as compared with positive control, but the difference observed between low dose and high dose of the extract was significant, see (Figure 4-19).

Depending on the vascularization of the cancerous nodule presented in Figure 4-20, Low dose of *F. religiosa* extract (3.8$g.Kg^{-1}$) was significantly increased in BVS value (7.13±0.42), while the combination between *F. religiosa* (3.8$g.Kg^{-1}$) + docetaxel (20 $mg.Kg^{-1}$) was significantly decreased when compared with positive control (docetaxel) (3.73±0.45*vs*.4.73±0.60). According to these results, significant difference was appeared in BVS values between the animals treated with *F. religiosa* extract (3.8$g.Kg^{-1}$) alone and animals treated with combination *F. religiosa* (3.8$g.Kg^{-1}$)+ docetaxel (20 $mg.Kg^{-1}$).

However, at high dose of *F. religiosa* extract (7.5$g.Kg^{-1}$) alone or when tested chemosenstizing effect of combination of *F. religiosa* extract (7.5$g.Kg^{-1}$)+ docetaxel (20 $mg.Kg^{-1}$), no significant difference was detected in BVS values (5.00±0.29 and 5.70±0.52) respectively, but further significant difference was detected between low and high dose of *F. religiosa* extract.

Table 4-4: Mitotic factor and Blood vessels in histopathological analysis of tumour-bearing nude mice treated with *F. religiosa* (FR) chloroform extract and docetaxel.

Groups		Dose	MF* Means±S.E.	BVS* Mean±S.E.
Positive Control (Docetaxel)		Doc (20 mg.Kg^{-1})	6.50±1.04^{a}	4.73±0.60^{bc}
FR Extract Treated Mice	FR extract Low dose	FR (3.8 g.Kg^{-1})	2.76±0.30^{b}	7.13±0.42^{a}
		FR (3.8g.Kg)+ Doc (20 mg.Kg^{-1})	3.13±0.49^{b}	5.70±0.52^{b}
	FR Extract High Dose	FR (7.5g.Kg^{-1})	7.26±0.87^{a}	5.00±0.29^{bc}
		FR (7.5g.Kg^{-1})+ Doc (20 mg.Kg^{-1})	8.00±0.43^{a}	5.70±0.52^{b}

*Different letters: Significant difference (P≤0.05), small letters between means of the same column.

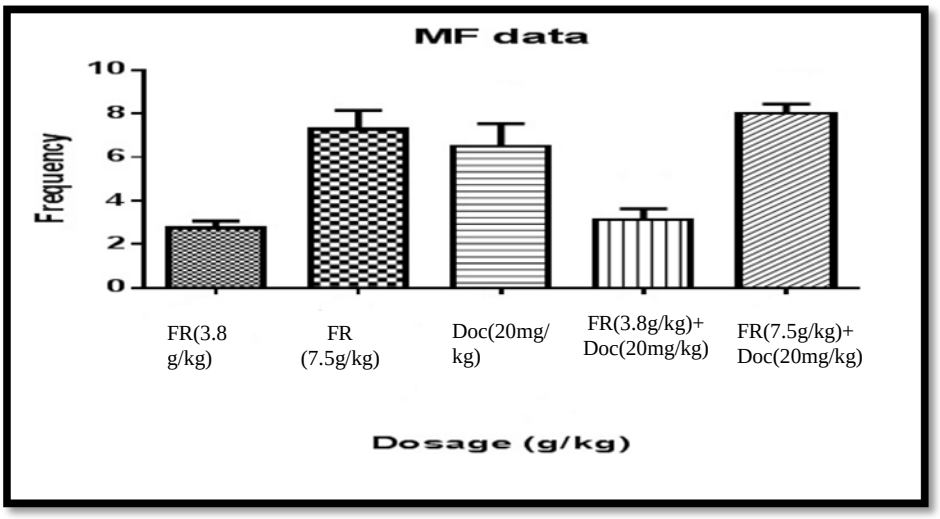


Figure 4-19: Mitotic factor (MF) in histopatholoical of tumour section in tumour-bearing on SCID mice treated with *F. religiosa* extract.

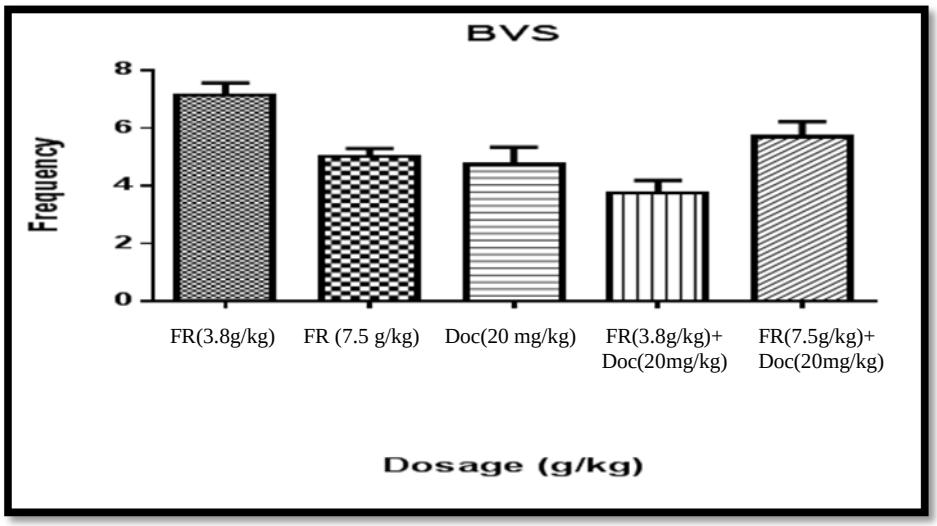


Figure 4-20: Blood vessels (BVS) in histopatholoical of tumour section in tumour-bearing on SCID mice treated with *F. religiosa* extract.

- **Histopathological finding**

Scoring on these features was based on examinations of tumour section of five groups (positive control (docetaxel), *F. religiosa* extract (3.8g.Kg^{-1} and 7.5g.Kg^{-1}), and combination of *F. religiosa* extract + docetaxel) which was viewed with power field (20X).

Mice treated with chloroform extract at doses of 7.5 g.kg^{-1} or 3.8 g.kg^{-1}, show many cells undergoing mitotic division and at different stages of growth. The mitotic figures are located in the center of the cancerous nodule as well as at the periphery (see Figures 4-21). The cancerous cells were well surrounded by growing blood vessels that sometimes engorged with the blood. A few adipocytes were also observed in the area among many infiltrated pleomorphic cells of the blood. Some inflammatory cells were also seen like plasma and macrophages.

The histological feature of the mouse cancerous cells injected with 20 mg.kg^{-1} docetaxel is presented in (Figure 4-22); it shows that the drug has an effect on the cancerous cells, depicted by more infiltration of the nodule by pleomorphic cells of the body. Neutrophilic infiltration with few blood vessels, few adipocytes and the mass were surrounded by connective tissue capsule that was made from collagen fiber. Few large cancerous cells in the center of the

mass were surrounded by few degenerated neutrophils.

Mouse treated with *F. religiosa* extract 7.5g.kg^{-1} and docetaxel 20 mg.Kg^{-1} showed few differentiated cells among few large blood vessels, low mitotic figures and scattered fibroblast (see Figure 4-23). Similarly mouse treated with 3.8 g.kg^{-1} *F. religiosa* extract with docetaxel has event less differentiated and smaller cells among few neutrophilic infiltration especially in the center of the mass (see Figure 4-24), whereas at the periphery, the cells were replaced by collagen fibers and few adipocytes, signs of chronic degeneration which also invaded with plasma cells and macrophages.

Mouse treated with *F. religiosa* extract 3.8 g.kg^{-1} only showed more signs of degeneration and chronic infections, few mitotic figures, many infiltrated neutrophils, and connective tissues surround the mass that is mainly from collagen type (see Figure 4-25).

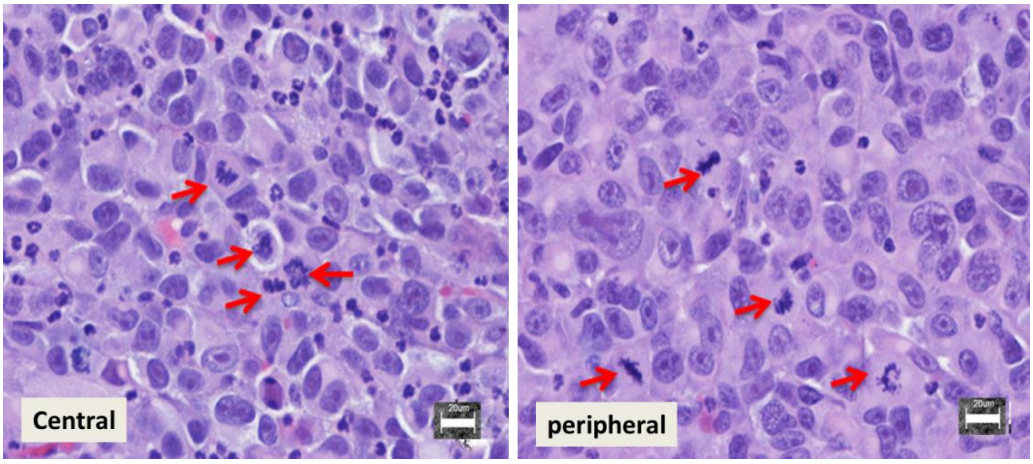


Figure 4-21: Section from the mass of treated mouse with 7.5 g.kg^{-1} *F. religiosa* only. Many cells are in different stages of mitotic figures (arrows) at the center and periphery of the mass. The cells in general are pleomorphic at both regions which viewing with power field (20X). H & E Stain. Bar is 20 µm.

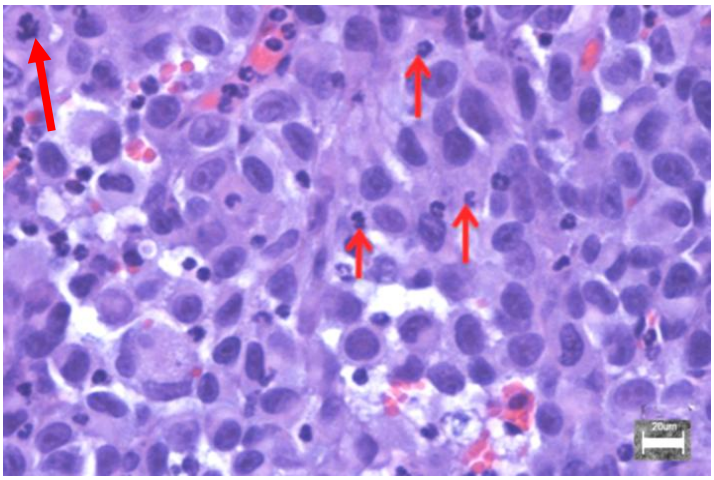


Figure 4-22: Section from the mass of treated mouse with docetaxel (20 mg.kg^{-1}) only. Well differentiated cells at the center and periphery of the mass. The mass was infiltrated with mononuclear cells (arrows), while the main mass was infiltrated with neutrophils which viewing with power field (20X). H & E Stain. Bar is 20 µm.

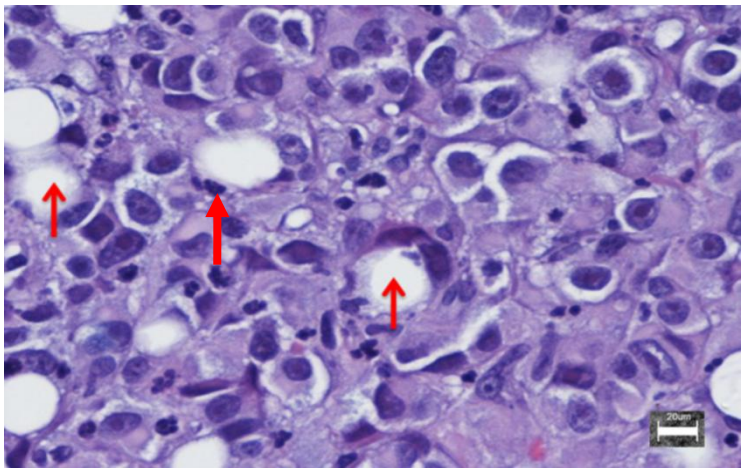


Figure 4-23: Section from the mass of treated mouse with (7.5 g.kg^{-1}) *F. religiosa* extract + docetaxel. Notice, well differentiated cells with relatively large number of blood vessels (arrow) among pleomorphic neutrophilic infiltration which viewing with power field (20X). H & E stain. Bar is 20 µm.

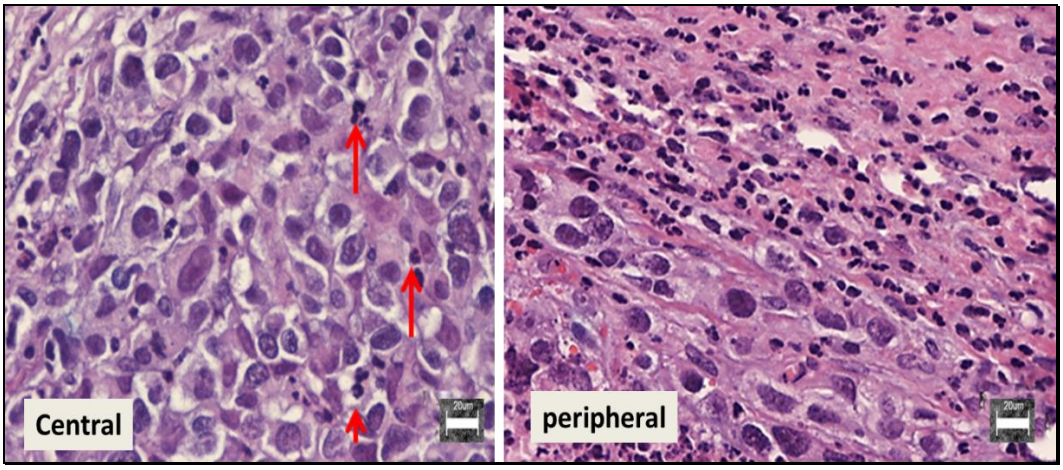


Figure 4-24: Section from the mass of treated mouse with (3.8 g.kg^{-1}) *F. religiosa* + docetaxel. Less differentiated and smaller size cells with few neutrophilic infiltration (arrow) in the center of the mass, while cells at the periphery replaced by connective tissue (mainly collagen fibers) which viewing with power field (20X). H & E stain. Bar is 20 µm.

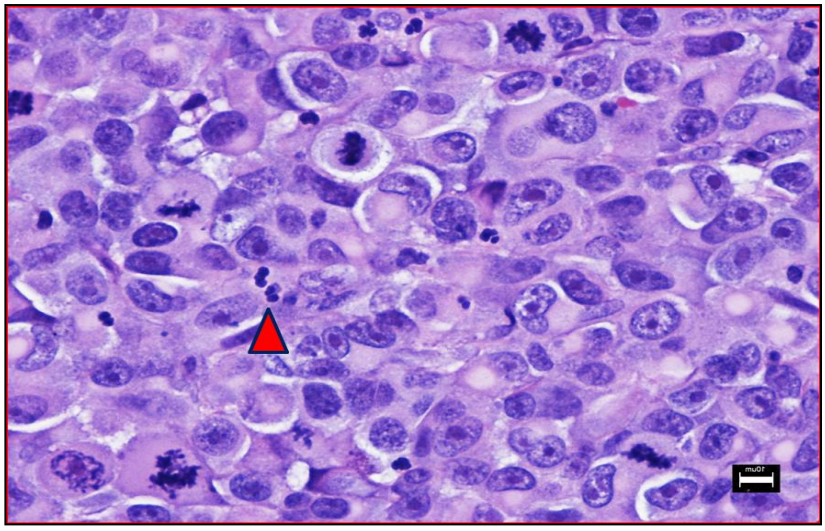


Figure 4-25: Section from the mass of treated mouse with (3.8 g.kg^{-1}) of *F. religiosa* extract. Notice large well differentiated cells in the center of the mass with few neutrophil infiltrations (arrow heads) which viewing with power field (20X). H & E stain. Bar is 20 µm.

4.5.3.2 Immunohistochemical findings

Control slides imaged after omission of primary antibody and Fluorescent –conjugated anti mouse secondary antibody were all negative (see Figure 4-26 and 4-27).

However, strong immunoflouresent reaction was observed on the cell membrane of the lining epithelium of the shrew small intestine that is known to contain P-glycoprotein (Figure 4-28).

Immunoflouresent localization of P-glycoprotein was noted in all samples obtained from different treatments. The immunoflouresent reaction (*P-gp* expression) in docetaxel (20 $mg.Kg^{-1}$) of the treated mouse was confined to the surface of cell membrane of the cancer cells, either completely covering the entire surface membrane or as single globule scattered on the surface of the cancer cells. In addition, some activities were observed on the surface of endothelial cells lining the blood vessels as in (Figure 4-29). The intensity of immunoreactive materials was varied according to their treatment. The lowest reaction (*P-gp* expression) was observed in mouse treated with *F. religiosa* only regardless of their dose (3.8 $g.Kg^{-1}$ and 7.5 $g.Kg^{-1}$), as Figures (4-30 and 4-31) respectively, The highest intensity was observed in mouse treated with a combination of *F. religiosa* (3.8 $g.Kg^{-1}$ or 7.5 $g.Kg^{-1}$) + docetaxel (20 $mg.Kg^{-1}$), as in (Figures 4-32 and 4-33).

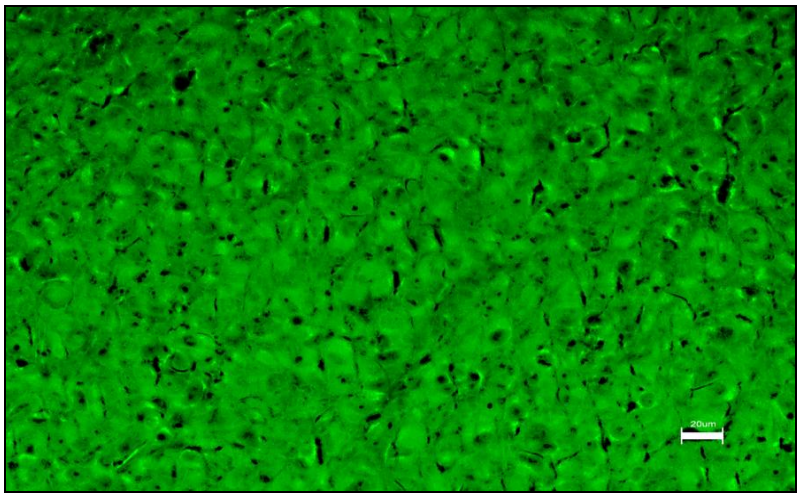


Figure 4-26: Immunohistochemistry of treated mice, lacking secondary HRP goat anti mouse (negative sample). Bar equal to 20µm.

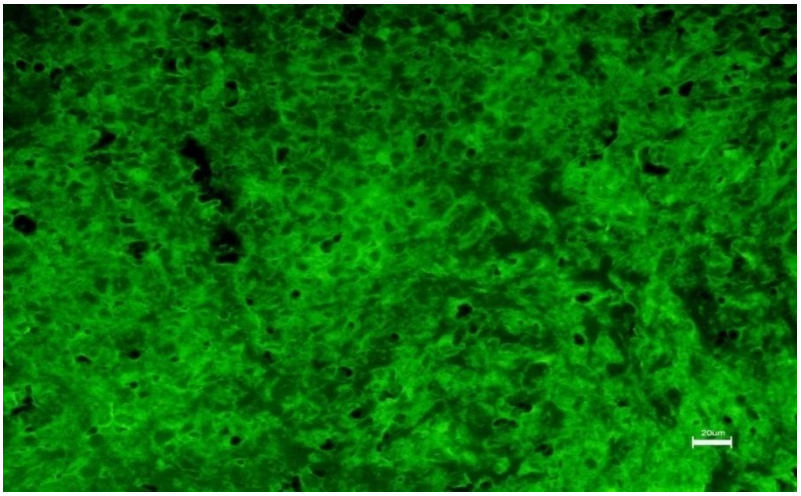


Figure 4-27: Immunohistochemistry of treated mice, lacking primary anti-P-glycoprotein (negative sample). Bar equal to 20µm.

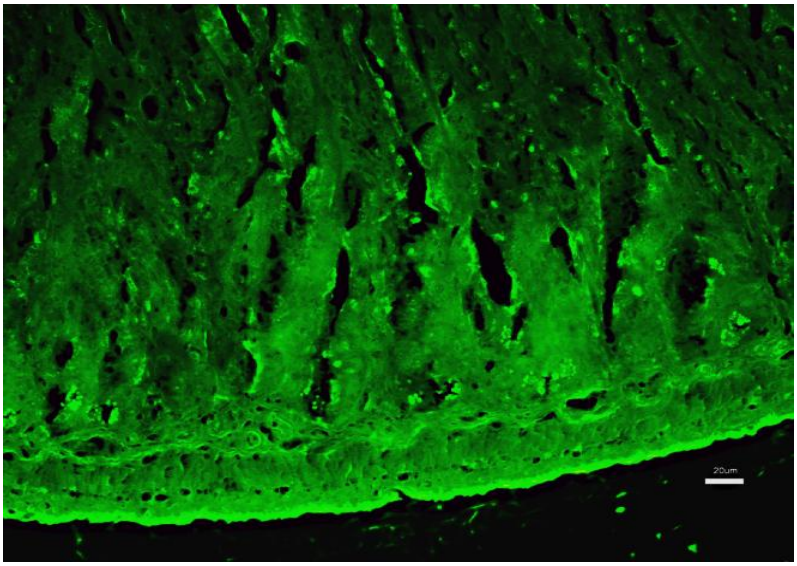


Figure 4-28: Immunohistochemistry of positive control sample taken from the shrew's small intestine (arrows) known to contained P-glycoprotein on the cells of surface epithelial lining of intestine. Bar equal to 20 µm.

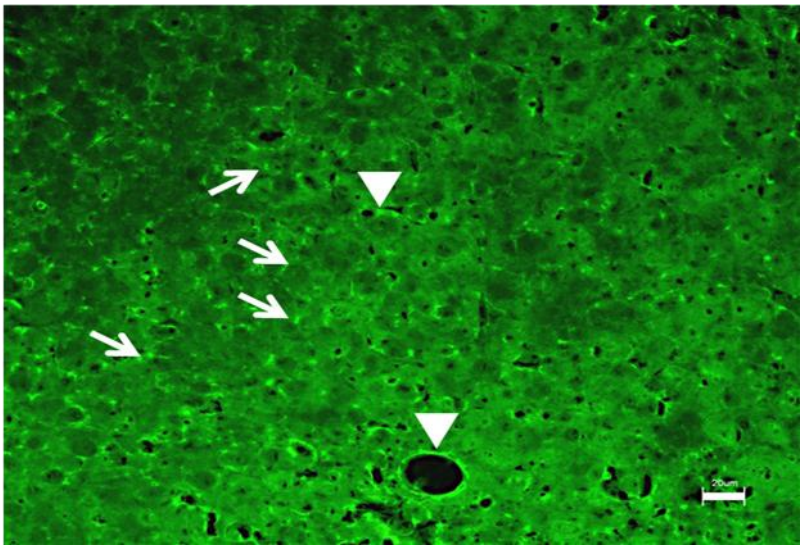


Figure 4-29: Immunohistochemistry of Docetaxcel (20 mg.kg^{-1}) treated mice, showing many positive immunoreactive materials on the surface of the cells (arrows) as well as the endothelium of blood capillaries (arrowhead). Bar equal to 20 µm.

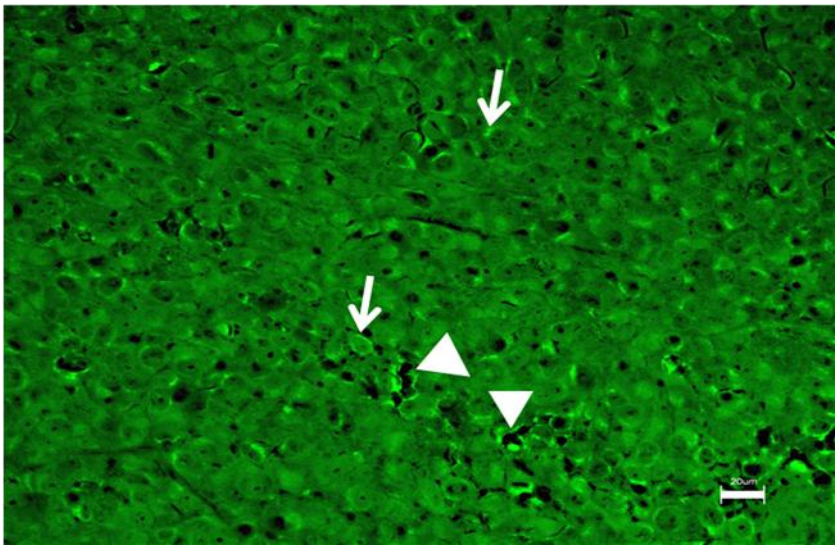


Figure 4-30: Immunohistochemistry of *F. religiosa* (7.5 g.kg^{-1}) treated mice, showing positive immunoreactive Materials on the surface of the cells (arrows) as well as the endothelium of blood capillaries (arrowhead). Bar equal to 20 µm.

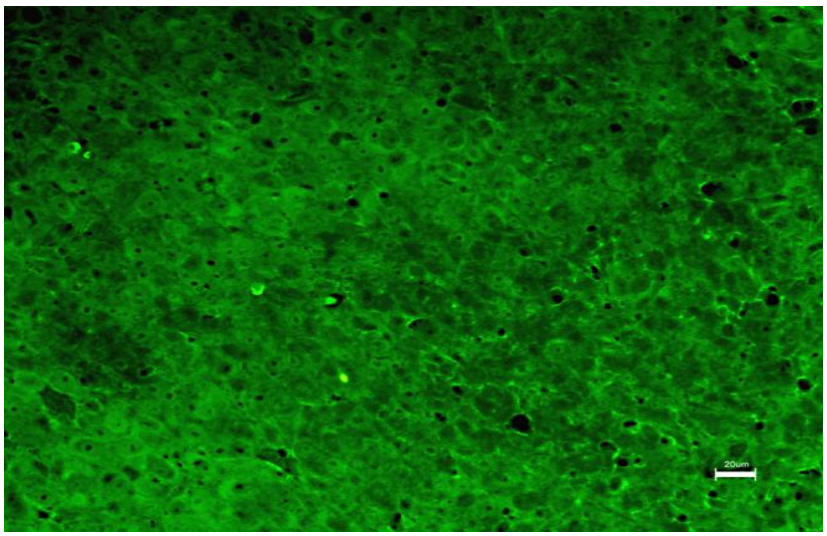


Figure 4-31: Immunohistochemistry of *F. religiosa* (3.8 g.kg^{-1}) treated mice, showing low immunoreactive labeled materials on the surface of the cells (arrows). Bar equal to 20 µm.

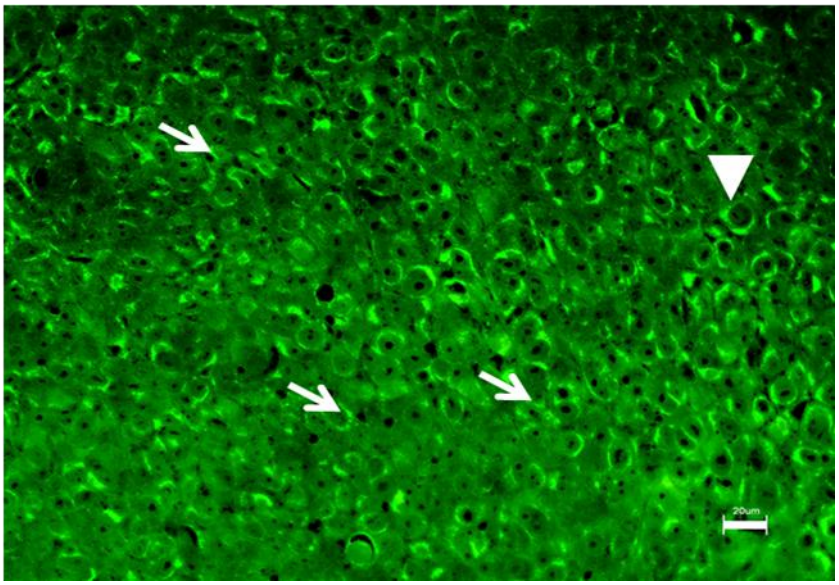


Figure 4-32: Immunohistochemistry of *F. religiosa* (7.5 g.Kg^{-1}) + docetaxcel (20 mg.Kg^{-1}) treated mice, showing extensive immunoreactive labeled materials on the surface of the cells (arrows) as well as the endothelium of blood capillaries (arrowhead). Bar equal to 20 µm.

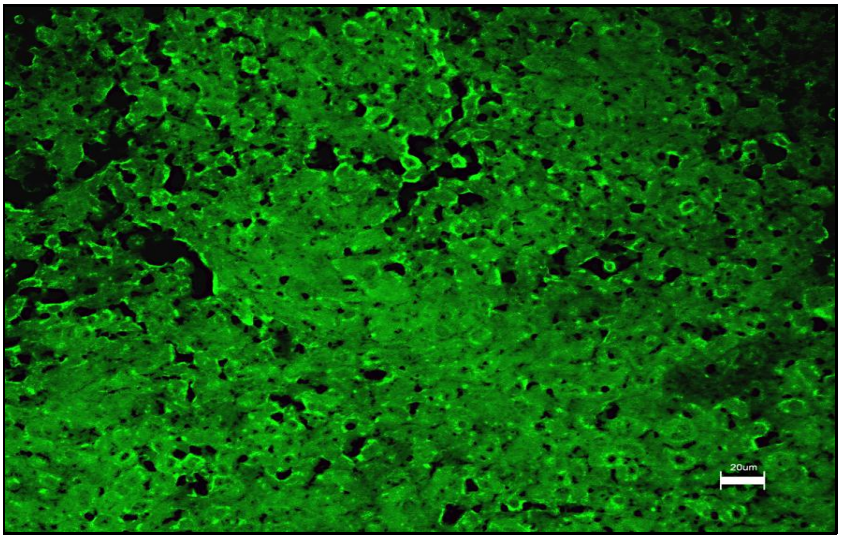


Figure 4-33: Immunohistochemistry of *F. religiosa* (3.8 g.Kg^{-1}) + docetaxel (20 mg.Kg^{-1}) treated mice, showing extensive immunoreactive labeled materials on the surface of the cells (arrows). Many blood vessels have labeled endothelium (arrowhead). Bar equal to 20 µm.

4.5.3.3 Gray Scale Analysis of Immunohistochemical of P-glycoprotein

The p-gp labeled protein was difficult to be estimated by counting, therefore this method was very precise to measure things that are uncountable by eyes like what you have (gel or bacteria growth in a test tube, etc). So graphs were generated from different slides for each treatment is making standing on a solid ground, as shown in Figure 4-34.

Data present in the Figure 4-35 confirms that animals treated with *F. religiosa* extract were significantly inhibited P-gp ($p \leq 0.05$). The P-gp gene showed a positive expression in the mice bearing-tumour xengraft with PC3-TxR cancer cells treated with docetaxel (20 mg.Kg^{-1}) as positive controlled group was (123.7±21.62), while the animals treated with *F. religiosa* extract (3.8 and 7.5 g.Kg^{-1}) were (114.56±17.42 and 89.21±12.24) respectively, non-significant inhibition of *P-gp* gene expression was observed, while when combined *F. religiosa* extract (3.8 g.Kg^{-1}) with docetaxel more

significant reduction in gene expression (25.21±1.39) was detected, than combined between *F. religiosa* extract (7.5 $g.Kg^{-1}$) with docetaxel (119.03±20.48), as compared with the controlled group.

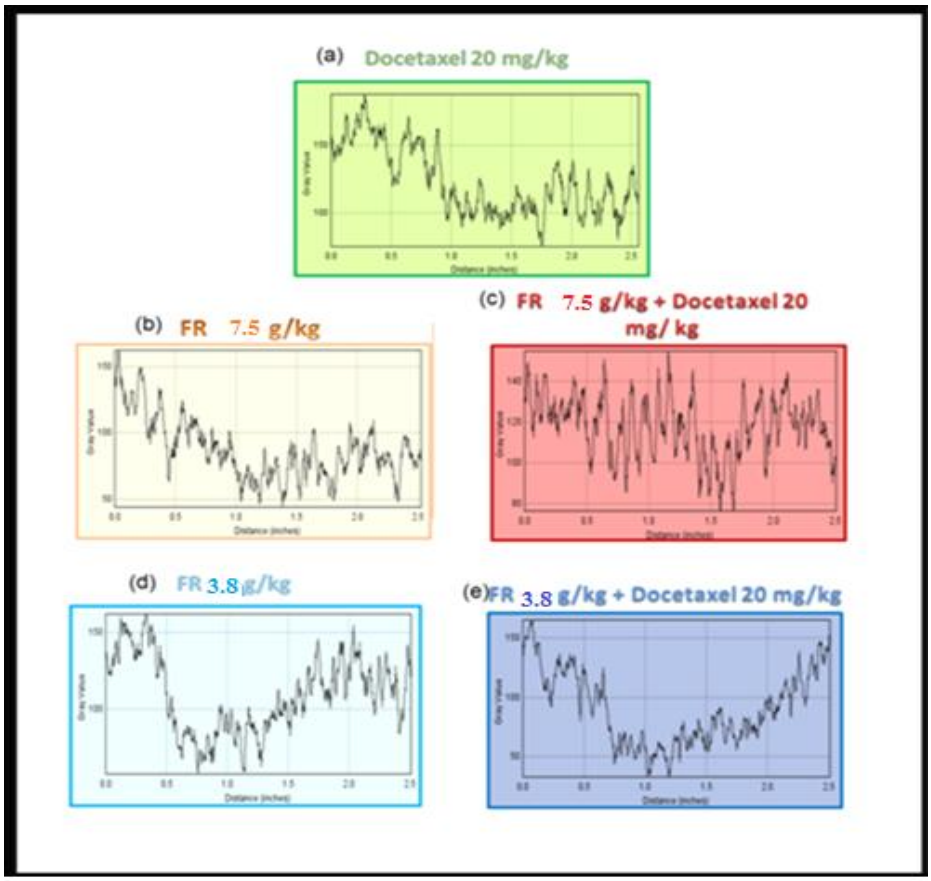


Figure 4-34: Graphs of P-glycoprotein in tumour section for tumour-bearing mice treated with *F. religiosa* extract and docetaxel drug, generated by Gray Scale Analysis.

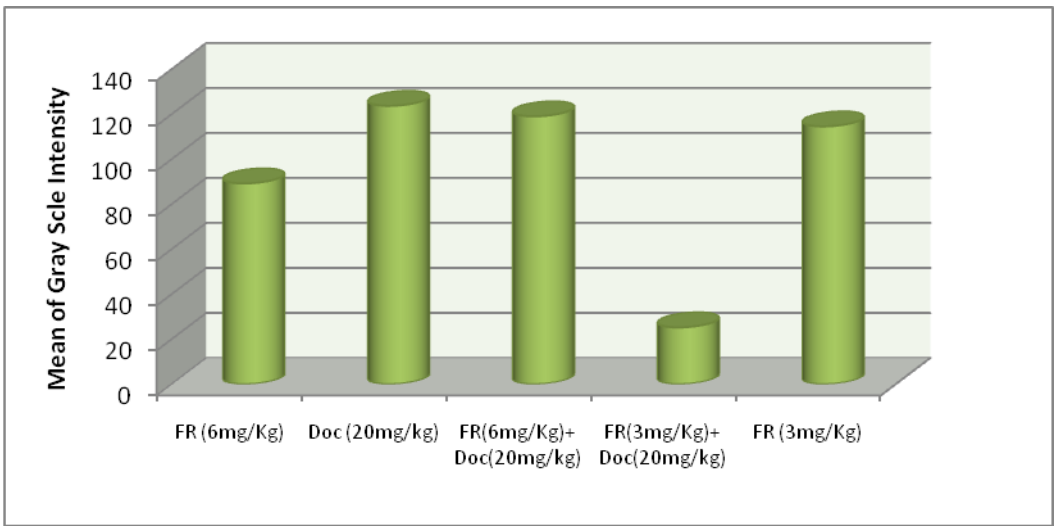


Figure 4-35: Gray Scale Analysis of *P-glycoprotein* gene expression in tumour section for tumour-bearing mice treated with *F. religiosa* extract +docetaxel drug.

5. Discussions

5.1 Cytotoxicity of *Ficus religiosa* Extracts

The *in vitro* cytotoxicity assessments of *F. religiosa* extracts in five human cancer lines (MCF7, PC3, PC3-TxR, K562 and K562Dox) revealed that the effects were dose- and time-dependent. Furthermore, the *F. religiosa* extracts target cell line were also interfered with these effects. In general *F. religiosa* chloroform and butyl alcohol extracts shared the most effective cytotoxicity, whereas *F. religiosa* methanol and crud aqueous extracts were less effective in this regard. According to these results, chloroform extract was selected as a best cytotoxic one for the next work. Such findings can be interpreted on the ground of their polyphenolic contents, because the highest concentrations of polyphenolics were presented in chloroform and butyl alcoholic extracts according to the method of extraction and among which the chloroform is the most non-polar one (Wang, personal communication). Therefore, the cytotoxicity of *F. religiosa* extracts seemed to be positively correlated with polyphenolic compounds. Many reported that flavonoids, phenolics and aromatics are the most important pharmacological active constituents of Ficus, and it is well known that these products are powerful anti-oxidants. For this reason, *F. religiosa* is considered a natural source of anti-oxidants, and the anti-oxidant activity of such extracts may play an important role in their anti-proliferative activities (Lansky *et al*., 2008).This view has been confirmed by *F. religiosa* extracts from India, in which it has been demonstrated that *F. religiosa* was effective in inducing growth inhibition and apoptosis in human breast cancer (MCF-7) (Haneef *et al*., 2012), *F. religiosa* extracts were screened for cytotoxic activity against healthy mouse fibroblast (NIH3T3) and three human cancer cell lines (gastric: AGS; colon:

HT-29; and breast: MDA-MB-4355) using the MTT assay (Uddin *et al*., 2009), the extract also showed anticancer potential against cervical cancer cell line (Gulecha *et al*., 2011, Choudhari *et al*., 2011).

Thus, the anti-proliferative effects of Iraqi *F. religiosa* could be related to an overall effect of the phenolic compounds present in the extracts, which were detected by HPLC-DAD analysis.

On the other hand, lipids and proteins present in biological membranes facilitate the solubility of polyphenols, and differences in cell membrane structures and metabolic activation of chemicals can also affect the activity of polyphenols (Szliszka *et al*., 2009). However, *F. religiosa* acetone extract showed in multiple apoptosis signalling in human breast cancer cells, irreversible inhibition of breast cancer cell growth with moderate toxicity to normal breast epithelial cells. This observation was validated using Sulforhodamine B assay, and showed cell cycle arrest in G1 phase and induction of sub-G0 peak. The extract induced chromatin condensation and displayed an increase in apoptotic population in Annexin V-FITC/PI (Fluorescein isothiocyanate/Propidium iodide) double staining. In addition, stimulated the loss of mitochondrial membrane potential in multiple breast cancer cell lines when compared to normal diploid cells (Haneef *et al*., 2012).

The inhibition concentration (IC_{50}) by *F. religiosa* extracts can result in apoptosis or necrosis, and such outcome may be subjected to the cell line under investigation. In cancer cells, *F. religiosa* extracts induced apoptosis. Therefore, it is possible to suggest that the chemical constituents of *F. religiosa*, especially phenolic acids and flavonoids, may have a selective cytotoxic effect against cells, and such effect is determined by the type of cells under investigation. Furthermore, and as *F. religiosa* was less cytotoxic to normal cells, it is also possible to consider this plant extract a potential anti-tumour agent that has no side effects (Uddin *et al*., 2009). However, the latter evaluation can be best defined on the ground of *in vivo* studies, included in this present study.

When the cytotoxic activity of Ficus extracts were tested by the SRB assay, all FR extracts marked differences in cytotoxic effect, but chloroform extract was the best in this regard. This results shared the activity reported for Indian FR extract with a little variation (Uddin *et al*., 2009), suggesting that the region and habitat of the plant have a quantitative effect on active constituent of the plant. Such potent activity seemed to be correlated with the total polyphenolic compounds, because the strong anti-oxidant activity occurs in *F. religiosa* extracts that are rich in phenolic compounds, and flavonoids are reported to be the most abundant and most effective anti-oxidant compounds found in *F. religiosa* plant (Hemant *et al*., 2011). Choudhari and his worker (2011) have also previously investigated the anti-oxidant activity of *F. religiosa* plant extract, and reported a significant correlation between flavonoid contents and antioxidant activity.

Flavonoids can significantly cause reduction of hydrogen peroxide which means scavengers of reactive oxygen species (ROS) in humans, flavonoid concentration in plasma and most tissues is too low to effectively reduce ROS. Instead, flavonoids may play key roles as signaling molecules in mammals, through their ability to interact with a wide range of protein kinases, including mitogen-activated protein kinases (MAPK), that supersede key steps of cell growth and differentiation (Brunitti *et al*., 2013).

The anti-oxidant activity of *F. religiosa* and its ability to sequester reactive oxygen species have been investigated by Choudhari and his collegues (2011), who studied the biological effects of different extracts, a correlation was observed between the anti-oxidant activity and chemical composition of its different fractions, with special emphasis on the presence of flavonoids and tannic acid derivatives. The study concluded that the components of *F. religiosa* act by different mechanisms sequestering reactive oxygen species. Additionally, several studies have confirmed that the pharmacological properties of *F. religiosa* are attributed mainly to the presence of favonoids as a result of their action against free radicals (Simtha *et al*., 2009), and these

polyphenols interfere not only with the propagation but also with the formation of free radicals both by chelating transition metals and by inhibiting enzymes involved in the initiation reaction (Brunetti *et al*., 2013).

The naturally occurring polyphenols are expected to help reduce the risk of various life-threatening diseases, including cancer and cardiovascular diseases, due to their antioxidant activities. Thus *F. religiosa* with antioxidant activity may protect humans from deleterious oxidative processes. Banskota *et al*. (2002) also reported that the anti-oxidative activity of *F. religiosa* is due to its phenolic constituents, which also possess anti-tumour and anti-hepatotoxic activities. However, phenols include many compounds; for instance, tannic acid and serotonin, and each compound may have different scavenging activity. Accordingly, the two compounds, tannic acid or its isomers and serotonin, were assessed and showed a more potent antioxidant activity in the *F. religiosa*. Overall, these results were findings of Naus *et al*., (2009) who reported that tannic acid extract from *Geranium Wilfordii Maxim* has potent chemotherapeutic activities.

5.2 Chemosensitizing Effect of *F. religiosa* Chloroform Extract on Resistance and Sensitive Cancer Cell Lines

The combination between chemotherapy and natural compounds showed significant reduction in cancer growth specially which resistance to chemotherapy, approximately 80% of patients with metastatic disease are cured using combination chemotherapy (Oudrad, 2012). In contrast, in most other types of metastatic cancer, treatment fails due to inherent or acquired drug resistance. Resistance can arise by spontaneous random mutation in mammalian cancer cells. The different response of tumour to therapies results from a low frequency of mutation to drug resistance (O'neill *et al*., 2011; Sternberg *et al*., 2009).

Prostate cancer acquired resistance to docetaxel and leukemic cancer acquired resistance to daunorubicin cell lines displayed chemosensitizing

effect when treated with combination of drug and *F. religiosa* chloroform extract. The findings demonstrated that phenolic compounds have potent anticancer promoting activity targeting MAPK/ERK kinase (MEK) (the mitogen-activated protein kinase (MAPK)/the extracellular signal-regulated kinase (ERK)) signaling (Lee *et al*., 2005a). It was also reported that these flavonoids have anti-oxidative (Terao, 2009), anti-proliferative (Wang *et al*., 2007; Zhang *et al*., 2008) and anti-inflammatory activities (Garcia-Lafuente *et al*., 2009). There have also been investigations on the protection of neurons (Bastianetto & Quirion, 2002), and effects on breast cancer (MCF-7), prostate cancer (PC-3) and endometrial cancer (Ishikawa) cells from various injuries by flavonoids (Tsiapara *et al*., 2009). Some studies have reported that phenols are able to protect hippocampal cells against toxic effects induced by Abeta peptides and rotenone (Molina-Jimenez, *et al*., 2004). Furthermore, with many studies suggesting that the potency of flavonoids in perturbing cell cycle and inducing apoptosis may be dependent on C2-C3 double bonds and number of hydroxyl groups in the 2-phenyl ring, which are important structural requirements for cytostatic effects of flavonoids (Rusakc *et al*., 2005).

Some naturally occurring chemopreventive compounds are known to act in synergy with other chemopreventive or anti-cancer agents. The anti-neoplastic agents have a dose limiting toxicity and drug resistance, thus limiting their clinical application. Development of novel strategies that overcome chemoresistance and sensitize cancer cells to anti-neoplastic agent can enhance the therapeutic effect of these drugs. Combination treatment with natural compounds and chemotherapeutic drug was synergistically cytotoxic and enhanced apoptosis in MDA-MB-231 human breast cancer cells and decreased tumor growth in a MCF-7 cell xenograft model (Scandlyn *et al*., 2008; Sartippour *et al*., 2006).

The combined treatment also resulted in synergistic effects by improving apoptosis resistance (Zhou *et al*., 2010), significantly decreased prostatic adenocarcinoma in prostate by effectively inhibiting cell growth and inducing

apoptosis (Narayanan *et al*., 2009). *In vivo* treatment altered expression of molecules involved in regulating cancer cell resistance to drugs and radiations (Prirgo *et al*., 2008), eliminates colorectal cancer cells growing *in vivo* leading to long term survival. Gene expression analysis of a Bcl-2 family of genes revealed that down-regulation of bcl-2 expression via inhibition of NF-κB activation, leading to inhibition of proliferation and angiogenesis (Kunnumakkara *et al*., 2008).

Recently, preclinical investigation revealed that combination therapy to be highly effective causing an over 95% regression of intestinal adenomas in *Apc*min/+ mice, which could be attributed to decreased proliferation and increased apoptosis (Nautiyal *et al*., 2011).

5.3 Chemical Analysis

By HPLC-DAD instrument detect for the first time, there was presenting serotonin as active compound as well as tannic acid or isomers of these compounds finding in *F. religiosa* chloroform extract, because such compounds are normally present in *F. religiosa* extract from Indian (Kapoor *et al*., 2011; Patil *et al*., 2011). This plant is rich in polyphenolic compounds and various diterpenes, which are reported from ficus. Singh and his colleagues (2011) reported *F. religiosa* leaves are rich with terpeniods and polyphenolic compounds.

Quantitative estimations (concentrations) of the identified compound were made by comparing the peak areas of extracted chromatograms of pseudo-molecular ions for the *F. religiosa* chloroform extract with the injected available reference standards (quricetin, myrecitin, tannic acid, gallic acid and serotonin).

An accurate assessment of the contents of bioactive compounds in extract requires the validation of certain analytical parameters such as precision, currency, linearity and limit of detection. To fulfill these obligations, the chloroform extract of *F. religiosa* were subjected to high-performance liquid

chromatography (HPLC). The technique is used to resolve and determine species in a variety of organic, inorganic, biological, ionic and polymeric materials, due to the fact that ficus contains many components that are found by using HPLC (Kapoor *et al*., 2011; Volpi and Bergonzini, 2006).

High-performance liquid chromatography represents the most popular and reliable analytical technique for the characterization of polyphenolic compounds as judged by the number of published papers in this subject (Gomez-Caravaca *et al*., 2006). Accordingly, HPLC column in the present study permitted a chemical analysis of *F. religiosa* chloroform extract with high sensitivity and specificity as demonstrated by the obtained results.

Detector in HPLC which is placed at the end of analytical column should have particular characteristics such as linear response as a function of concentration of the solute, wide linear dynamic range, high signal to noise ratio. A robust detector with good sensitivity works approximately in range of 0.01-100 of compound in elutes. Following such lead, all the chromatographic peaks of different substances were clearly separated by combining retention times and high resolution extracted ion chromatograms of pseudo-molecular ions. Moreover if more and more sample amount is applied to the column, the peak height and peak area increases but the peak symmetry and the capacity factor remain unchanged (Rathore, 2003). Such analysis was further presented in term of $[M-H]^-$ ion peak to quantify the identified flavonoids in *F. religiosa* chloroform extracts. Under this negative mode and at an energy level of 100% and a drying temperature at 350^{o}C, the flavonoids were identified as the major $[M-H]^-$ ion peaks, thereby allowing these ions to be selected for use in the quantitative analysis (Volpi and Bergonzini, 2006). Accordingly, this method can be considered very sensitive and accurate in achieving linearity over a wide range of concentrations and the findings of the present study concentration range (0.1 - 2000 μg ml^{-1}) can justify such conclusion (Huber, 2006).

5.4 Effect of *F. religiosa* Chloroform Extract on Gene Expression

In the present study, results *F. religiosa* chloroform extract showed more toxicity and chemosensitizing on human cancer cells which are resistant to drug and it was proved that *F. religiosa* has polyphenolic compounds which cause anti-tumour activity and the pathway of effect is through the induction of apoptosis. Inhibition of apoptosis is an important mechanism for tumour formation and progression, and therefore, several chemotherapeutic compounds have been reported to induce apoptosis and this may be the primary mechanism for their anti-tumour activity (Tait, 2008). To test this hypothesis, the gene expression was evaluated *in vitro* in tumour cell (PC3-TxR) lines after a treatment with *F. religiosa* chloroform extract through types of assays, which were regulated by *P-gp, EGFR, Nrf2, Patch1, Gli2, Id1, Id2* and *Id3* gene expressions. Then P-gp protein was assessed by using western blot. The results pointed out that the tested substances showed a potent inhibitory cytotoxic effect against the proliferation of PC3-TxR cells through either apoptosis or necrosis.

Treated PC3-TxR cells with *F. religiosa* extract showed a clear apoptotic manifestations characterized by Inhibition concentration of growth (chromatin condensation and DNA fragmentation), and down-regulation of *P-gp, Id1* and *Id2* but up-regulation of *EGFR, Nrf2, hatch1, Gli2* and *Id3* gene expressions, additionally inhibition P-gp protein synthesis.

By using real time-PCR can detect the ability of *F. religiosa* extract for regulation of gene expression was tested, the basic goal of real-time PCR is to precisely distinguish and measure specific nucleic acid sequences in a sample even if there is only a very small quantity. Real-time PCR amplifies a specific target sequence in a sample then monitors the amplification progress using fluorescent technology. During amplification, how quickly the fluorescent signal reaches a threshold level correlates with the amount of original target

sequence, thereby enabling quantification. In addition, the final product can be further characterized by subjecting it to the increase of temperatures to determine when the double-stranded product "melts." This melting point is a unique property dependent on product length and nucleotide composition. To accomplish these tasks, conventional PCR has been coupled to state-of-the-art fluorescent chemistries and instrumentation to become real-time PCR (Valasek and Repa 2005). The latter features were encountered in the present study, but they were dependent on the sample investigated and its concentration, incubation period and the target cell line. With respect to the first dependent factor, FR chloroform extract was the most effective in inducing apoptosis in PC3-TxR cells.

Prostate cancer cells were acquired chemotherapy resistance; several mechanisms of drug resistance have been examined. Over expression of a membrane efflux transporter, P-glycoprotein (*P-gp*) is one of the most consistent alterations in drug resistance (Gottesman, 2002; Gottesman et al., 2001). *P-gp* has become an important clinical target and the object of numerous studies (Sikic *et al*., 1997; Sandor *et al*., 1997). Drug resistance can also be caused by over expression of multidrug resistance (MDR)-associated protein (Krishnamachary and Center, 1993; Grant *et al*., 1994), although many studies suggest that chemotherapy resistance in cancer cells is in some instances associated with an enhanced cellular capacity to glycosylate ceramide (Lavie *et al*., 1996; Liu *et al*., 2001). This implies that chemotherapy toxicity would be blunted in cancer cells that up-regulate ceramide clearance. This places glucosylceramide synthase, the enzyme that catalyzes ceramide glycosylation, in a key position, especially when considering the large number of drugs that elicit ceramide formation (Senchenkov *et al*., 2001).

The apoptotic effects of *F. religiosa* extract were better at the higher dose, and therefore the dose can be considered a further factor in the induction of apoptosis. In this regard, it has been demonstrated that the anti-tumour activity of some phenol compounds is highly dependent on dose, and accordingly it has

been suggested that the higher doses of phenols can interfere with cell processes such as enzyme and glutathione levels, and this may induce cells death or apoptosis (Ramos, 2008).

The inhibitory mechanisms of flavonoids and other polyphenols on *P-gp* function have been studied. Inhibition of substrate binding to *P-gp* and inhibition of ATPase activity have been reported. For example, flavonoids have been reported to inhibit *P-gp* substrate azidopine binding to *P-gp* (Zhang and Morris, 2003) Inhibition of ATPase activity by phenol has also been reported (Mei *et al*., 2004), also found significant inhibitory effects of polygalloylglucose on verapamil-stimulated *P-gp* ATPase activity. Flavonoids have been suggested to be modulators with bifunctional interactions at vicinal ATP-binding sites and steroid-interacting regions, which are expected to be in close proximity to the ATP-binding site, within a cytosolic domain of *P-gp* (Wang *et al*., 2002; Boumendjel *et al*., 2002; Conseil *et al*., 1998). Flavonoids may induce their binding affinity towards NBD2 of *P-gp* through their ability to mimic the adenine moiety of ATP. Therefore, hydrophobic moiety of polyphenols may be important for interaction at the steroid-interacting hydrophobic sequence of *P-gp* (Kitakawa, 2006) Hydroxyl groups in polyphenols such as those in the gallic acid moieties and alkyl gallates may be important in polar interactions with *P-gp* (Klopman *et al*., 1997) possibly at the ATP-binding site. In addition, the direct interaction with *P-gp* may cause down regulation of *MDR1* gene expression by polyphenols (Mei *et al*., 2004).

These collective observations may qualify the *F. religiosa* extract as a potent apoptosis-inducing agent, and its action is through *P-gp* regulation, but it has to be confirmed on *in vivo* based studies.

For more interesting chloroform extract of the plant down-regulation of inhibition DNA/ differentiation (*Id*) gene expression which plays an important role in tumourigenesis in certain types of human cancer was reported (Ouyang *et al*. 2002). Ouyang *et al*., (2001) reported that *Id* was up-regulated during sex hormone- induced prostate carcinogenesis in a Noble rat model, and Ouyang *et*

al. (2002), investigated the direct effect of *Id* expression on human prostate cancer cell proliferation by transfecting an *Id* expression vector into a prostate cancer cell line LNCaP. They found that *Id* expression induced phosphorylation of RB and down- regulation of $P16^{INK4a}$ but not $P21^{Waf1}$ or $P27^{Kip1}$.Therfore *Id* induced inactivation of $P16^{INK4a}$/pRB pathway may be responsible for the increased cell proliferation in prostate cancer cells.

The possible explanation of better effects of chloroform *F. religiosa* extract on down regulation is their richness in polyphenols, which were significantly detected in chloroform extract. Polyphenols and bioflavonoids have been demonstrated to reverse the effects of DNA hypermethylation which cause cancer and defect the genes involved in cell cycle regulation (*p16INK4a, p15INK4a, Rb, p14ARF*), genes associated with DNA repair (*BRCA1, MGMT*), apoptosis, drug resistance, detoxification, angiogenesis, and metastasis are susceptible to hypermethylation (Aggarwal and Shishodia, 2006) .

Polyphenols have been shown to induce apoptosis in some cancer cell lines and although the molecular mechanisms by which flavonoids induce apoptosis have not yet been clarified, several mechanisms may be involved, including inhibition of DNA topoisomerase I/II activity (Bailly, 2000), regulation of heat shock proteins expression (Rong *et al*., 2000), decrease of reactive oxygen species (Khan, 2002), modulation of signaling pathways (Kim *et al*., 2005), release of cytochrome C with a subsequent activation of caspase-9 and caspase3 (Michels *et al*., 2005), down-regulation of *Bcl-2* and *Bcl-X*(L) expression but promotion of Bax and Bak expression (Lee *et al*., 2005a), nuclear transcription factor kappaB (NF-kappaB) (Kanno *et al*., 2006), activation of endonuclease (Kook *et al*., 2007), and suppression of myeloid cell leukaemia-1 (Mcl-1) protein (Siegelin *et al*., 2008). More recently, preliminary evidence suggested that apoptosis induced by flavonoids in HL-60 cells may be associated with activation of caspase-3 and mediated through Fas and cytochrome C pathways, as well as, regulated through the inactivation of NF-kappaB (Naoghare *et al*., 2010).

5.5 Anti-tumour Assessment *in vivo*

The anti-tumour potentials of *F. religiosa* chloroform extract, which were assessed *in vitro*, were extended for a further *in vivo* evaluation in SCID mice xenografted with PC3-TxR cells and treated orally (p.o.) with the extract. In these *in vivo* experiments, a dose-dependent inhibition of tumour growth was observed with a significant tumour regression at dose 7.5 $g.kg^{-1}$, but less tumour inhibition at dose 3.8 $g.Kg^{-1}$, However mice tolerance was observed and manifested by lack of animals death or body-weight loss in addition to death of some animals (two out of 6 mice), and such consequences made the investigator to end the observation period on day 12. The unusual results appeared that losing of mice weight and death in the groups were not clarified in this experiment which may be related to use high doses of *F. religiosa*. The latter side effects may be also related to the PC3-TxR cancer cells resistance to docetaxel, because group treated with saline 0.9%, as well as, 20 $mg.kg^{-1}$ docetaxel treated mice also showed more than 20% weight loss and more than 20% animal death. Therefore, the observed side effects may be not related to toxic effects of *F. religiosa* only; especially after giving saline 0.9% for 12 days because such side effects were observed from first days of treatment. However, the study has demonstrated that *F. religiosa* is not toxic to humans or mammals unless it exceeds LD_{50} value which estimated equal to measure 11.3 $g.kg^{-1}$. These results are confirmed by Pandit and his colleagues (2010) who reported that the aqueous bark extract of *F. religiosa* was found to be safe up to 2 $g.kg^{-1}$; p.o. dose in acute toxicity studies carried out using OECD guidelines on Swiss female albino mice.

All studies were demonstrated that *F. religiosa* found to exhibit anti-ulcerogenic potential by decreasing the level of malondialdehyde (MDA), superoxide dismutase (SOD). Further it can stimulate the synthesis of endogenous prostaglandin (PG's) and thus act as ulceroprotectant (Saha and Goswami, 2010, Khan *et al*., 2011), and mice did not observe mortality or

severe symptoms alteration after daily intake of *F. religiosa* 7.5 g.Kg^{-1} in the diet of mice during 12 days period during gavage FR extract of normal CD-1 mice which used to measure multiple tolerance dose.

As mentioned, the investigated *F. religiosa* extract was involved in an *in vivo* reduction of the xenografted tumour. To shed light on such potential and understand the pathway of tumour degeneration, a histological examination of tumours on groups was carried out which revealed that a treatment with *F. religiosa* extract was associated with several histopathological changes that are generally considered as signs of tumour degeneration. Labeling protein 'antigen' in different cells of tissues by produce antigen-antibody interaction particular antigen very specific. This procedure is widely used as a diagnostic tool for different diseases like cancer. The most important encountered changes were reduced mitosis and vascularization (Al-Tikriti-personal communication).

The mitotic division in tumour sections of FR treated mice was significantly decreased, and the effect was dose-dependent. In 3.8 g.kg^{-1} the reduction was significant, while in dose 7.5 g.kg^{-1} it was less significant when compared with a positive control (docetaxel 20 mg.Kg^{-1}) value. Therefore, such cells were arrested at the metaphase stage of mitosis, and such arrest may be related to *F. religiosa* effects on the mitotic-spindle microtubules of dividing cells. Microtubules are extremely important in the process of mitosis, during which the duplicated chromosomes of a cell are separated into two identical sets before cleavage of the cell into two daughter cells. Their importance in mitosis and cell division makes microtubules an important target for anti-cancer drugs and their dynamics are the targets of a chemically diverse group of anti-mitotic drugs (with various tubulin-binding sites) that have been used with a great success in the treatment of cancer (Schmidt and Bastians, 2007; Jackson *et al*., 2007; Zhou and Giannakakou, 2005; Jordan and Wilson, 2004). Microtubules seem to be a favourite target of naturally occurring, presumably self-protective, toxic molecules that are produced by a large

number of plants and animals and most microtubule-targeted compounds have been discovered in large-scale screens of natural products (Schmidt and Bastians, 2007).

The end-stage of metaphase arrest halts the cell cycle in mitosis, and when this occurs, cells may follow either of two possible pathways: commitment to a genetic program that results in apoptotic cell death, or a pathway involving the skipping of anaphase, initiating a new cell cycle (endocycle), and forming polyploid or endoreduplicated cells. Cells taking the latter pathway undergo a further round of DNA replication, with the result that at the next mitosis they produce metaphases made up of diplochromosomes (Cortes and Pastor, 2003a; Cortes *et al*., 2003b). Endoreduplication in eukaryotes is a process that involves DNA amplification without corresponding cell division. While the molecular mechanisms which are responsible for endoreduplication are poorly understood, it is a useful endpoint for assessing failure in the proper decatenation of replicated chromosomes before mitosis (Pastor *et al*., 2005). The latter outcome was observed as histopathological changes in tumour sections of mice treated with *F. religiosa* and accordingly, the *F. religiosa* extract is rich in compounds that are able to induce such effects. One of the most encountered constituents in *F. religiosa* extract was polyphenols (flavonoids and phenolic acid). In this regard, it has been demonstrated that flavonoids are very strong inducers of endoreduplication as compared with other investigated chemicals (Cantero *et al*., 2006), and accordingly the authors suggested that the most likely hypothesis for the induced endoreduplication is that the flavonoids action on both DNA and the enzyme topoisomerase II itself results in a highly negative effect on the enzyme performance for chromosome segregation.

The histopathological examinations were extended further but in terms of immunohistochemical evaluation for the expression of regulatory gene, which was P-glycoprotein.

Multi Drug Resistance (MDR) is one of the major mechanisms

responsible for failure of cancer chemotherapy. In cancer cells, the over expression of P-glycoprotein (*P-gp*), a member of the ATP-Binding Cassette (ABC) superfamily transporters implicated in drugs efflux, increases the mechanism of resistance. *P-gp* recognizes a wide range of a polar compound including anticancer agents and extrudes them outside the cell (Tahara *et al*., 2009; Sauna and Ambudkar, 2007).

The results showed a significant increased expression in tumour sections of mice treated with Saline 0.9% and mice treated with docetaxel 20 $mg.Kg^{-1}$, the treatment of prostate cancer resistant to docetaxel represents a significant challenge due to the accumulation of multiple mechanisms of drug resistance and includes changes in classical multiple drug resistant pathways, expression of different b-tublin isotypes, mutations in tumour suppressor proteins and altered expression of pro- and anti-apoptotic protein (Pollock and Challaghan, 2011). Chemoresistant PC-3 cells over express *P-gp*, as a result, these cells may increase their resistance to the selective drugs (Crowe, 2012), which would reduce intracellular concentrations of the drug through increased efflux. Apart from chemical compounds, physical stress, such as X-irradiation, ultraviolet light irradiation and heat shock, are also essential for the induction of *P-gp* expression (Stockner *et al*., 2012). Global gene analysis results have confirmed that expression of some genes is altered associated with take up of docetaxel for long period time in prostate cancer cells (O'neill *et al*., 2011) and developing MDR have been recurrence and rapid growth of tumor, leading to multiple metastases and high morbidity and mortality. Various membrane efflux pumps, including *ABCG2, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, P-gp (ABCB1*) and the prostate resistance protein (PRP) have been reported to play a role in reducing drug intracellular concentrations leading to MDR in tumor cells in vitro (Marquez and Van Bambeke, 2011).

Drug resistance can also be caused by changes in topoisomerase II activity (Braunhut *et al*., 2005), modifications in glutathione S-transferase (Takada et al., 2005), and altered expression of apoptosis-associated protein

Bcl-2 (Reed, 1995), and tumor suppressor protein p53 (Mueller and Eppenberger, 1996). Apoptosis is an essential element in the cytotoxic effect of many anticancer agents (Lutzker and Levine, 1996) and the neutral lipid, ceramide, has been shown to play a role in this response (Hannun and Obeid, 1997).

Depending on histochemistry results' data showed that mice treated with *F. religiosa* extract signed with significantly decrease *P-gp* gene expression, dose depended in manner, as a result of the free radical scavenging property which may be one of the mechanisms by which this plant is effective as anticancer agent. Most of tannins and flavonoids are phenolic compounds responsible for antioxidant properties of many plants (Lee *et al*., 2005b; Lee *et al*., 2005c). Details on the biochemistry of *F. religiosa* structure and compositions, including defense-related proteins have been extensively reviewed (Wititsuwannakul and Wititsuwannakui, 2001). Most of proteases derived from *F. religiosa* plants have been classified as cysteine proteases and more rarely belong to aspartic proteases (Sharma *et al*., 2012). The name of ficin has been used to describe the endoproteolytic enzyme activity in tree latex of the genus *Ficus* (Kaur *et al*., 2011). The ficins isolated from different *Ficus* trees possess different characteristic properties.

Furthermore, *F. religiosa* possesses the anti-oxidant properties, which contain polyphenols exert interesting biological abilities in animal experiments and *in vitro* systems. The compounds are able to trap and scavenge free radicals, decrease leukocyte immobilization, induce apoptosis, inhibit cell proliferation and angiogenesis, and exhibit phytoestrogenic activity (Kuno *et al*., 2012; Higdon and Frei, 2003). Dietary polyphenols interfere with signal transduction pathways related to the carcinogenesis process, thereby acting as chemopreventive agents. They include the suppression of NF-κB and activating protein (AP-1) activation, inhibition of the mitogen-activated proteins (MAPKs)-, protein kinases and growth factor receptor mediated pathways, cell cycle arrest, induction of apoptosis, anti-oxidant and

anti-inflammatory effects, and suppression of angiogenesis (Fresco *et al*., 2006).

Some effective MRP1 inhibitors were identified among the compounds naturally occurring in plants. The ability of flavonoids to modulate MRP1 transport activity has been recognized for some time (Lania-Pietrzak *et al*., 2005; Bobrowska *et al*., 2003; Leslie *et al*., 2001). The ability of phenol compounds to inhibit *P-gp*, another *MDR*-associated *ABC* transporter, has been tested (Duarte *et al*., 2006), additionally; it has been demonstrated to exhibit a synergistic antiproliferative effect when used in combination with doxorubicin, to promote the apoptosis of cancer cells (Duarte *et al*., 2007), and to act as an antitumor promoter in cancer cells infected by human cytomegalovirus (Pusztai *et al*., 2007). helioscopinolide B (Cheng *et al*., 2004) have been found to be effective inhibitors of *P-gp* mediated transport of rhodamine 123 in resistant mouse T lymphoma cells (Ferreira *et al*., 2006).

A number of natural or synthetic compounds have been discovered to inhibit *P-gp* and resensitize resistant tumor cells *in vitro* (Wink, 2007; He and Liu, 2002; Genne *et al*., 1992; Chauffert *et al*., 1990). Although these agents work successfully in some patients, most results of clinical trials were disappointing (Dantzig *et al*., 2001; Solary *et al*., 2000). Some of these reversal agents did not work *in vivo* or some had too severe side effects. Most modulators of ABC transporters act by binding to membrane transport proteins (especially *P-gp, MRP1*, and *BCRP*) as competitive inhibitors, or by indirect mechanisms related to phosphorylation of the transport proteins ,or the expression of the mdr1 and mrp1genes.Other inhibitors not only act at the level of the transporter gene but influence their expression; for example, the alkaloid piperine lowered the expression levels of *ABCB1*, *ABCC1*, and *ABCG2* genes which encode *P-gp*, *MRP1*, and *BCRP* (Li *et al*.,2011).

Lipophilic secondary metabolite, such as monoterpenes, diterpenes, triterpenes (including saponins), steroids (including cardiacglycosides), and tetraterpene function as substrates for *P-gp* in cancer cells. Because of their

lipophilicity, these terpenoids most likely are substrates for *P-gp* and other *ABC* transporter. Flavonoids are characterized by relatively low lipophilicity, despite this difference, in phenol groups of compounds, the derivatives with higher log P-values were weaker inhibitors of BCECF efflux from erythrocytes. The lipophilicity of the inactive compounds was diverse. It seems likely that factors other than lipophilicity (such as the spatial arrangement of electron donor groups in the molecule) (Xia *et al*., 2012) are important for the ability of compounds to inhibit MRP1 transport activity.

Among the structurally heterogenous group that *P-gp, MRP1, BCRP*, and *OATP* in cancer cells with MDR inhibite a large number polar phenolic compounds (phenolicacids, flavonoids, catechins, chalcones, xanthones, stilbenes, anthocyanins, tannins, anthraquinones, and naphthoquinones). Some of them can reverse *MDR* when given in combination with cytotoxic agents (Guz *et al*., 2001; Falcao-Silva *et al*., 2009). Some of the phenolics are lipophilic enough to be competitive inhibitors of *ABC* transporters. Polyphenols are exciting the ring compounds of proteins. They can effectively interact directly with proteins by forming hydrogen and ionic bonds with amino acid side chains. They can thus interfere with the 3D structure of proteins (conformation) and inhibit their activities (Wink, 2008; Wink and Schimmer, 2010). Therefore, that the inhibition seen in polyphenolsis caused by a direct binding and complex formation (not necessarily the active side) of *ABC* transporters. Since many polyphenols have a very low toxicity (e.g., many of them are ingredients of our food, such as flavonoids or tannins), these experimental data can be regarded as a proof of concept that plant secondary products could be interesting candidates for chemosensitization (even if they did not interfere with *ABC* transporters) of cancer.

At the end of this discussion, it is possible to address the following question: *Is it possible to use F. religiosa as a biological therapy in the form of a natural supplement in cancer treatment alone or with chemotherapy drug*? It is difficult to answer such a question, but the present obtained data may augment

the view for an understanding of the biological potential of *F. religiosa*. This can be firstly scoped on a ground of the identified chemical constituents, which are compounds that were presented with important anti-oxidant and anti-tumour potentials both *in vitro* and *in vivo*. If we consider such scenes in a more prospective thinking, it is possible to augment the view that *F. religiosa* is a promising anti-tumour agent, but the *in vivo* evaluation may limit such optimism, because the inhibition rate did not reach 100%, and in best evaluation, it was approximately 30%- 40%, which cannot be ignored. Other evaluations of the implemented tumours may however direct the attention to the pathway of action for *F. religiosa* to reach its 40% inhibition rate; it is possible to introduce *F. religiosa* as an adjuvant with chemotherapy enhancing the anti-tumour non-specific immunity.

Conclusions and Recommendations

I. Conclusions

Based on the obtained results, it is possible to reach the following conclusions:

1. *Ficus religiosa* extracts which have a powerful antioxidant activity were the most cytotoxic against the investigated tumour cell lines (MCF7, PC3, PC3-TxR, K562 and K562Dox) and chloroform *F. religiosa* extract possessed the highest potency in inhibiting growth of cancer cells.
2. Chloroform extract has chemosensitizing effect on prostate cancer cells which acquired resistance against docetaxel (PC3-TxR) and on leukemic cancer cells which acquired resistance against daunorubicin (K562Dox) when companied with drug.
3. Chloroform extract of *F. religiosa* leaves extract is rich in chemical constituents that have important biological potentials; Such as flavonoids and phenolic acids and their isomers.
4. Chloroform extract has potent apoptosis effect agent against PC3-TxR cells, and its action was through down regulation of *P-gp* gene and inhibition P-gp protein synthesis.
5. *F. religiosa* chloroform extract showed no toxic effect which has LD_{50} $11.3 g.Kg^{-1}$ on CD-1 mice, and revealed no cytotoxic effect or reduce in the body weight during period of treatment but chloroform extract was involved in reducing the progression of xenografted tumour in SCID mice.
6. The obtained results from *in vitro* and *in vivo* evaluations suggested the selectivity of *F. religiosa* against cancer cell lines, and it is possible to speculate that *F. religiosa* is promising plant as a dietary supplement or for pharmaceutical production for reducing cancer risk because of their anti-proliferative potential and its cytotoxic effects against cancer cells.

II. Recommendations

The recommendations of these studies are as follows:

1. Study of cytotoxic effect of tannic acid and strotonin compounds on different cancer cells.
2. Investigations are required to define the precise mechanisms that can explain how chemosensitizing effect modifies resistance cancer cell to sensitive one with the application of other chemotherapies used in cancer treatment.
3. Purification and identification of all active compounds found in polyphenolic fraction using GC –MS HPLC.
4. The anti-mutagenic effects of *Ficus religiosa*, future study is recommended to use other new techniques such as: DNA microarray to examine the gene expression profile in *Ficus religiosa* treated tumour cells, *in vitro* and *in vivo*.
5. Study of the immune regulatory effect of *Ficus religiosa* on the basis of functional immunological properties by evaluations of the immune responses employing CD markers (CD4 and/or CD8) and cytokines as tools for such evaluations.
6. Study the other plant extract materials (hot water or hot alcohol extract) for different part of *Ficus religiosa* plant (bark, root, stem, etc) and identified the stage of growth for this plant.

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا
عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ

صدقَ الله العَظيم

سورة البقرة ألاية "٣٢"

الخلاصة

يعد نبات لسان العصفور من النباتات الطبية الهامة و التي تستخدم عادة لعلاج مختلف الأمراض بما في ذلك التهاب الضرع ، التهاب الأذن الوسطى ، التهاب البلعوم و الحنجرة ، التهاب الإحليل، وعسر الطمث و السكري. أجريت هذه الدراسة لتقييم المضادات الورمية لأوراق لسان العصفورالتي جمعت من بغداد ، العراق ، وكما يلي:

1- تم اختبار مستخلصات مختلفة من نبات لسان العصفور (كلوروفورم ، بوتيل الكحول ، الميثانول المطلق و مستخلص الخام مائي) لتحديد تأثيراتها السامة على الخلايا ،سرطان الثدي (MCF7) ، وسرطان البروستات حساسة للدوسيتاكسيل (PC3) ، ومقاومة لدوسيتاكسيل (TXR - PC3) ، وسرطان الخلايا اللوكيميا حساسة للداونوروبيسين (K562) والمقاومة للداونوروبيسين إلى (K562Dox) خطوط الخلايا السرطانية باستخدام فحص Sulforhodamine-B .ثم اختيار افضل مستخلص سبب تثبيط في نمو الخلايا السرطانية لدراسة تاثير التحسس الكيميائي على الخلايا السرطانية باستخدام فحص SRB و تم تشخيص المركبات الفعالة في المستخلص الاكثر سمية بأستخدام HPLC-DAD و دراسة قدرة المستخلص في تثبيط التعبير الجيني لجينات: *P-gp, Nrf2, EGFR, Patch1, Gli2, d1, Id2 and Id3* على خلايا PC3- TXR باستخدام تقنية RT-PCR و Western blot.

2- قد اجري تقييم التأثيرالسام للمستخلص في الجسم الحي بواسطة تحديد LD_{50} وتقييم المضادات للورم (histopathologically و immunohistochemically لجين P-gp) في الفئران SCID .

أظهرت النتائج في الزجاج أن التركيز التثبيطي (IC_{50}) ضد خطوط الخلايا التي عوملت باربعة مستخلصات لنبات لسان العصفور ،انخفاض (IC_{50}) للخلايا معنويا ($P \leq 0.05$) وكان التأثيرمعتمدا على مقدار الجرعة. يتبين أن مستخلص الكلوروفورم عالي السمية ضد PC3- TXR بينما كان المستخلص المائي الخام أقل سمية . وأجري التحليل الكيميائي لمستخلص الكلوروفورم باستخدام (HPLC -DAD)، وفقا لذلك ، شخصت خمسة مركبات مختلفة (quercitin, myrecitin, tannic acid, gallic acid and serotonin) . وفضلا عن ذلك فقد كشف عن حمض التانيك و السيروتونين في المستخلص، في حين لم يتم الكشف عن بقية المركبات النقية الأخرى ، مع وجود مجموعة من المركبات في المستخلص والتي اعتبرت غير معروفة . وقد لوحظ أعلى تركيز لحمض التانيك في المستخلص بينما كان أقل تركيز للسيروتونين . أتضح أن مستخلص الكلوروفورم انه يتمتع بتحسس كيميائي عالي ضد خط الخلية PC3- TXR مقارنة بخطوط الخلايا السرطانية الاخرى. مضافا الى ذلك ، يمتلك مستخلص الكلوروفورم قوة تثبيطية سامة ضد نمو الخلايا السرطانية PC3- TXR ، من خلال

التنظيم المثبط لتعبير جينات *P-gp*، *Id2* و *Id1* و كان التأثير بشكل ملحوظ بعد 24 ساعة من المعاملة. أكدت نتيجة Western blot أن مستخلص الكلوروفورم مثبط لانتاج P-gp بروتين في خطوط الخلايا السرطانية PC3- TXR . وكانت الجرعة المميتة (LD_{50}) لمستخلص الكلوروفورم من أوراق لسان العصفور 11.3 غم/كغم واظهرت النتائج أن مستخلص الكلوروفورم فعال بشكل واضح في أختزال حجم الورم بطريقة تعتمد على الجرعة ، بالمقارنة مع الفئران المحقونة بالوريد بدوسيتاكسيل (20 ملغم/كغم) مرة واحدة أسبوعيا والذي يرتبط بآثار جانبية ضارة وكذلك ملاحظة أنخفاض في وزن الفئران أكثر من 30 ٪ مقارنة مع المجموعات الأخرى. كشفت فحوصات التقطيع النسجي في مقاطع الأورام من الفئران التي جرعت بالمستخلص أنخفاضا في الانقسام الخيطي وزيادة في الأوعية الدمية في الخلايا. أظهرجين *P-gp* جين إيجابية التعبير في الخلايا السرطانية التي عوملت مع دوسيتاكسيل لوحده (سيطرة موجبة) ، في حين أنها أظهرت انخفاض التعبير في مقاطع الورم من الحيوانات المعالجة عن طريق الفم مع جرعة 3.8 غم/ كغم و على جرعة 7.3غم/ كغم.

أعتمادا على النتائج يمكن أن نستنتج أن نبات لسان العصفور يمتلك نشاطا مضادا للخلايا سرطانية في الحي والزجاج .

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

التحري عن التاثيرات ضد السرطانية لاوراق نبات لسان العصفور (*Ficus religiosa*)

أطروحة

مقدمة إلى مجلس كلية العلوم جامعة النهرين وهي جزء من متطلبات

نيل درجة دكتوراه فلسفة في علوم التقانة الإحيائية

من قبل

نيران علاء ابراهيم

بكالوريوس في التقانة الاحيائية الجامعة النهرين - 2003

ماجستير في التقانة الاحيائية جامعة النهرين–2007

اشرف عليها

الاستاذ الدكتور
ناهي يوسف ياسين

الاستاذة الدكتورة
خلود وهيب عبود

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2013

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