IN VITRO AND *IN VIVO* STUDIES OF SOME NEW QUINAZOLINONE–BASED COMPOUNDS IN BREAST CANCER

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ABSTRACT

The synthesis of quinazolinone-Schiff bases compounds attracted great attention over the past few decades as an alternative mean to produce analogues of natural products. Quinazolinone compounds, sharing the main principal core structure, are currently announced in the clinical trials and pharmaceutical markets as anti-cancer agent. Therefore, there is a high clinical interest to identify new drugs that could be used to control the growth and expansion of cancer cells. In the present study, the cytotoxicity effect of some new quinazolinone compounds were tested on MDA-MB-231 and MCF-7 human breast cancer cell lines. This study also evaluated the induction of apoptosis by these compounds and their possible mechanisms of action on MCF-7 cell line. Cell Morphological changes, ROS generation, cytochrome *c* release, caspases activity and inhibition of NF-κB were also analyzed. MTT cytotoxicity test showed all five compounds demonstrated a potent anti-proliferative effect in MCF-7 cells, with IC_{50} value of a range of 3-6 μg/ml after 72 h of treatments. However, they showed no significant effect on MDA-MB-231 human breast cancer cell and MCF-10A human normal breast cell line compared to MCF-7 cell line. Most apoptosis morphological features in treated MCF-7 cells were observed by AO/PI staining. All compounds were found to possess a significant effect on perturbation in mitochondrial membrane potential and cytochrome *c* release from the mitochondria to the cytosol. They all triggered activation of caspase-9 and caspases-3/7 which imply the involvement of intrinsic pathways in the observed apoptosis. Compound 1, 2 and 3 also triggered expression of caspase-8 which exhibited the involvement of extrinsic pathway. However, treated MCF-7 cells with compounds 4 and 5 showed no activation of caspase-8 and nor suppression effect on NF-κB translocation, indicating the excluding of the involvement of extrinsic apoptosis pathway. Moreover, the toxicity assessment of quinazolinone compounds were also performed on the renal and hepatic function of ICR

female mice at 250 mg/kg and the results revealed no adverse effects on the organ weight, body weight, serum biochemistry, and histopathology. Chemopreventive effect of compounds 2 and 4 against LA7-induced cancer in rats were also evaluated. The assessment of enzymatic antioxidants showed significant elevations of superoxide dismutase and catalase activities and a reduction in the level of malondialdehyde in treated groups. In addition, the histopathological assessments revealed that the rat mammary glands were protected from the carcinogenic effects of LA7 cells by compounds. Treatment with 2 and 4 also up regulated the expression of Bax and P53, however; down- regulated expression of Bcl-2 and PCNA in the breasts of LA7-induced rats in immunohistochemistry assay. Our results demonstrate a significant role of quinazolinone-based compounds as anti-proliferative agent toward human breast cancer which triggered apoptosis *in vitro* and *in vivo.*

ABSTRAK

Pensintesisan sebatian bes quinazolinone-Schiff telah mendapat perhatian yang meluas sejak beberapa abad yang lalu sebagai alternative dalam penghasilan produk-produk semulajadi. Sebatian quinazolinone yang merupakan teras tunjang struktur amat giat digunakan dalam ujian klinikal dan pasaran farmaseutikal sebagai ejen anti-kanser. Oleh yang demikian, wujudnya keperluan dan kepentingan yang tinggi dalam ujian klinikal dalam mengenalpasti drug baru yang boleh digunakan dalam mengawal pertumbuhan dan perkembangan sel-sel kanser. Dalam kajian semasa, kesan sitotoksik pada segelintir sebatian quinazolinone yang baru telah diuji ke atas titisan sel MDA-MB-231 dan MCF-7. Kajian juga dijalankan untuk menilai penginduksian apoptosis yang disebabkan oleh sebatian-sebatian ini serta tindakan mekanisme yang berkemungkinan akan berlaki ke atas titisan sel MCF-7. Perubahan ke atas morfologi sel, generasi ROS, pelepasan sitokrom c, aktiviti kaspase dan penindasan NF-κB turut dianalisa. Ujian sitotoksi MTT menunjukkan kesemua lima sebatian mempunyai kesan anti proliratif yang kuat dalam cell MCF-7 dengan nilai IC50 dari 3-6 μg/ml pada rawatan selama 72 jam. Namun demikian, tiada kesan signifikan yang diperhatikan pada sel kanser MDA-MB-231 dan sel normal MCF-10A apabila dibandingkan dengan sel MCF-7. Kebanyakan sifat-sifat morfologi apoptosis dalan sel MCF-7 yang dirawat dapat diperhatikan dengan pewarnaan AO/PI. Kesemua sebatian didapati memiliki kesan signifikan terhadap gangguan potensi membran mitokondria dan pelepasan sitokrom c dari mitokondria ke sitosol. Kesemuanya memulakan pengaktivasian kaspase-9 dan kaspase-3/7 yang membuktikan penglibatannya dalam laluan intrinsic dalam apoptosis yang dikaji. Sebatian 1, 2 dan 3 turun mencetuskan pengekspressan kaspase-8 yang membuktikan penglibatan laluan esktrinsik. Namun demikian, sell MCF-7 yang dirawat dengan sebatian 4 dan 5 tidak menunjukkan sebarang pengaktivasian kaspase-8 dan penindasan kesan terhadap translokasi NF-κB, yang

menunjukkan pengecualian penglibatan laluan ekstrinsik apoptosis. Selain itu, penilaian toksisiti sebatian quinazolinone juga dijalankan ke atas tikus betina untuk mengkaji fungsi renal dan hepatik pada 250 mg/kg. Keputusan menunjukkan tiada kesan sampingan yang didapati pada berat organ, berat badan, serum biokimia dan histopatologi. Kesan kemopreventif sebatian 2 dan 4 terhadap tikus aruhan-kanser LA7 turut dikaji. Penilaian antioxidant enzim menunjukkan elevasi signifikan pada superoksid dismutase dan aktiviti katalase serta penurunan kadar malondialdehyde pada kumpulan yang diuji. Tambahan, penilaian histopatologi menunjukkan kelenjar mamari tikus dilindungi dari kesan karsinogen oleh sebatian dalam sel LA7. Rawatan dengan sebatian 2 dan 4 telah menaikkan paras regulasi Bax dan P53 serta menurunkan paras ekpresi Bcl-2 dan PCNA dalam mamari tikus-teraruh LA7 yang ditunjukkan dalam asai immunihistokimia. Keputusan kajian kami menunjukkan bahawa sebatian bersifat quinazolinone mempamerkan anti-proliferatif terhadap kanser payu dara manusia yang telah mencetuskan apoptosis secara in vitro dan in vivo

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Abbreviation	Description
%	Percentage
/	Divide by
µl	Microlitre
µm	Micrometer
˚C	Degree Celsius
<	Less than
±	Plus minus
Abs	Absorbance
ACUC	Animal Care and Use Committee
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2
BW	Body weight
CAT	Catalase
CO2	Carbon dioxide
DHE	Dihydroethidium
DCFH-DA	2′, 7′-dichlorofluorescin diacetate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

dTMP	deoxythymidine monophosphate
et al.	and other people
EDTA	Disodium Ethylene Diaminetetracetate
EtOH	Ethanol
ER	Estrogen recepotor
FFPE	Formalin Fixed and Parraffin Embbeded
FBS	Fetal bovine serum
GGT	Gamma-glutamyl transpeptidase
H	Hour
HCS	High content screening
H&E stain	Hematoxylin-eosin stain
H2O	Water
HIV-1	Human immunodeficiency virus
HD	High dose
I.P	Intraperitoneal
IC_{50}	Inhibitory Concentration (50%)
IHC	Immunohistochemistry
Kg	Kilogarm
LD	Low dose
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
MDA	Malondialdehyde
mg	Milligram
Min	Minute/s
ml	Milliliter
mM	Micromole

Mm	Millimeter
mmol	Millimole
MMP	Mitochondrial membrane potential
MTT	3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NC	Normal control
NF-κB	Nuclear factor-kappa B
OECD	Organization for Economic Cooperation and Development
NCCLS	National committee for clinical laboratory standards
NGOs	Non-governmental organizations
nm	nanometer
PBS	Phosphate buffer saline
PCNA	Proliferating cell nuclear antigen
ROS	Reactive oxygen species
SD	Standard deviation
S.C	Subcutaneous
SD	Sprague Dawley
SEM	Standard error of the mean
SOD	Superoxide Dismutase
T	Tumour
TAM	Tamoxifen
TC	Tumour control
TBARS	Thiobarbituric acid reactive substance
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TNF-α	Tumour necrosis factor alpha
UM	Universiti of Malaya

UV	Ultraviolet
v/v	Volume over volume
w/v	Weight over volume
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 Cancer

Cancer is the second largest cause of death in developed countries and third largest cause of death in the developing world representing a leading public health problem (Thongchai, 2014). Cancers are assemblies of cells that originate from a single cell in a part of body which start to grow out of control and can be defined by a deficiency of normal growth regulation (Saslow et al., 2007; Menzies et al., 2014). Cancerous cells often are transportable to other parts of the body, where they initiate to grow and generate new tumors and consequently replace normal tissue. This process is called metastasis. So far, more than one hundred types of cancer have been diagnosed and categorized based on types of tissue being affected and the main cause of cancer, if it is a genetic factor or viral infection or a combination of both (Vollset et al., 2013).

1.2 Breast cancer

Breast cancer is the most common form of cancer in women worldwide (Koduru et al., 2007). Among women, breast cancer has the highest occurrence, forming 31.1% of newly diagnosed cancer cases (Protani et al., 2010). Approximately, one million women develop breast cancer and almost 600,000 die per year worldwide (Tew et al., 2014). Over the past few decades, the number of cases has increased significantly in Asia because of the increase in life expectancy, the advance in lifestyle and the enormous changes in epidemiological features such as decrease in the birth and breast-feeding rates. The proportion of Asian cancer deaths has increased every year at a more rapid rate than the world average. Particularly, the incidence of breast cancer in Malaysian women has increased annually. Out of each 100,000 females 47.4 of the females are diagnosed with breast cancer as reported by the Malaysian National Cancer

Registry Report for the year 2005 and accordingly the percentage of breast cancer mortality upsurges of the middle-aged Malaysian women increased over the last century (Ahmadian & Abu Samah, 2013). The five-year survival rate is becoming decreasingly prevalent and unfortunately many physicians do not investigate for breast cancer till it is diagnosed by mammography. The sudden rise of symptoms appearance at the postmenopausal age is a significant problem, as they are often attributed to normal ageing.

Cancer is a group of cells, characterized as lack of normal growth regulation. This regulation is known as apoptosis or programmed cell death, a vital well-regulated process that play vital role in the maintenance of tissue homeostasis as well as elimination of damaged cells. In cancer cells, apoptosis is suppressed and required to be triggered which is a key factor in area of anticancer drug development (Elmore, 2007; Hunter, 2007). Breast cancer is a malignant tumor that initiates in the cells of the breast where cancer cells can invade into surrounding tissues or metastasize to distant areas of the body (Saslow et al., 2007). The aim of treatment is reliant upon the stage of the cancer at the time of diagnosis (Shupe et al., 2014). The survival percentage ranges from 84% for the early stage of illness to 18% for cases at the advanced stages of cancer. Therefore, the main purpose of the treatment at early breast cancer (EBC) is to prevent its recurrence and lengthen overall survival without causing complications (Sainsbury, 2013). Despite the implication of different treatments for advanced breast cancer, the aim of treatment mainly is to attain a sustainable statement of response to the therapy and increase the quality of life of patients with minimum toxicity effects involved with treatment (Tryfonidis et al., 2013).

Quinazoline nucleus is an interesting molecule among the most fundamental classes of aromatic bicyclic compounds with two nitrogen atoms in their structure. It is consisting

of aromatic benzopyrimidine system made up of two fused six member simple aromatic rings benzene and pyrimidine ring (Figure 1.1) (Faraj et al., 2014).

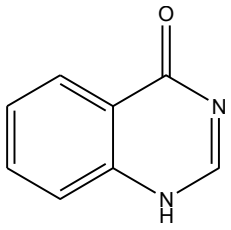


Figure 1.1: Quinazolin-4-one

More attention has recently been paid to quinazolinones and their derivatives due to the wide range of their uses in medical chemotherapy (Manasa et al., 2011). A brief survey about biological importance of quinazoline and their derivatives revealed that a large number of publications began to appear after 1960s. They have been identified with wide range of biological and pharmaceutical activities such as: anticancer (AL-Zubiady & Ibrahim, 2013), antioxidant (Vagdevi et al., 2012), antiviral (Krishnan et al., 2011), and anticonvulsant (Patel et al., 2010), anti-inflammatory (Saravanan et al., 2010), anti-HIV (Alagarsamy et al., 2006) and etc.

Although the current anticancer quinazoline-based agents have revealed great clinical benefits in cancer treatment (Selvam & Kumar, 2011), we still need to establish better anticancer agents from quinzoline derivatives with minimum adverse side effects (Kranz & Dobbelstein, 2012) that provides much more hope to mankind. The aim of this study was to develop potential anticancer agents against breast cancer cell line and screen for their possible mechanism either intrinsic or extrinsic mitochondrial pathways possessed by five newly quinazolinones derivatives that could be excellent candidates for chemoprevention and treatment of breast cancer, with possibly less or without side-effects as compared to generally used chemopreventive agents.

1.3 Hypothesis of the research

The present study might offer important facts in order to solve the existing problems to treat human breast cancer in Malaysia. It is expected that the newly synthesized quinazolinones will affect breast cancer cells through the induction of apoptosis without damaging normal breast cells. Finding out the mechanism of the compounds as anti-cancer proposes the induction of apoptosis through either cellular mitochondrion signaling or extrinsic signaling pathways. The present research hypothesized that synthesized quinazolinones possess cytotoxic effect on two human breast cancer cell lines (MCF-7 and MDA-MB-231) and an anti-proliferative effect on LA-7-induced tumour in rat mammary glands.

1.4 The objectives of this study

Main objective:

- To assess the effect of quinazolinone-based compounds as anti-proliferative agents toward human breast cancer to trigger apoptosis *in vitro* and *in vivo*.

Specific objectives:

1. To assess the *in vitro* cytotoxic activity of compounds on the human mammary cancer cell lines (MCF-7 and MDA-MB-231).
2. To assess the mechanism of cell death induced by compounds.
3. To determine the acute toxicity of compounds on ICR mice.
4. To study the chemoprotective effect of selected quinazolinone-based compounds against breast carcinogenesis in Sprague Dawley rats.

CHAPTER 2: LITRATURE REVIEW

2.1 Breast cancer overview

Aside from being the most common cancer affecting women, breast cancer is also the major cause of death among women globally (Thongchai, 2014). Breast cancer represents 31.1% of newly diagnosed cancer cases among women (Protani et al., 2010). Based on the latest reports, approximately one million women are diagnosed with breast cancer globally every year (Tew et al., 2014). In 2008, the National Cancer Registry (NCR) stated that the risk of Malaysian women to develop breast cancers in their lifespan is almost one in twenty women which is still considered to be low compared to one in eight in Europe and the United States (Porter, 2009). During 2003 to 2005, the occurrence of breast cancer among Malaysian women has surged, with a frequency of 47.4 per 100,000 females, as estimated by the National Cancer Registry Report (Ahmadian & Samah, 2013). In 2012, The International Agency for Research in Cancer (GLOBOCAN) estimated the rate of breast cancer in Malaysian women as 38.7 per 100,000 (Yip & Bhoo, 2014). Since, these incidence rates are closely associated with the lifestyle and environment factors, women should consider protective measures to prevent this life-threatening disease. It has been showed that up to 70% of the development of breast cancer in women result from environmental factors and lifestyle and only 30% from genetic factors (McPherson et al., 2000).

Besides the consequence of secondary diseases or metastasis, late diagnosis commonly is considered as major contributing factor in most breast cancer deaths. Therefore, early detection of disease prior the invasion of cancer to the surrounding tissue or metastasize to distant sites can significantly reduce the possibility of death risk and, eventually, improve the quality of life of patients (Ceber et al., 2013). Moreover, it might also

decrease the suffering and expenses of treatment. As the key to declining risk of death is generally via early detection, the main focus of most researchers in oncology and cancer therapy is now on prevention, screening, early detection and consequently prompt action to the breast cancer (Adesunkanmi et al., 2006; Ceber et al., 2013).

Recently, great attention has been recently paid concerning the awareness of breast cancers in Malaysia. The Ministry of Health and several non-governmental organizations (NGOs) including National Cancer Society of Malaysia and National Cancer Council (MAKNA), enthusiastically are involved in cancer welfare services (Lim, 2002). In addition, many accomplishments have been attained, such as a cancer education program, cancer counselling services, psychological support for cancer patients and welfare services in order to raise people's awareness about breast cancer as well as women's health issues. Nowadays, more information about breast cancers are provided via articles in local newspapers, women's magazines, and on television.

2.2 Human normal breast

The female breast comprises mainly of lobules (milk-producing glands), ducts (tiny tubes that carry the milk from the lobules to the nipple), and stroma (fatty tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels) (Hassiotou & Geddes, 2013). Lobules and ducts are formed by epithelial cells whose role is to produce and to secrete the different constituents of milk. The epithelial cells are enclosed by a stratum of myoepithelial cells, which are attached to a basal membrane and play role in maintenance of the tubular structure of the ducts and lobules (Hondermarck, 2003). The lobules and ducts are surrounded by a large amount of connective and fat tissues that shape the form of breast (Figure 2.1).

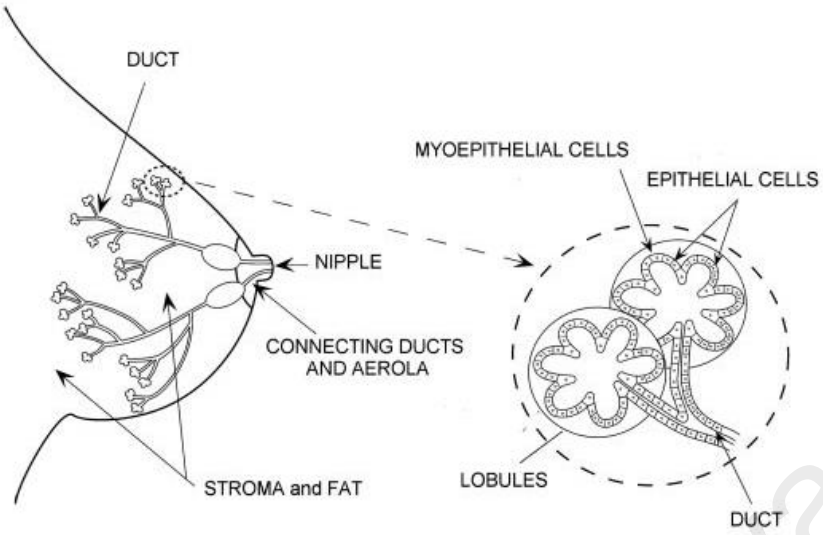


Figure 2.1: Anatomy of the female breast (Hondermarck, 2003)

2.3 Types of breast cancer

Most breast tumours initiate from epithelial origin, and thus, the great numbers of malignant breast tumorus are considered as carcinomas (malignant epithelial tumors). Sarcomas (malignant tumors arising from connective tissue) are rarely detected in the breast The term "breast cancer" covers many types of tumors that are categorized based on their origin and to their histological properties (Hondermarck, 2003).

There are mainly two classes of the breast tumours: *in situ* carcinomas (Figure 2.2) which are characterized by tumor cells originated either in the ducts (ductal carcinomas in situ, DCIS) or the lobules (lobular carcinomas in situ, LCIS) (Figure 2.3), without invasion over the basement membrane into the surrounding stroma. In contrast, invasive carcinomas, the basement membrane is partially or completely destroyed and cancer cells gradually attack surrounding tissues, which ultimately leading to metastatic event (Weigelt & Reis-Filho, 2009). The class of invasive carcinomas comprises more than 10 diverse types. The invasive ductal carcinomas include 65–80% of all breast cancers,

however; the invasive lobular carcinomas possess 5–15% of the cases, whereas other breast cancer types such as mucinous, medullar, tubular, and apocrine carcinomas are less common (0.1–4%) (Hondermarck, 2003).

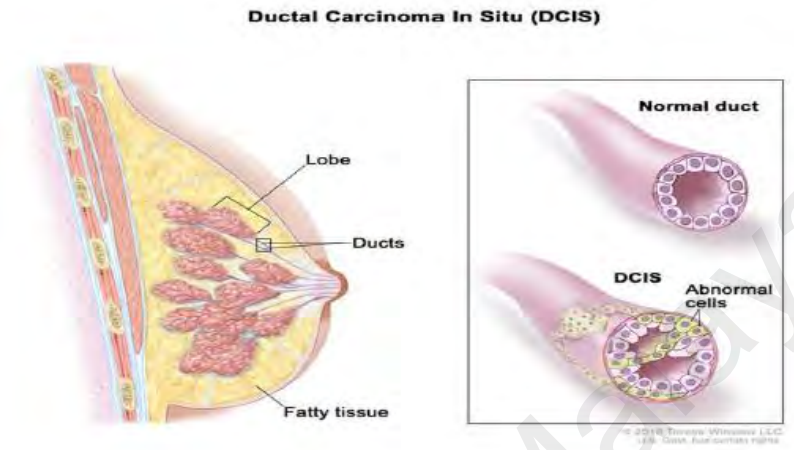


Figure 2.2: Ductal carcinoma in situ (DCIS). (Adopted from National Cancer Institute, 2012).

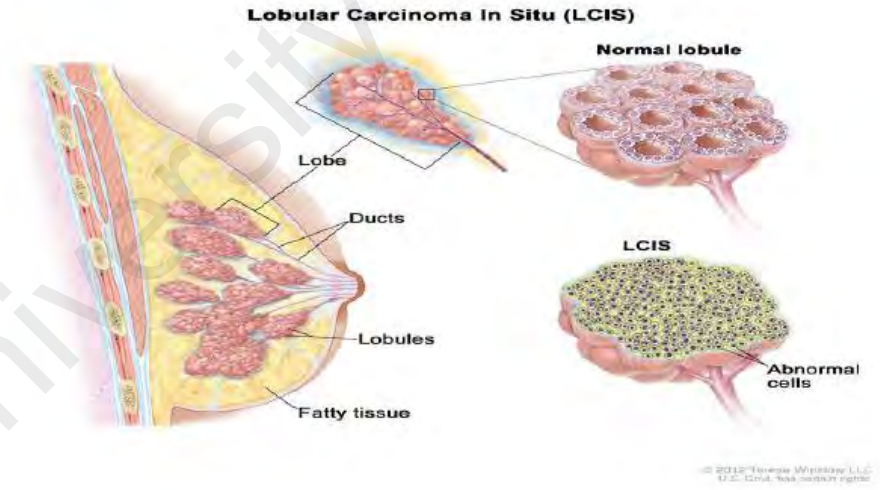


Figure 2.3: Lobular carcinoma in situ (LCIS). (Adopted from National Cancer Institute, 2012).

Besides in situ and invasive carcinomas, Paget's disease of the nipple is a particular form of malignant epithelial tumour, where malignant cells infiltrate the epidermis.

Lastly, a number of tumors are known as benign (precancerous form) which is expected that around 6-8 years' period spend between the appearance of the primary cellular changes leading to hyperplasia and the detection of breast tumour by mammography. Moreover, normal epithelial, myoepithelial, fibroblastic, and endothelial cells are also trapped in breast tumours and consequently remain their development in a transformed environment. (Hondermarck, 2003).

2.4 Risk factors for breast cancer

Breast cancer is a complex disease that several factors contribute to cause the disease and a number of risk factors have been documented to affect its development. The most important risk factors are age, family history of breast cancer, reproductive history and sustained exposure to endogenous steroid hormones (Chlebowski et al, 2013). Other risk factors include exposure to radiation, use of hormonal contraception, duration of breastfeeding, alcohol consumption and smoking, menopausal hormone therapy and diet (Hulka & Moorman, 2001; Ellberg, 2011).

2.4.1 Gender

Being a woman is the main risk factor for developing breast cancer. Men can also develop breast cancer, but this disease is about 100 times more common among women than men. This is most probably because of hormones estrogen and progesterone which can enhance breast cancer cell growth (Helmrich et al., 1983; Aren et al., 2012).

2.4.2 Aging

On average, women over 60 are more likely to be diagnosed with breast cancer. However, this may vary for different races or ethnicities (Yancik et al., 1989).

2.4.3 Genetics and inheritance

Genetic susceptibility is a crucial factor to promote the development of breast cancers. In general, around 10 to 15% of all breast cancer cases result from genetic predisposition (Ghoussaini et al., 2013). Epidemiological investigations identified several risk factors that lead to causing genetic mutation including radiation, environmental pollutants and viruses, failure of the immune system to eliminate the malignancy at an early phase, irregular expression of growth factors involving epithelial and stromal cells, and lastly, inherited genetic defects particularly in the DNA repair genes (Irigaray et al., 2007).

Family or hereditary history of breast cancer could be an essential risk factor to cause the cancer. Almost 25% of all cases of the disease taking place in women aged less than 30 years (Ceber et al., 2013). The most common cause of hereditary breast cancer is a mutation in the BRCA1 and BRCA2 genes which are characterized as tumor suppressor genes. Approximately 90 to 95% of familial breast cancer cases are due to abnormalities of either BRCA1 or BRCA2 genes. The women with an inherited BRCA1 or BRCA2 mutation might be affected by 80% chance of developing breast cancer over their lifetime However, the remaining may be caused by other major tumour suppressor genes, such as p53 and ras (Keen et al., 2003; Mavaddat et al., 2012).

Beside the tumour suppressor genes, oncogenes are also known as risk factor of breast cancer. Mutation in the oncogenes such as HER2, src, myc and ras have been extensively related to breast cancers Modifications of these genes definitely lead to abnormal expression of oncoproteins and consequently result in malignant transformation (Slamon et al., 1989).

2.4.4 Exposure to estrogen

Exposure to endogenous estrogen plays a significant role in the development and growth of breast cancer which is known as main reason of high incidence rate of breast cancer in females. Although the exact mechanisms remain to be completely clarified, the alkylation of cellular molecules and the generation of free radicals that can damage DNA together with the potential genotoxicity of estrogen and some of its metabolites (e.g., the catechol estrogens) have been associated (Clemons et al., 2001). Consequently, DNA Damaging lead to triggering mutations in the epithelial cells in the breast and consequently which resulting in unregulated cell proliferation and differentiation (Arens et al., 2012). Intakes of alcohol, fat, antioxidant vitamins, and fiber may influence exposure to estrogen and the risk of breast cancer (Clemons et al., 2001). Furthermore, it has been proved that childless women and women having children later in life are at an increased risk of developing breast cancer. However, women having a first child before 20 years of age have a 50% reduction in lifetime breast cancer risk when compared with women who do not have children. This protective effect is mainly owing to estrogen receptor positive breast cancer (Britt et al., 2007).

2.5 Breast cancer screening

The primary principle for breast cancer screening is to detect the breast cancers before they become palpable. Early detection means the application of a technique or strategy that resulting in earlier diagnosis of nonpalpable (Saslow et al., 2003). Breast cancer is a progressive disease, and minor tumors are more likely to be early stage, and are more successfully treated (Tabár et al., 1999). Breast cancers are commonly detected using different screening approaches such as mammography, clinical breast examinations (CBE) and breast self-examinations (BSE) (Saslow et al., 2007).

2.5.1 Mammography

Mammography has been recognized for detection of breast cancer at an early stage and when followed up with suitable diagnosis and treatment, to decrease mortality from breast cancer (Saslow et al., 2007). The advantages of mammographic screening are generally recommended for women aged over 40 years old with the average risk of breast cancer. In older women, screening decisions should be personalized concerning the potential benefits and risks of mammography according to current health status and estimated life expectancy (Saslow et al., 2003). Women should be aware of limitations, and potential harms involved in regular screening. There are some limitations in this method. Firstly, it has been announced that X-rays can potentially induced carcinogenesis, and secondly, a breast tumor should be at least a few millimeters in size, while a tumor of this size already contains several hundred million cells. Thus, it is already late when a breast tumor is detected by mammography. However, X-ray mammography is still the best approach available for the early detection of breast cancers (Hondermarck, 2003).

2.5.2 Breast self- and clinical examinations

Women in their 20s should be informed about the advantages and limitations of breast self-Examination (BSE). The prompt recording of any new breast symptoms to a health expert can be significant action. For women in their 20s and 30s, it is advised to do clinical breast examination (CBE), preferably at least every three years and those aged 40 and over should remain clinical breast examination annually (Saslow et al., 2003).

2.5.3 Imaging

Numerous dominant imaging diagnostic approaches have been developed in order to detect breast cancers. These approaches comprise digital mammography, magnetic resonance imaging (MRI), positron emission tomography (PET), magnetic resonance

spectroscopy (MRS), scintimammography and ultrasonography (Hendrick et al., 2010). The application of these progressive tools demonstrates a potential for powerful innovations in cancer diagnosis that will enhance efficiency in the detection of breast cancers. In 2007, American Cancer Society Guidelines indicated that women at high risk of breast cancer could benefit from additional screening strategies, such as MRI other than mammography and physical examination. The disadvantage of this method is that they are still too expensive to be used by most people. Among all cancers, breast cancer is apparently the easiest to be detected over physical checkup; however most of the proved breast cancer cases are at progressive stages and have metastasized to distant organs (Saslow et al., 2007).

2.5.4 Tumour markers

Serum tumour markers are glycoproteins, which can be identified by monoclonal antibodies. These markers include mucins, such as cancer antigen (CA) 15.3 and CA 27.29; carcinoembryonic antigen (CEA), α-fetoprotein (AFP), oncoproteins, such as c-erbB2, c-myc and p53; and cytokeratins, such as tissue polypeptide antigen (TPA) and tissue polypeptide specific antigen (TPS) (Yerushalmi et al., 2012). Although increases of these proteins in plasma are extensively associated with primary breast cancers, the clinical efficacy in early detection of breast cancer are limited because of their low sensitivity or specificity (Duffy, 2013). Thus, none of the serum biomarkers have been used for the early detection of breast cancers (Khatcheressian et al., 2013). Among all of serum tumour markers, mucins and CEA have been suggested in monitoring the disease recurrence after therapeutic treatment in advanced breast cancers (Aebi et al., 2011).

2.6 Modern techniques in breast cancer detection

2.6.1 Molecular approaches

Recently, molecular approaches have been proposed more sensitive and reliable techniques for the screening and detection of breast cancers. Enzymes Linked Immunosorbent Assay (ELISA) is widely used as diagnostic tools for breast cancer which can be applied to breast nipple discharges. Ambrosi and colleages (2009) developed an optical enzyme-linked immunosorbent assay (ELISA) immunoassay for the analysis of CA15-3 antigen, characteized in mucins class, and useful for the follow-up of the clinical therapy of breast cancer. ELISA is also able to detect a new serium biomarker namely mammoglobin which possess remarkable development in detection of breast cancer (Batta et al., 2012).

In aera of chromosomal analysis, either numerical or structural modifications in the diploid state of chromosomes in breast epithelial cells can be signs of pre-cancerous and cancerous predisposition of the breast cancers (Nik-Zainal et al., 2014). There are two commonly used methods for detection of chromosomal changes regarding the breast cancers; Comparative genomic hybridization (CGH) and Fluorescence in situ hybridization (FISH) (Lin et al., 2012).

2.6.2 Immunohistochemistry

Immunohistochemistry (IHC) is a protein-based method used to detect malignant cells which is based on antigen–antibody interaction with high sensitivity and specificity. Among all well known breast cancer markers that can be detected by IHC, Human Epidermal Receptor Protein-2 (HER-2) is commonly use (Wulfkuhle et al., 2012). HER-2 oncogene protein is a transmembrane glycoprotein in the epidermal growth factor receptor family which is expressed at low levels in breast duct epithelium, but

amplification of the HER2 gene and protein overexpression are present in 10–20% of primary breast cancers. Besides HER-2, the presence of estrogen receptors (ERs) and progesterone receptors (PRs) can also be detected by IHC using particular antibodies. Estrogen and progesterone receptors are weak prognostic markers of upshot; however, they are strong predictive markers of response to endocrine, for example, tamoxifen-based therapy (Allred, 1998; Fagan et al., 2012). Morover, IHC is also used for detemination the proliferative status of the malignant cells in breast cytology specimens via the detection of proliferation markers, such PCNA, Ki-67, cyclins and telomerase (Malkas et al.,2006; Bojovic & Crowe, 2013). Immunihistochemistry asssay is also can assess the expression level of pro-apoptotice and anti-apopotoic proteins such as Bax, Bcl-2 and caspase 3 which are implicated in the induction of apoptosis through intrinsic apoptosis pathways (Rubio et al., 2005; Karimian et al., 2015).

In spite of all current methods for screening, detection and diagnosis of breast cancer, none has shown independently potential as a gold standard for cancer detection. Therefore, a multidisciplinary approach including pathology, physiology, cytology and biochemistry should be considered as the diagnostic profile for cancer detection (Mach et al., 2013).

2.7 Treatment of breast cancer

Most women with breast cancer require some type of surgery. It depents on the stage of disease, patients mainly undertake two type of surgury including lumpectomy or breast-conserving surgery and mastectomy which lead to removing the whole breast. Fortunetly, nowadays there is a reconstuctive surgery to replace the excised breast tissue in order to prevent tragic psychological consequences (Cochrane et al., 2003; Smith et al., 2013).

Chemptherapy is a type of cancer treatment that uses chemical substances containing one drug or combination of different anti-cancer drugs. It has been discovered that hormone-sensitive and -insensitive breast cancers may have different metastatic patterns and consequently sensitivities to chemotherapy. This stauts led to new strategies that include the assessment of the extent and pace of disease spread, the location of metastasis, and the estrogen and progesterone receptors of the cancer cells (Baselga et al., 2012; Anders et al., 2013). Patients with significant expression in a particular hormone receptor are normally suggested to undertake endocrine therapy. The most commonly used endocrine treatments for breast cancers are the selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene (Jordan, 2004); selective estrogen receptor down-regulators (SERDs), such as fulvestrant (Howell et al., 2004); gonadothropin analogues, such as goserelin and leuprolide (Mounsey et al., 2006); and selective aromatase inhibitors, such as anastrozole, and lestrozole (Rose et al., 2003). The majority of these agents have been revealed to be more effective than previously used endocrine interventions, and their safety profiles are also much greater than other therapeutic agents used in cytotoxic chemotherapy (Wolff et al., 2013; Stone et al., 2013).

For women with estrogen receptor-positive, treatment with tamoxifen for 5 years significantly reduces the breast cancer mortality rate throughout the first 15 years after diagnosis (Davies et al., 2013), whereas the aromatase inhibitors are 18 to 43% greater more effective treatment for metastatic breast cancer than tamoxifen for post-menopausal women (Freedman et al., 2010; Johnston et al., 2013). For pre-menopausal women, the best choice is SERMs only or in combination with gonadothrophin agents (Palmieri et al., 2014). The main factor of the drug development is concerning the inhibition of specific cellular growth pathways. Among all well-konwn chemotherapeutic drugs, docetaxel and paclitaxel from the taxane group are ideal due to

their high activity against breast cancers (Singla et al., 2002; Chang et al., 2003; Tryfonidis et al., 2013). In addiotion, some other cytotoxic agents that showed great activity against breast cancers are doxorubicin and epirubicin which classified in the anthracycline group (Poole et al., 2006; Bao et al., 2012).

Unfortuently, cytotoxic agents can cause acute side-effects within lifespan. Most patients treated with current therapeutic treatments are afflicted with severe side effects such as hair loss, immunosuppression, diarrhoea and bleeding (Kranz and Dobbelstein, 2012). Therefore, there is an urgent demand in using inorganic chemistry to identify novel chemotherapeutic agents that are more effective and have minimal adverse side effects. Previous studies of some quinazolinones and their derivatives in cancer drug development attrected great attention to invesitage our newly synthesized compounds to annonce them as promising agents with better anticancer properties and less side effects.

2.8 *In vitro* Experiment

2.8.1 Breast cancer cell lines

So far, 51 breast cancer cell lines have been characterized based on different factors such as estrogen receptor (ER) or progesterone receptor (PR) positivity, HER2 overexpression, and TP53 protein levels and mutational status (Neve et al., 2006). Among all well-known breast cancer cell lines, MCF-7 is the most commonly used cell line that established in 1973 at the Michigan Cancer Foundation (Soule et al., 1973; Holliday & Speirs, 2011). The popularity of MCF-7 is mainly thanks to its exquisite hormone sensitivity through expression of estrogen receptor (ER), introducing it a supreme model to study hormone response (Levenson & Jordan, 1997; Simstein et al., 2003). Due to difficulties in culturing homogeneous populations and severe ethical

regulations surrounding obtaining human tissue for research, few breast cancer cell lines have been relatively established over the past decades (Holliday & Speirs, 2011).

ER/PR-positive cell lines such as MCF-7 and T47D are only form tumours in the presence of estrogen which cell growth can be certainly inhibited by anti-estrogen therapy such as Tamoxifen and Fulvestrant. Other breast cancer cell lines such as BT474, MDA-MB-468 and MDA-MB-231 have been also shown to be tumourigenic (Holliday & Speirs, 2011). MDA-MB-231 is known as invasive ductal carcinoma and considered as a hormone- independent *in vitro* model (ER/PR-negative cell line) which has been widely used in drug development research (Kim et al., 2010; Karimian et al., 2014). As MDA-MB-231 is hormone-independent cell line and showed difference cellular properties compared to MCF-7 cell line, we hypothesized to assess the effect of newly synthesized compounds on different type of breast cancer cells.

2.8.2 Apoptosis

Apoptosis or progarmmed cell death (PCD) is a fundamental process in normal development, tissue homeostasis and integrity of multicellular organisms. Thus, failure to this fundamental process contributes to the development of cancer cells as these cells have lost their ability to undergo cell death "naturally" (Elmore 2007). In cancer cells, apoptosis is suppressed and required to be triggered which is a key factor in area of anticancer drug development (Hunter et al., 2007; Faraj et al., 2014). Generally, apotosis is regulated by a variety of extracellular and intracellular signals (LeBlanc, 2003). Under critical physiologic conditions, apoptosis is initiated in specific cell types by endogenous tissue-specific agents and exogenous cell-damaging agents (Neuman et al., 2002). Numerous exogenous events of apoptosis, physical agents (such as radiation, physical trauma, cold shock and chemotherapeutic drugs) and infection agents (such as viruses and bacterial toxin) effect the most types of cells (Duckett et al., 1998). In

addition, internal imbalance can also cause apoptosis such as growth factors withdrawal, ablation of trophic hormone and loss of matrix attachment (Caron-Leslie et al., 1991, Neuman et al., 2002).

The features of apoptosis are noticeable in morphological changes of apoptotic cell such as chromatin condensation, plasma membrane blebbing, cell shrinkage and DNA fragmentation (Elmore, 2007; Platonova et al., 2012). These morphological changes can be simply visualized under light microscopy. However, some other details such as changes in organelles including mitochondria and endoplasmic reticulum can only be observed via electron microscopy. The majority of these changes are the caused by activation of protease-mediated cleavage of a nuclease inhibitor, inhibitor caspase-activated DNase (ICAD) that issues the caspase-activated DNase (CAD) (Veldhoen et al., 2013).

2.8.2.1 Apoptosis pathways

Apoptosis is mainly trigggered by two pathways; the extrinsic pathway and the intrinsic pathway. The extrinsic receptor-mediated pathway activated by the death receptor and the death signal proteins. In contrast, the intrinsic pathway is initiated via the release of mitochondrial signaling factors within the cell. These two pathways are both connected, but triggered from two distinct mechanisms (Fulda & Debatin, 2006; Elmore, 2007; Zhao et al., 2013).

Basically, extrinsic pathways are closely associated with the activating of death receptors (DR), on the cell surface (Elmore, 2007). The largest family of cell death receptors are member of tumour necrosis factor (TNF) receptor, including TNF-R1, Fas (Apo-1/CD95), TRAIL (Apo-2/R1/R2), D3 and D6 (Locksley et al., 2001; Fulda & Debatin, 2004). These death receptors are characterized by a cytoplasmic domain of 80 amino acids called “death domain”. This death domain plays an important role in

conveying the death signal from the cell surface to the intracellular signaling pathways (Debatin & Krammer, 2004).

The extrinsic pathway can be initiated though binding death ligands to their relavant death rceptors under various extracellular stimuli, such as UV or gamma-irradiation, chemotherapeutic drugs and heat shock (Mor et al., 2002; Fulda & Debatin, 2006). The mechanism of each death ligand seems to be slightly different from one to another concering the the complexity of the recruitment of apoptotic proteins. Tumour necrosis factor alpha (TNFα) is formed by the T-lymphocytes and triggered macrophages during infection and systemic inflammation (Fujiwara & Kobayashi, 2005).Upon ligant binding, tumour necrosis factor receptor-1 (TNFR1) produces trimeric clusters which will recruit TNFR-associated death domain intracellular adaptor protein (TRADD) to form the death inducing signaling complex (DISC). If TNF-associated factor 2 (TRAF2) attach to the TRADD, it ultimately will lead to the activation of NFκB pathway and N-terminal kinase (JNK) pathway which suppress apoptosis activation (Debatin, 2004); however the attachment of Fas-associated death domain protein (FADD) will resut in the activation of procaspase-8 and consequently activation of caspase-8 (Bao & Shi, 2007). Beside the TNF-α, FAS and TRAIL signaling pathways have been extensively characterized (Hu and Kavanagh, 2003). Fas signaling is slightly different from TNFα signaling. Fas receptor ligand (FasL) binds to the TNFR1 receptor and FADD is recruited without TRADD (Berglund et al., 2000). Activation by FasL leads to the activation of caspase-8. Once caspase-8 is activated, the execution phase of apoptosis is triggered via activation of effectors caspases such as caspase -3, -6 and -7 and commit the cell to apoptosis (Werner et al., 2002; Elmore, 2007). TRAIL activation is comparable to Fas activation. TRAIL ligands are regulated by five receptor subtypes; of which only TRAIL-R1 and –R2 lead to activation caspase-8 and downstream apoptosis

(Fulda & Debatin, 2004). Morover, after the activation caspase-8, BH3 interacting domain death agonist (Bid) proteins (from the Bcl-2 protein family) are cleaved to truncated-Bid (tBid) proteins which induce Bax mediated mitochondrial cytochrome *c* release and consequently involved in mitochondiral events (Werner et al., 2002).

In contrast, The intrinsic pathways are mediated by the mitochondria events. The intrinsic or mitochondrial pathway includes the pro-apoptotic members of Bcl-2 family proteins which are responsible for the activation of caspase-9 (Fulda & Debatin, 2006; Elmore, 2007; Shamas-Din et al., 2013). Several signals converge on mitochondrial, including growth factor withdrawal, hypoxia, DNA damage and oncogene induction (Reed & Pellecchia, 2005). In this pathway, the proapototic protein from the bcl-2 family such as Bax, Bak, Bid are invovled, which activate the release of apoptogenic factors such as cytochrome *c*, Smac/DIABLO, Omi/Htr A2 and apotosis- including factor (AIF) into cytosol. Cytochrome *c* then bind to an adaptor protein, Apaf-1 (apoptotic protease-activating factor 1) with engaging procaspase-9 form a complex named the apoptosome complex. Ultimately, caspase-9 is activated and then induce activation effectors caspases. Generally, Caspase-3, -6, and -7 act as effector or "executioner" caspases, cleaving various substrates including cytokeratins, PARP, the plasma membrane cytoskeletal protein alpha fodrin, that ultimately cause the morphological and biochemical changes seen in apoptotic cells (Boatright & Salvesen, 2003; Elmore, 2007; Ouyang et al.,2012).

In apoptotic cells, activated executioner cleaves ICAD to release CAD, resulting in degredation of chromosomal DNA within the nuclei and causes chromatin condensation. In the meantime, Omi/HtrA2 and smac/DIABLO are involved in promote caspase activation by counteracting to inhibitor of apoptosis protein (IAP) (Okada & Mak, 2004). The death signals conveyed over the mitochondrial pathway activate the

effector caspases which lead to mitochondrial dysfunction through depolarization, increased permeability transition (PT) and the geneation of reactive oxygen species (ROS) (Elmore, 2007; Masgras et al., 2012).

Interestingly, MCF-7 cells do not express caspase-3 due to lack of 47 base pair of the associated gene in the exon region resulting in gerenation of a stop codon that terminate the translation of this protease (Janicke et al., 1998). Recently, Jenicke re-emphasized the findings stated in 1998 and indicated that MCF-7 is susceptible to cell death induced by TNF, staurosporine and DNA damaging agents and stressed that these cell deaths are not accompanied by caspase-3 induced DNA fragmentation (Janicke, 2009). Figure 2.4 represents a schematic diagram of the extrinsic and intrinsic pathways of apoptosis.

Extensive plasma membrane blebbing arises followed by separation of cell fragments into apoptotic bodies during a process known "budding." Apoptotic bodies contain of cytoplasm with tightly packed organelles with or without a nuclear fragment. The organelle integrity is still conserved within an intact plasma membrane. Afterward, these bodies are phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded within phagolysosomes. There is no inflammatory reaction associated with the process of apoptosis nor with the removal of apoptotic cells because: (1) apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue; (2) they are quickly phagocytosed by surrounding cells thus likely preventing secondary necrosis; and, (3) the engulfing cells do not produce anti-inflammatory cytokines (Kurosaka et al., 2003, Elmore, 2007). Future understanding of the cell death signaling pathways will provide a molecular source in order to develop the novel agents via apoptosis-associated targets (Ghobrial et al., 2005). Therefore, the novel agents might control some resistance form of cancer and also enhance the efficiency of conventional chemotherapy regiments.

2.8.2.2 Distinguishing apoptosis from necrosis

Necrosis is the alternative process to apoptosis, which is considered to be an uncontrolled and accidental cell death that usually affects large fields of cells which is triggered by poor nutrient supply and ATP depletion and ultimately cause infammatory responses. However, apoptosis is controlled and energy-dependent and can affect individual or clusters of cells without causing an inflammatory responses (Elmore, 2007; Liang et al., 2010; Sosna et al., 2014). There are two main factors that will convert an ongoing apoptotic process into a necrotic process: 1) a decrease in the availability of caspases and 2) intracellular ATP. Cell is comitted to undertake either necrosis or apoptosis based on the nature of the cell death signal, the tissue type, the developmental stage of the tissue and the physiologic milieu. Since both process can happen simultaneously, it is not always easy to differentiate apoptosis from necrosis. Some of the major morphological changes that occur with necrosis include cell swelling; formation of cytoplasmic vacuoles; distended endoplasmic reticulum; formation of cytoplasmic blebs; condensed, swollen or ruptured mitochondria; disaggregation and detachment of ribosomes; disrupted organelle membranes; swollen and ruptured lysosomes; and eventually disruption of the cell membrane (Zeiss, 2003; Zong & Thompson, 2006; Chaabane et al., 2013). This loss of cell membrane integrity result in the release of the cytoplasmic contents into the surrounding tissue, sending chemotatic signals with eventual involvment of inflammatory cells (Chaabane et al., 2013). Since apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue and are rapidly engulfed by macrophages or adjacent normal cells, there is essentially no inflammatory reaction (Dajas, 2012). It is also important to note that pyknosis and karyorrhexis are not exclusive to apoptosis and can be a part of the spectrum of cytomorphological changes that occurs with necrosis (Elmore, 2007). Unlike apoptosis, the change in necrosis at the molecular level is poorly

understood. Therefore, the most reliable approach to distinguish apoptosis from necrosis is relied on morphological features (Whelan et al., 2012). Table 2.1 indicates several distinct morphological features of apoptosis and necrosis.

Table 2.1: Morphological features of apoptosis versus necrosis (Elmore, 2007).

Apoptosis	**Necrosis**
Single cells or small clusters of cells	Often contiguous cells
Cell shrinkage and convolution	Cell swelling
Pyknosis and karyorrhexis	Karyolysis, pyknosis, and karyorrhexis
Intact cell membrane	Disrupted cell membrane
Cytoplasm retained in apoptotic bodies	Cytoplasm released
No inflammation	Inflammation usually present

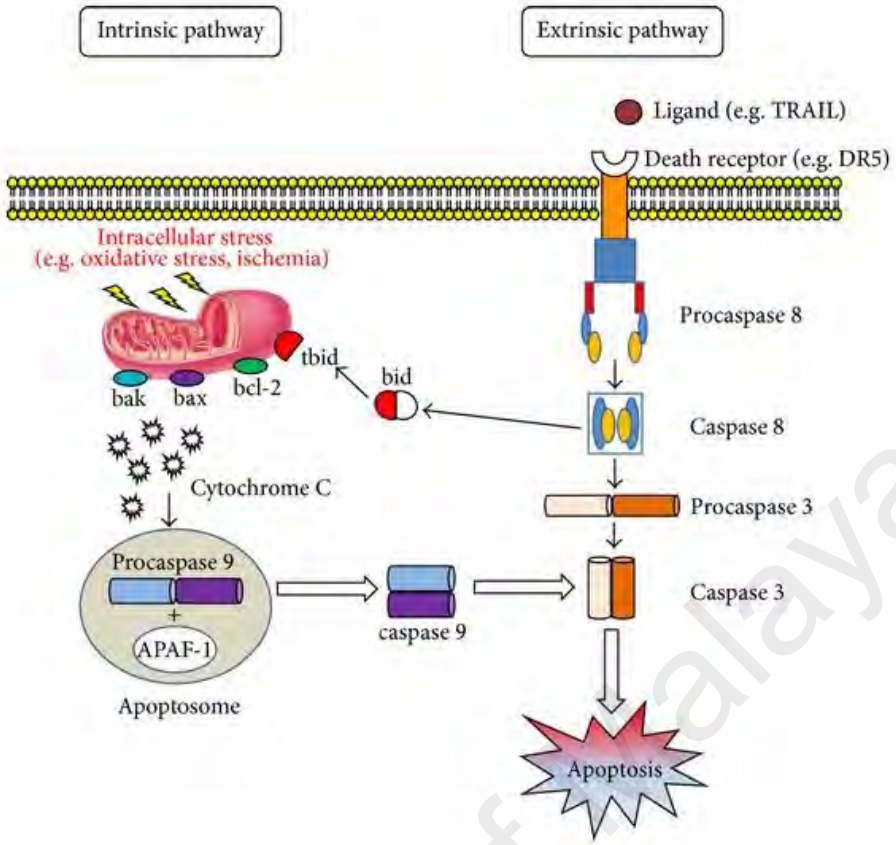


Figure 2.4: Extrinsic and intrinsic pathways of apoptosis (Loreto et al., 2014)

2.9 Free radicals and reactive oxygen species

Free radicals are chemical compounds that hold unpaired electrons in their outer electron orbit. The free radicals are energetic and extremely unsteady which they consequently seek other electrons to pair with to gain stability and ultimately attack and steal electrons from other molecules such as lipids, proteins, DNA and carbohydrates. Therefore, they can damage DNA and cause mutation and chromosomal damage. Damaged molecules lose its electron and converts free radicals itself which lead to uncontrolled chain reaction and damage the natural function of the living cell, and resulting in various diseases (Valko et al., 2006).

Reactive oxygen species (ROS) play an important role in apoptosis induction. They includes free radicals, such as hydroxyl and superoxide radicals and non-radicals including hydrogen peroxide and singlet oxygen. Generally, ROS can be found in all

aerobic cells and are produced by various endogenous metabolic events such as mitochondrial respiration or oxogenous sourcs, which contains UV light, ionizing radiation, inflammatory cytokines, smoking, alcoholic beverages, food and carbonated drinks (Waris & Ahsan, 2006). When there is a great generation of ROS associated with the reduced levels of antioxidants in the body, this phenomenon is named oxidative stress. Accumulating evidences showed the role of oxidative stress and antioxidant status in breast malignant cancer (Ray et al., 2000; Tas et al., 2005; Valko et al., 2006). There are two sources of free radicals namely endogenous and exogenous sources. Endogenous sources comprise free radicals formed during nutrient metabolism and energy production in the mitochondria. Another endogenous source of ROS, specifically in the liver, is a group of enzymes called the cytochrome P450 mixed–function oxidases. The biochemical reactions catalyzed by the cytochrome P450 molecules use molecular oxygen, and during these reactions small amounts of ROS are produced. The level of ROS generation may vary greatly relianing on the compound to be degraded and on the cytochrome P450 molecule involved. Cytochrome P450 2E1 (CYP2E1) is a type of cytochrome molecule that is particularly active in generating ROS (Lieber, 1997). The exogenous sources originate from the environmental contaminants such as smoking, toxic chemicals, radiation, air pollution, organic solvents and pesticides (Irigaray et al., 2007).

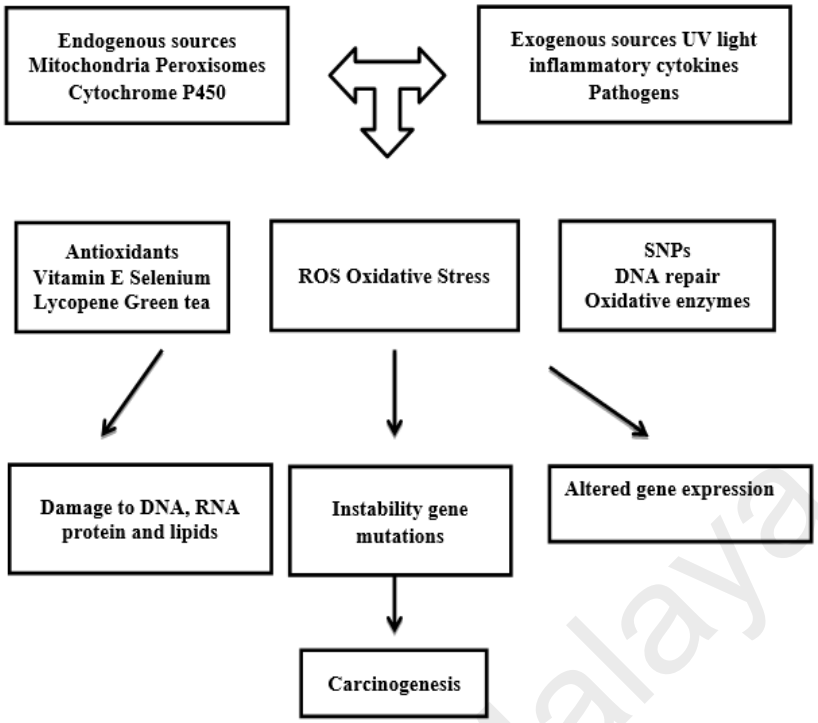


Figure 2.5: Role of reactive oxygen species (ROS) in the development of cancer (Klaunig et al., 2010).

2.10 *In vivo* experiment

2.10.1 Appropriate animal models in breast cancer research

Currently, animal studies play a significant role in the development of new cancer treatment regimens and the evaluation of the efficacy and safety of novel anti-cancer drugs (Dixit & Boelsterli, 2007; Zadnik et al., 2013). Certainly, carcinogenesis studies using laboratory animals provide the most reliable means offered to identify the potential public health hazards (Patlolla et al., 2012). Among laboratory rodents animals, mice and rats have been widely used for human cancer studies, including the research in cancer biology and the mechanisms, modulators and pathogenesis of the disease (Cook et al., 2012). Rats are appropriate model due to easily accessible, having short life-span and cost-effectiveness (Johnson & Tomarev, 2010) and they also present the most appropriate model for breast cancer studies thanks to the great similarities to humans. These resemblances include physiological properties, anatomy, biochemistry,

metabolism and genetics, which will upsurge the chance in expecting results for human research areas (Pan et al., 2013).

2.10.2 Rat mammary gland tumour

Rat mammary tumors have many points of interest and differ from those of the mouse in many ways. Histologically, both benign and malignant tumors occur spontaneously in the rat, or may be induced by a number of means. The mammary glands of rats are susceptible to transformation induced by chemical carcinogens (Siddiqui et al., 2013). Over the past few decades, high incidence mammary tumour has been reported in Sprague Dawley rats. Chemically-induced mammary gland carcinogenesis models are widely used in rats to evalute the biology of cancer and for development of strategies for the prevention of cancer (Dias et al., 2013). There are two most widely used active tumour-inducing chemical agents namely 7, 12-dimethylbenz (a) anthracene (DMBA) and N-methyl nitrosurea (MNU) (Budan et al., 2009; Mafuvadze et al., 2013).

Human and rat mammary gland tumours microscopically share the similar criteria of malignancy (Mafuvadze *et al*., 2013). Numerous resaerch have shown that mammary gland carcinoma induced in rats by DMBA possess a histogenetic pathogenesis similar to that of human breast cancer (Nandhakumar et al., 2012; Ouhtit et al., 2014). For instance, the mammary tumours in rats where root from the epithelium of the terminal end buds, are comparable in arrangement to the terminal ductal lobular units in human breast cancer (Marongiu et al., 2012).

Recently, LA7 cells-induced mammary carcinogenesis has been reported as a new mammary cancer model and revealed to be the most comparable to the human breast cancer form concering the hormone sensitivity and histopathology. LA7 cells were originated from a mammary adenocarcinoma induced in rats using DMBA. In this mode, implanting of LA7 cells into the mammary pad in the left or right flank will

develop the malignant tumours during 7 to 10 days period. In addition, this tumour induction techniqe offer more benefits such as ease of inoculation, continuity and reproducibility of tumour growth, safeness and economic advatntages (Abbasalipourkabir et al., 2010; Karimian et al., 2015).

2.11 Biological potential of quinazolinone derivatives

Quinazolinones is a class of fused heterocycles that are of extensive interest due to the diverse variety of their biological properties (Connolly et al., 2005). Numerous substituted quinazolinone derivatives possess a wide range of bioactivities such as antimalarial, anticancer, antimicrobial, antifungal, antiviral, antiprotozoan, anti-inflammatory, diuretic, muscle relaxant, antitubercular, antidepressant, anticonvulsant, acaricidal, weedicide, and many other biological activities (Rajput & Mishra, 2012; Pati & Banerjee, 2013; Vijayakumar et al., 2013; Asif et al., 2014). Morover, quinazolinone compounds are applied in preparation of different functional materials for synthetic chemistry (Abida et al., 2011).

Quinazolinones are categorized into the following five categories, depending on the substitution patterns of the ring system (Mhaske & Argade, 2006). These are 2-substituted-4(3H)-quinazolinones, 3-substituted-4(3H)-quinazolinones, 4-substituted-quinazolines, 2,3-disubstituted-4(3H)-quinazolinones, and 2,4- disubstituted-4(3H)-quinazolinones. Based on the position of the keto or oxo group, these compounds may categorize into three types (Mahato et al., 2011). Out of the three (2(1H)quinazolinones, 4(3H)quinazolinones and 2,4(1H,3H)quinazolinedione) quinazolinone structures, 4(3H)-quinazolinones are most dominant, either as intermediates or as natural products in various proposed biosynthetic pathways. This is partly thanks to the structure being derived from the anthranilates (anthranilic acid or various esters, isatoic anhydride,

anthranilamide, and anthranilonitrile) while the 2(1H)-quinazolinone is mainly a product of anthranilonitrile or benzamides with nitriles (Mahato et al., 2011).

2.11.1 Anticancer activity

Quinazoline nucleus has attracted the attention of medicinal chemists thanks to its well known anticancer activity and many substituted quinazoline derivatives have recently earned great interest in chemotherapy as antitumor drugs (Selvam et al., 2011). A series of novel 4,6- disubstituted quinazoline derivatives exhibited significant anti-inflammatory and anticancer activity against U937 leukemia cell lines (Chandrika et al., 2008). The 6-Arylbenzimidazo [1,2-c]quinazoline derivatives were act as a tumor necrosis factor alpha (TNF- α) production inhibitors. These compounds were analysed for their *in vitro* ability to prevent the lipolysaccharide (LPS) induced TNF-α secretion in the human promyelocytic cell line HL-60 (Galarce et al., 2008). In addition, 3-(3-methylisoxazol-5-yl) and 3-(pyrimidin- 2-yl)-2 styrylquinazolin-4(3H)-ones were also prepared by refluxing in acetic acid the corresponding 2- methylquinazolinones with the benzoic aldehyde and confirmed anti leukemic activity against L-1210 (murine leukemia), K-562 (human chronic myelogenous leukemia), and HL-60 (human leukemia) cell lines showing in some cases good activity (Kaushik et al., 2009). A new 2,3-di-substituted 8- arylamino-3H-imidazo[4,5-g] quinazoline derivative as a potent antitumor agent possessed the highest activity on the A549 cell line (Chen et al., 2011). Morover, some new 3- substituted quinazolin-4(3H)-ones and 3,4-dihydro-quinazolin-2(1H)-one derivatives have been reported that compounds 2-[2-(4-chlorophenyl)-2-oxo-ethylthio]-3-(4-methoxyphenyl) quinazolin-4(3H) one (1) and 3-(4-chlorophenyl)-2-[2-(4-methoxyphenyl)-2-oxo-ethylthio]quinazolin-4(3H)-one (2) as broad-spectrum antitumors show effectiveness toward numerous cell lines that belong to different tumor subpanels (Gawad et al., 2010). A series of novel quinazoline derivatives containing

thiosemicarbazidemoiety has been evaluated and confimed their biological activity as antitumor agents (Wang et al., 2012). Recently, a comprehensive review paper have been published and annonnced the most potential anticancer quinazolines-based agents patented during 2007 to 2010 (Marzaro et al., 2012).

CHAPTER 3: METHODOLOGY

3.1 Materials

Tween 20 and 95% (v/v) ethanol were purchased from (Sigma-Aldrich, UK). Filter paper (Whatmann No.1, Fitchburg, WI, USA), Genie-2 vortex (Scientific Industries Inc., USA). DMSO (dimethyl sulphoxide) purchased from (Merck, Darmstadt, Germany). Neubaur haemocytometer (Weber, Teddington, UK), IR Jacketed incubator (from NUAIRE laboratory equipment supply, Plymouth, MN, USA). PBS (phosphate buffer saline), xylazine, ketamine, Formalin, Hematoxylin & Eosin were obtained from (Sigma-Aldrich, Gillingham, UK). Automated tissue processing machine, Leica Tissue Microtome (Leica, Germany). Homogenizer (DAIHAN Sci., Seoul, Korea), Jouan C312 centrifuge (Santa Fe Springs, CA, USA) and Rotofix 32 refrigerated centrifuge (Hettich Zentrifugen, Germany). Power wave X 340 ELISA plate reader from (BIO-TEK instruments, Winooski, VT, USA). Homogenizer (DAIHAN Sci., Seoul, Korea), Jouan C312 centrifuge (Santa Fe Springs, CA, USA).

Commercially available kits from Cayman (USA) were used to Malonialdehyde (MDA) (cat. #10009055), Catalase (CAT) (cat. #707002) and superoxide dismutase (SOD) (cat. #706002) activities. RPMI 1640 cell culture media, Foetal Bovine Serum (FBS) and Penicillin/Streptomycin solution (PS) were purchased from Biowest, France and trypsin ethylenediaminetetraacetic acid (EDTA), MTT (3-(4,5-dimethyl thiazol-2-yl-2,5-diphenyl tetrazolium bromide), Steril Tissue culture flasks, ethanol analytical grade were obtained from (Merck, Germany). Sterile cell culture plates, Sterile centrifuge Tubes, Phosphate buffer saline, Dimethyl sulfoxide, reagent was purchased from Sigma Aldrich, Germany, Cell culture flasks and plates were purchased from (Jet Biofil). Cell culture work was done under steril conditions in class II bio-safetycabinat (ESCO, USA).

3.2 Quinazoline-Schiff bases

All five Quinazoline-Schiff based compounds were provided from Prof. Dr. Hapipah Mohd Ali, Chemistry Department, Faculty of Science, and University of Malaya. In addition, the general procedure for synthesis quinazoline-Schiff based compounds were recently published (Faraj et al., 2014; Zahedifard et al., 2015a; Zahedifard et al., 2015b) and chemical structure of all compounds have been showed in Figure 3.1. As shown in figure 3.1, the difference between all five compounds is due to the difference in their substitution attached to the base structure (compound 1 contains nitrogen dioxide, compound 2 contains bromide, compound 3 contains chloride, compound 4 contains bromine and methoxy groups and compound 5 contains methoxy groups on the quinazoline base structure).

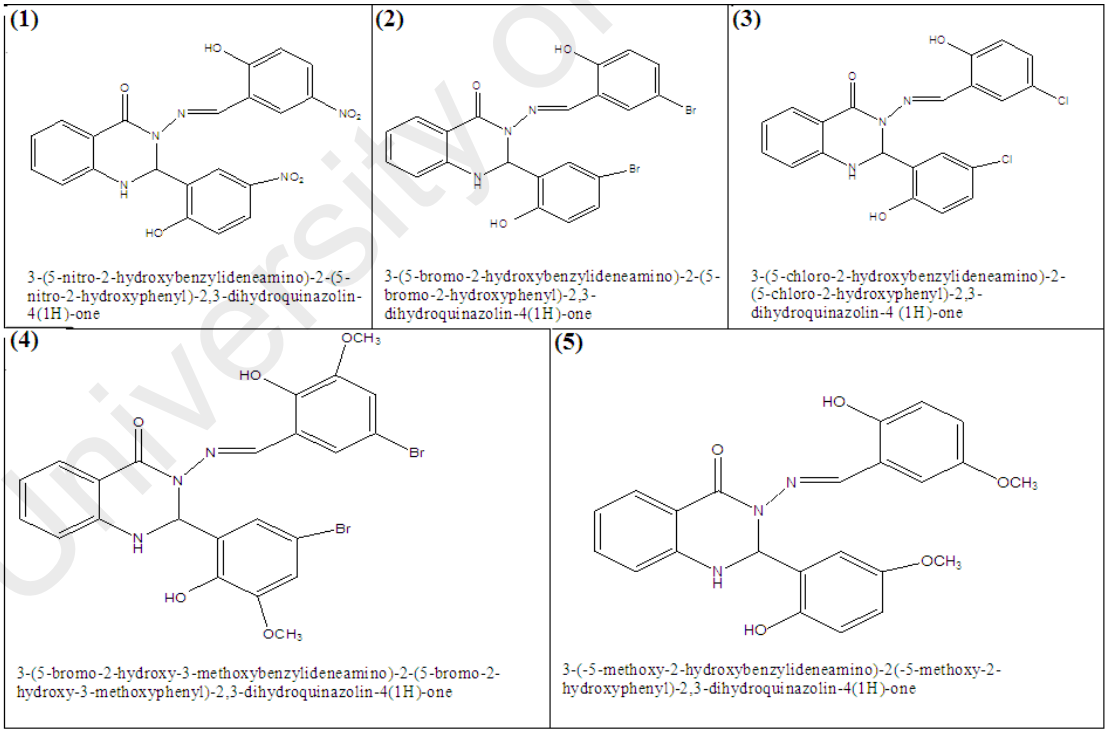


Figure 3.1 : Chemical structures of Quinazoline Schiff Bases

3.3 *In vitro* study of anticancer properties of five quinazolinone-based compounds

3.3.1 Cell culture

Human mammary cancer MCF-7, MDA-MB-231 cell lines and human normal breast cells MCF-10A, were purchased from American Type Cell Collection (ATCC) and were kept at 37°C in an incubator with 5% CO_2 saturation. They were maintained in RPMI-1640 medium with 10% Fetal Bovine Serum (FBS) and 100 µg/mL streptomycin and 100 U/mL penicillin G at 37°C in a humidified atmosphere of 5% CO_2/95% air. Dulbecco's modified Eagle's medium (DMEM) was used to maintain the human normal breast cells MCF-10A under same condition.

3.3.2 MTT cell viability assay

The cytotoxic effect of the compounds was assessed by MTT cell viability assay against MCF-7, MDA-MB-231 and MCF-10A cell lines. Cell lines were seeded in triplicate into 96-well transparent flat bottom plates (Greiner Bio-One) at the concentration of 5×10^5 cells/well. On the next day, the cells were treated with a serial dilution for different concentrations of compounds (1.563, 3.125, 6.25, 12.5, 25, and 50, 100 μg/mL). After 24, 48 and 72 hours of incubation, 20 µL of a solution containing 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazoliumbromide (MTT, Santa Cruz) at a concentration of 5 mg/mL was added to each well of the microplate. After 4 hours, media with MTT reagent was removed before adding 100 µL of dimethylsulfoxide (DMSO) to solubilize the formazan crystals. The optical density was measured with an ELISA microplate reader at an absorbance of 570 nm. The assay was performed in triplicate to calculate the half maximal inhibitory concentration (IC_{50}) value. MCF-7 cells were also treated with two standard drugs, Doxorubicin and Tamoxifen.

3.3.3 LDH release Assay

The cytotoxicity of the compounds was also determined by lactate dehydrogenase (LDH) release assay on MCF-7 cells (Choi et al., 2012). LDH release in the medium is due to the loss of membrane integrity either due to apoptosis or necrosis. Briefly, MCF-7 cells were treated with various concentration of the compounds for 24 h. Then, the supernatant of the treated cells was relocated into 96-well plates, and 100 µL of the LDH reaction solution (PierceTM LDH Cytotoxicity Assay Kit, Thermo Scientific™, Pittsburgh, PA) was added for 30 min. Finally, the intensity of red colour in the samples indicating the LDH activity was measured at 490 nm using a Tecan Infinite®200 Pro (Tecan, Männedorf, Switzerland) microplate reader. Increase in the LDH release of treated MCF-7 cells with dose-dependent manner of compounds will compare with untreated cells.

3.3.4 Morphological assessment of apoptotic cells by (AO/PI) double staining

A combination of a cell-permeable DNA-binding dye, that is, acridine orange (AO) with plasma membrane-impermeable, and DNA-binding dye propidium iodide (PI) was used to assess the morphological changes in treated MCF-7 cells. Briefly, MCF-7 cells were seeded and then treated with IC_{50} concentration of compounds for 24, 48, 72 h treatment period. In this experiment, untreated MCF-7 cell also was stained as a negative control. After each period of treatment, cells were washed twice using PBS to remove the remaining media. Then, cells were stained by adding equal volume of AO and PI (10 μg/mL) (Sigma, UK). 10 µL of the cell suspension was placed onto a glass slide and morphologically assessed with a fluorescence microscope within 30 min prior to the fluorescence fading (Wahab et al., 2009).

3.3.5 Measurement of reactive oxygen species generation (ROS)

ROS assay was performed to determine the effect of compounds on the generation of ROS in treated MCF-7 cells and measured using a Cellomics Oxidative Stress 1 HCS Reagent Kit (Thermo Scientific, Pittsburgh, PA), according to the manufacturer's protocols. Briefly, 1×10^4 cells per well were seeded into 96-well plate and incubated overnight at 37°C under condition of 5% CO_2. The cells were then treated with different concentrations of the compounds. After 24 hours, dihydroethidium (DHE) dye was added into live culture for 30 min. Cells were fixed and washed with wash buffer. The DHE dye probe is oxidized to ethidium in the presence of superoxides. The fluorescence intensity was measured using a fluorescent plate reader at an extension wavelength of 520 nm and an emission wavelength of 620 nm (Liu et al., 2012). The values are represented as means ± SD of three sets of experiments**.**

3.3.6 Multiple cytotoxicity assay

A Cellomics multiparameter cytotoxicity 3 kit (Thermo Fisher Scientific, Waltham, MA, USA) was used as described in detail previously (Cheah et al., 2013). This kit simultaneously measures some independent parameters in the same cell including mitochondrial membrane potential (MMP) change, cytochrome *c* release, and cell membrane permeability. Briefly, 1×10^4 cells per well were seeded into a 96-well plate under incubation condition. On the next day, cells were treated with different concentrations of compounds as well as dimethylsulfoxide (negative control) for 24 h. Next, MMP dye and cell permeability dye were added to live cells and incubated for 30 min at 37°C. Cells were then fixed, permeabilized, and blocked with 1X blocking buffer prior to probing with primary cytochrome *c* primary antibody and secondary DyLight 649 conjugated goat antimouse immunoglobulin G for 1 h each. Then, Hoechst 33342 was added into the staining solution to stain the nucleus. Finally, the plates were

analyzed using ArrayScan high content screening system (Thermo Fisher Scientific). The ArrayScan high content screening system is a computerized automated fluorescence imaging microscope that automatically identifies stained cells and reports the intensity and distribution of fluorescence in individual cells. In each well, 1,000 cells were analyzed. Images were acquired for each fluorescence channel using suitable filters. Images and data regarding intensity and texture of the fluorescence within each cell, as well as the average fluorescence of the cell population within the well, were stored in a Microsoft SQL database for easy retrieval. Data were analyzed with ArrayScan II Data Acquisition and Data Viewer version 3.0 software (Thermo Fisher Scientific). The values are represented as the means ± SD of three sets of experiments.

3.3.7 Measurement of Caspase-3/7,-8 and -9 Activities

A Caspase-Glo® 3/7, 8 and 9 kits (Promega, Madison, WI) was used to measure the activation of caspase-3/7, -8 and -9 according to the manufacturer's protocols. Briefly, MCF-7 cells were treated with compounds at IC_{50} concentrations of 48 h treatment in a time-dependent manner (6, 12, 18, 24 and 30 h) exposed to caspase-Glo reagent (100 µL) for 30 min. Finally, the caspases activity were determined as the degree of aminoluciferin-labelled synthetic tetrapeptide cleavage and luciferase enzyme substrate release from the cells as operated by Tecan Infinite®200 Pro microplate reader (Tecan, Männedorf, Switzerland).

3.3.8 Detection of NF-kB activity

Cellomics NF-κB activation HCS kit (Thermo Scientific, Pittsburgh, PA) was used for measuring the cytosol-to-nucleus translocation of nuclear factor-κB (NF-κB). Briefly, cells were treated with different concentrations of compounds for 3 h and stimulated with 1 ng/mL tumor necrosis factor alpha (TNF-α) for 30 min. Then, medium was removed and the cells were fixed and stained with nuclear factor kappa B (NF-κB)

activation kit from Thermo Fisher Scientific according to the manufacturer's instructions. The plate was analyzed by an ArrayScan high content screening reader. Calculation of the cytoplasmic and nuclear NF-κB intensity ratio was performed using Cytoplasm to Nucleus Translocation BioApplication software (Thermo Fisher Scientific). The average intensity of 200 objects (cells) per well was quantified. The ratios were then compared between TNF-α-stimulated, treated, and untreated cells (Mohan et al., 2012).

3.4 Acute toxicity study

3.4.1 Chemicals and reagents

All chemicals were acquired from Sigma Chemical Co., St. Louis, MO, USA. Moreover, all the reagents, solvents and chemicals were of analytical grade.

3.4.2 Animals

ICR female mice (25 - 30 g body weight) were purchased from the Animal House Unit, Faculty of Medicine, University of Malaya (UM). The animals were kept at 20 ± 2ºC using a 12 h light/dark cycle and relative humidity of 50–60%. Free access to water and food was allowed at all times. Animals were housed in each sterilized plastic cage using homogenized wood shavings as bedding. Human care to animals was conducted according to the criteria mentioned in the "Guide for the care and use of laboratory animals" prepared by the National Academy of Sciences (Garber et al., 2010). All the experimental protocols were performed based on the rules set by the Institutional Animal Care and Use Committee, Faculty of Medicine (Ethic number PM/27/07/2010/MAA (R)).

3.4.3 Acute toxicity test

The single-dose acute oral toxicity test was performed on ICR female mice based on OECD 420 (Chemical D., 2005; Garber et al., 2010). The animal were divided equally into 2 groups (n = 6 per group) labeled as vehicle control group (5% Tween 20), and experimental group (250 mg/kg of compounds), respectively. Prior to the experiment, all mice were fasted for 24 h. After treatment, the animals were observed for the first 30 min and 4–5 times at intervals of 48 hr to detect any signs of abnormality. After 14 days, the animals were sacrificed by an overdose of xylazine and ketamine anesthesia. Blood samples were then collected for serum biochemical examination. In addition, kidney and liver histological analysis was performed using hematoxylin and eosin (H&E) staining. All values are mean of three experiments (Figure 3.2).

3.4.4 Assessment of kidney and liver functions

The biochemical tests were conducted by spectrophotometer (Hitachi-912 Auto analyser, Mannheim, Germany) with the kits provided by Roche Diagnostics (Mannheim, Germany). Total protein, albumin, globulin, Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transpeptidase (GGT) levels were measured to evaluate liver function As indicators of kidney function, blood sodium, potassium, chloride, carbon dioxide, anion gap and urea levels were measured. The serum samples were analyzed in triplicate.

3.4.5 Histopathological examinations

Renal and hepatic tissues were fixed in 10% formalin, and the fixed samples were embedded in paraffin, sectioned at 5 μm and stained with haematoxylin-eosin. All sections were observed using a photomicroscope (Olympus BH-2, Japan).

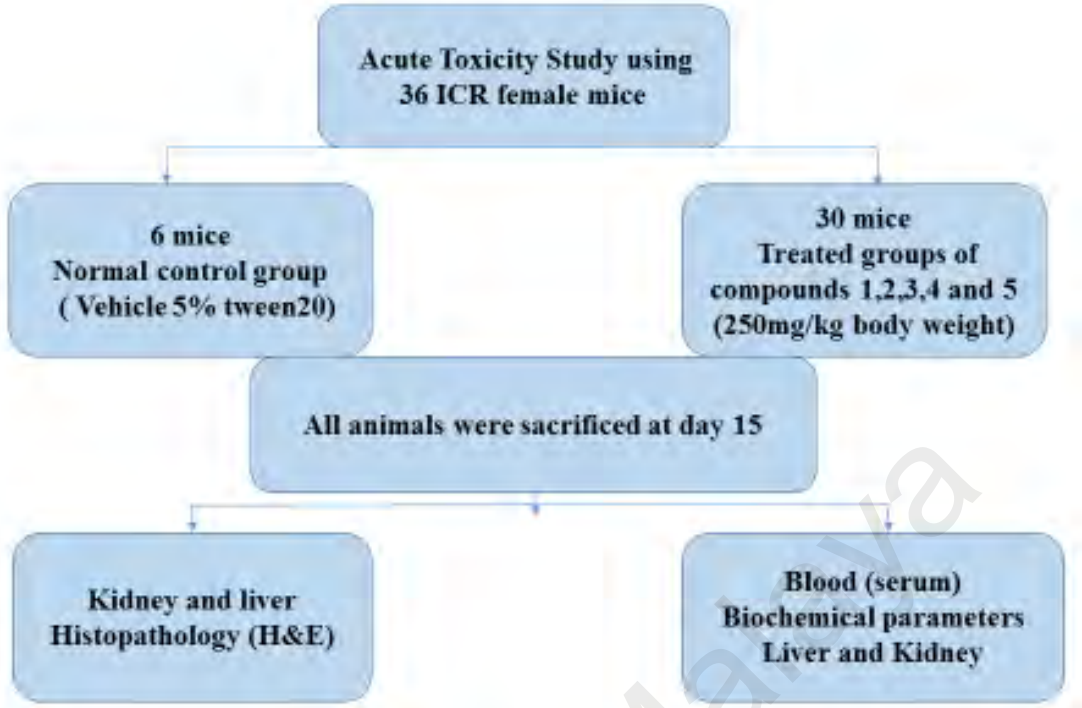


Figure 3.2: Acute toxicity flow chart of quinazolinone-based compounds in female mice (250mg/kg).

3.5 *In vivo* study of anticancer properties of 2 and 4

3.5.1 Animals

Animals used in the present study were provided by the Animal House of Faculty of Medicine, University of Malaya, Kuala Lumpur and the study was approved by the Institutional Animal Ethical Committee (2014-10-14/IBS/R/MZF). Pathogen free female Sprague Dawley (SD) rats were maintained at 25±3 ºC, 12 h light/dark cycle and relative humidity 55-60ºC. All the animals were fed standard food pellets and tap water ad libitum.

3.5.2 Cell preparation

LA7 (rat mammary tumor cells) cell line was purchased from American Type Cell Collection (ATCC, Manassas, VA, USA). The cells were maintained in DMEM (Sigma, St. Louis, MO, USA) and supplemented with 10% foetal bovine serum (FBS, Pasching, Austria) at 37 ºC incubator with 5% CO2 saturation. Upon reaching 90% confluency, dead cells were removed using fresh medium. After 24 h, the medium was removed and phosphate buffered saline (PBS) was used to rinse the cells. Following washing, the cells were detached using trypsin ethylenediaminetetraacetic acid (EDTA) and centrifuged at 100 g for 10 min at 4ºC. The cells were then subjected to two washes using PBS followed by dispersion, staining by trypan blue and finally counting by the hemocytometer. An amount of $6x10^6$ cells were suspended in 300 µL PBS to be used within one hour of preparation in inducing mammary glands tumour.

3.5.3 Induction of mammary gland tumours

After one-week period of acclimatization, the rats were anesthetized with a mixture of ketamine-HCl (150 mg/kg body weight) and xylazine (10 mg/kg body weight), and a dose of 300 µL containing 6 x 10^6 LA7 cells were subcutaneously injected into the mammary fat pad of each rat using 26-Gauge needle and tuberculin syringe. (Abbasalipourkabir et al., 2010; Karimian et al., 2015).

3.5.4 Experimental design and animal treatment

A total of 35 female SD rats (6–10 weeks old, 180-200 g body weight) were used in this study, which were randomly divided into five groups. Among all five compounds, compounds 2 and 4 were selected as they showed most significant cytotoxicity effect towards MCF-7 breast cancer cell line.

Group I animals (n=5) were kept as the normal control group labeled as (NC, vehicle only 5 % Tween 20); Group II animals (n=5) were classified as LA7 induced non-

treated or mammary tumor control group and labeled as cancer control (TC); Groups III animals (n=5) treated with low dose (25 mg/kg) of compound 2 (T+2-LD); Group IV animals (n=5) treated with high dose (50 mg/kg) of compound 2 (T+2-HD); Group V animals (n=5) treated with low dose (25 mg/kg) of compound 4 (T+4-LD); Group VI animals (n=5) treated with high dose (50 mg/kg) of compound 4 (T+4-HD); and Group VII animals (n=5) treated with Tamoxifen (10 mg/kg) as standard drug control group (T+TAM).

The animals were hosted for one week of acclimation period before conducting the experiment. The animals were then fed orally using gastric tube daily with compound 2 and 4 in two dosages (25 and 50 mg/kg dissolved in 5% Tween 20) as well as Tamoxifen (10 mg/kg dissolved in 5% Tween 20) from two weeks before LA7 cell injection. After 6 weeks of treatment, all rats were sacrificed with an overdose of CO2. Samples of mammary tissue were removed, rinsed twice with ice-cold 0.1 M phosphate buffer saline (PBS) for biochemical, histopathological and immunohistochemical analyses.

3.5.5 Determination of tumor size

The latency period and tumor incidence was determined as tumor growth landmarks (Rivera et al., 1994). All the animals were observed for mammary tumour development. Tumour diameters were measured every week. The tumor mass was measured horizontally and vertically using a digital caliper. The volume of tumour (V) was calculated by the formula determined by Carlsson: V= (ab2)/2, where ‘a’ and ‘b’ is the longest and shortest diameters of the tumour, respectively (Carlsson et al., 1983).

3.5.6 Assessment of antioxidant in breast tissue

The organ of the breasts was removed. The tissues were carefully rinsed using ice-cold normal phosphate buffer saline, pH 7.2 (PBS, 0.9%) before being cut into little pieces

with a heavy-duty blade. The tissues were homogenized using Wise MixTM HG 15A homogenizer (DAIHAN Sci., Seoul, Korea) in cold PBS, before centrifuging at 4,000 rpm for a period of 10 min in -4°C using refrigerated centrifuge Rotofix 32 (Hettich Zentrifugen, Germany). Next, the supernatant was removed and kept in -80°C freezer until the assays was performed according to the instruction manual from the manufacturer. Commercially available kits from (Cayman Chemical Co., USA) were used to determine the supernatant catalase (CAT) (Appendix D), superoxide dismutase (SOD) (Appendix E) and Malondialdehyde (MDA) (Appendix F).

3.5.7 Hematoxylin and Eosin staining

Macroscopic mammary tumours were fixed in 10% buffered formalin and implanted in paraffin utilizing tissue-processing machine (Leica, Germany). The tissues were then embedded in paraffin blocks and each block was cut into 5 μm thick slices and located on the histology glass slide to be dried for 1 overnight at 40°C before staining with hemotoxylin-eosin. The sections were then stained with hematoxylin and eosin (H&E) and were observed with a light microscope (BX51, Olympus, Japan).

3.5.8 Immunohistochemistry

Formaldehyde was used to fix the samples, before being dehydrated using a graded alcohol series. After embedding the samples into paraffin, sections (3-5 μm) of implanted specimens were mounted on to poly-L-lysine coated slides, and positioned in oven at 58°C for 2 h. The sections were deparaffinized, hydrated, as well as subjected to antigen retrieval by immersion in 10 mM citrate buffer (pH 6.0) at 92–95°C for 20 min; subsequent to the cooling of the slides to room temperature, they were washed in phosphate-buffered saline (PBS). Each section was then incubated with primary antibodies against Bax, Bcl-2, P53 and PCNA (1:100, 1:50, 1:50 and 1:50 respectively, dilution, Santa Cruz Biotechnology). The entire incubations were completed in a

humidified hall. Subsequent to being rinsed with PBS, the sections were incubated with Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulins in Tris-HCl buffer containing stabilizing protein and an anti-microbial agent. at 37°C for 30 min. Then, the chromogenic reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride solution for 3–5 min. A light Mayer hematoxylin counterstain was utilized. DAB detection kit (Boster Biological Technology Co. Ltd., Wuhan, China) was used for the colorimetric detection and all sections were observed using light microscope and images were then obtained (BX51, Olympus, Japan) (Karimian et al., 2015).

3.5.9 TUNEL assay

The Flourometric TUNEL assay was used to determine the fragmented DNA of the apoptotic cells by flourescein-12-dUTP at 3′-OH DNA ends utilizing the terminal recombinant deoxynucleotidyl transferase enzyme (rTdT). The assay was conducted following the company's manual (Promega Inc., Madison, USA). Briefly, the deparaffinised breast tissue slides were washed in 0.85% NaCl, followed by 5 min immersing in PBS and fixing in 4% methanol-free formaldehyde supplemented with 100 µL of proteinase K (20 µg/mL). The slides were then incubated for 8–10 min at ambient temperature and rinsed in PBS for 5 min. 50 µL of fresh rTdT buffer was applied on the slides and re-incubated for 60 min in the dark with at 37°C for the tailing reaction to occur. The reaction was ended by submerging the slides in saline-sodium citrate solution at room temperature for 15 min. The slides were washed twice followed by staining with propidium iodide solution (1 µg/mL in PBS) and kept in the dark and for 15 min before rinsing in deionized water. The slides were mounted and cover slipped and left to dry for 10 min. The slides were examined using confocal microscope

(ZIESS, LSM 70) with standard fluorescein filters and DNAase as positive control (Karimian et al., 2015).

3.6 Statistical analyses

Predictive Analysis Software (PASW) version 18 was used to analyze the data of this study. One-way ANOVA was conducted using the Prism statistical package (GraphPad Software, USA and Tukey's post-hoc test). The data are displayed as the means ± SD and the level of significance was set at $P<0.05$.

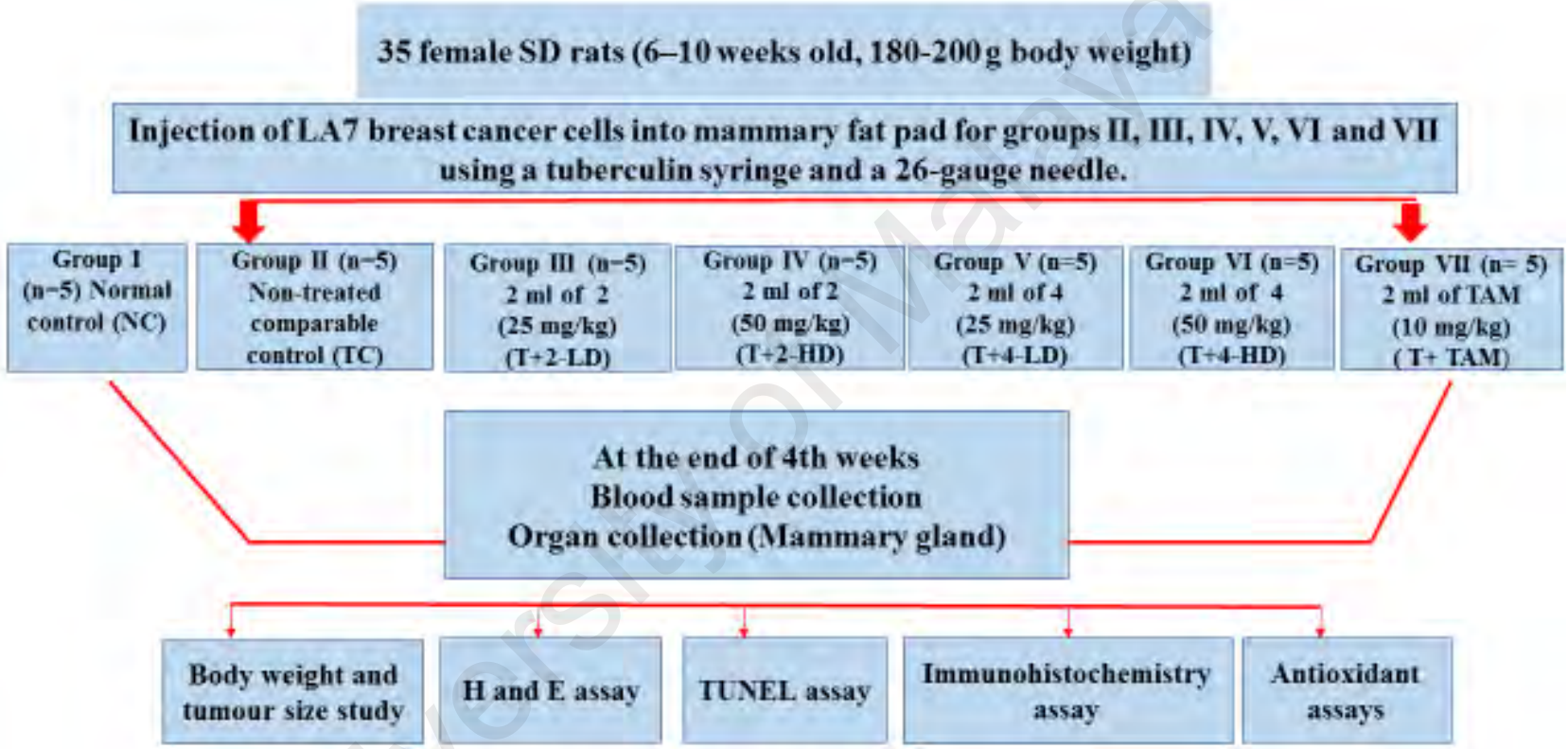


Figure 3.3: Animal chemoprevention study flow chart. Animals experimental for chemoprevention effect of quinazolinone-Schiff based compounds on LA7-induced breast cancer in Rat

CHAPTER 4: RESULT

4.1 *In vitro* results

4.1.1 MTT cell viability assay

The cytotoxic effects of five quinazolinone-based compounds on the MCF-7 and MDA-MB-231 cells were determined using MTT assay. As shown in Table 4.1, all compounds significantly inhibited the growth of MCF-7 cells after 24, 48 and 72 h of treatment with IC_{50} value that are genrally less than 6 μg/mL. However, they all could not induce significant cytotoxicity effect on MDA-MB-231 cells (> 50 μg/mL). In addition, they did not show cytotoxicity effect remarkably toward the normal breast cell line MCF-10A exhibiting an IC_{50}> 25 μg/mL. Furthermore, Doxorubicin and Tamoxifen were used as positive control. Therefore, breast cancer MCF-7 cell line was used for further investigation into apoptotic features and mechanism of treated cells with quinazolinone-based compounds.

Table 4.1: MTT cytotoxicity assay. IC_{50} values of the quinazolinone-based compound against MCF-7 cell line after 24, 48 and 72 h of treatment. Doxorubicin and Tamoxifen were used as positive control.

Compounds	Cell lines	IC_{50} (µg/ml)		
		24 h	48 h	72 h
1	MCF-7 MDA-MB-231 MCF-10A	10.67±0.55 > 50 > 25	5.87±0.40 > 50 > 25	4.36±0.21 > 50 > 25
2	MCF-7 MDA-MB-231 MCF-10A	4.17±0.93 > 50 > 25	3.93±0.44 > 50 > 25	3.23±0.67 > 50 > 25
3	MCF-7 MDA-MB-231 MCF-10A	7.42±0.42 > 50 > 25	3.76±0.25 > 50 > 25	3.27±0.17 > 50 > 25
4	MCF-7 MDA-MB-231 MCF-10A	4.39±0.46 > 50 > 25	3.82±0.31 > 50 > 25	3.41±0.62 > 50 > 25
5	MCF-7 MDA-MB-231 MCF-10A	10.39±1.04 > 50 > 25	7.26±0.58 > 50 > 25	5.85±0.71 > 50 > 25
Doxorubicin	MCF-7 MDA-MB-231 MCF-10A	2.43±0.24 2.84±0.12 > 20	2.28±0.33 2.32±0.15 > 20	2.08±0.16 1.76±0.23 > 20
Tamoxifen	MCF-7 MDA-MB-231 MCF-10A	1.85± 0.22 2.93±0.35 > 20	1.66 ± 0.12 2.44±0.16 > 20	1.53±0.18 1.95±0.3 > 20

4.1.2 LDH release assay

The cytotoxicity effect of the quinazolinone-based compounds was also determined by the LDH release assay, was quantified on MCF-7 cells treated with various concentrations of the compounds for 24 h. All compounds triggered a significant elevation in LDH release, revealing cytotoxicity at different concentrations (4-15 µg/ml) compared to the control cells (Figures 4.1, 4.2, 4.3, 4.4 and 4.5).

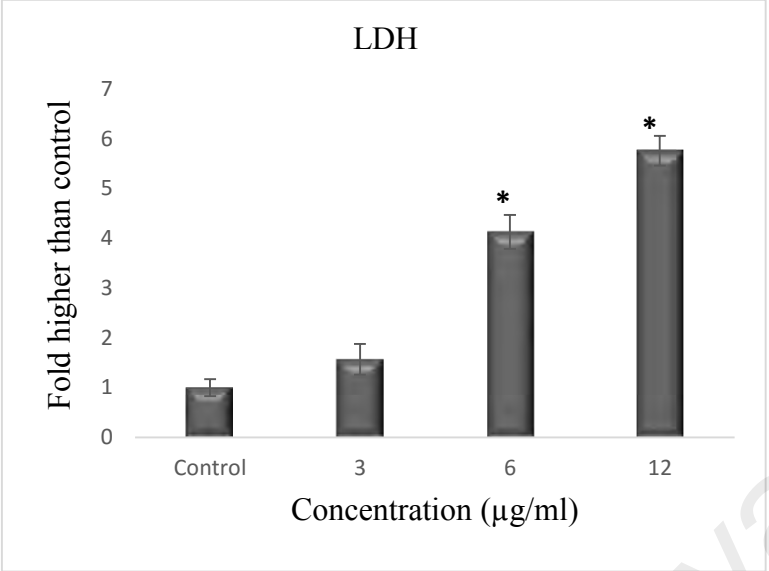


Figure 4. 1: Lactate dehydrogenase (LDH) assay of compound 1. The release of LDH was significant when MCF-7 cells were incubated with quinazolinone based compound at concentrations of 6-12 µg/mL after 24h. The data indicate the means ± SD of three independent experiments (significant values: $p<0.05$).

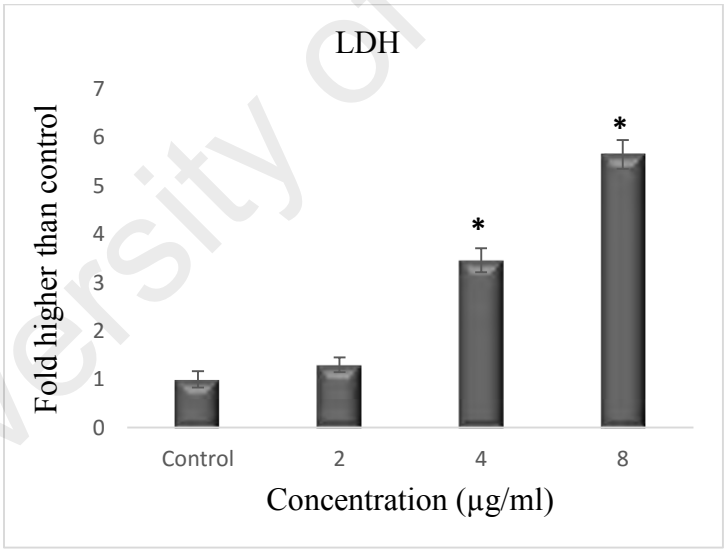


Figure 4. 2: Lactate dehydrogenase (LDH) assay of compound 2. The release of LDH was significant when MCF-7 cells were incubated with quinazolinone based compound at concentrations of 4-8 µg/mL after 24h. The data indicate the means ± SD of three independent experiments (significant values: $p<0.05$).

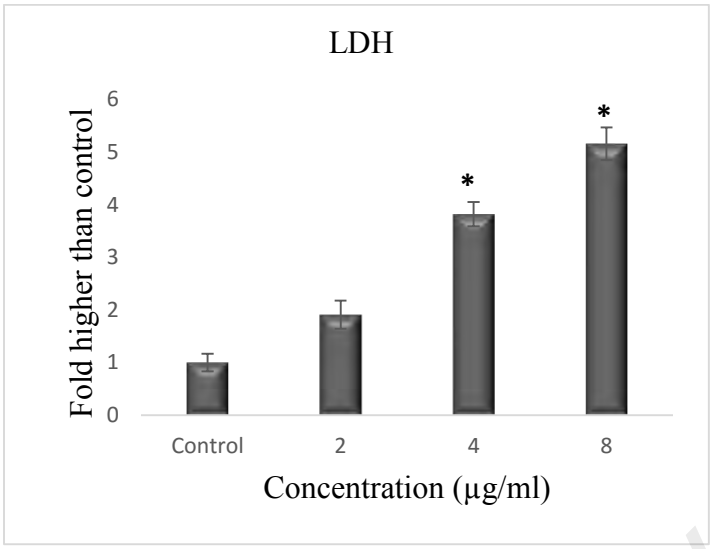


Figure 4. 3: Lactate dehydrogenase (LDH) assay of compound 3. The release of LDH was significant when MCF-7 cells were incubated with quinazolinone based compound at concentrations of 4-8 µg/mL after 24h. The data indicate the means ± SD of three independent experiments (significant values: $p<0.05$).

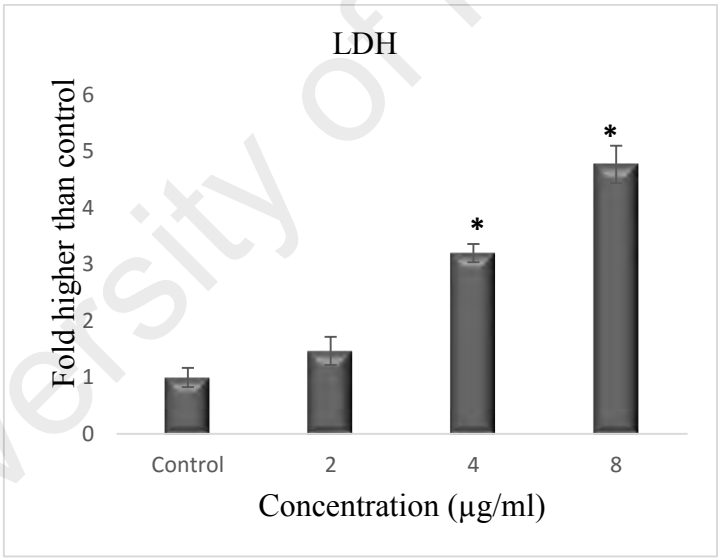


Figure 4. 4: Lactate dehydrogenase (LDH) assay of compound 4. The release of LDH was significant when MCF-7 cells were incubated with quinazolinone based compound at concentrations of 4-8 µg/mL after 24h. The data indicate the means ± SD of three independent experiments (significant values: $p<0.05$).

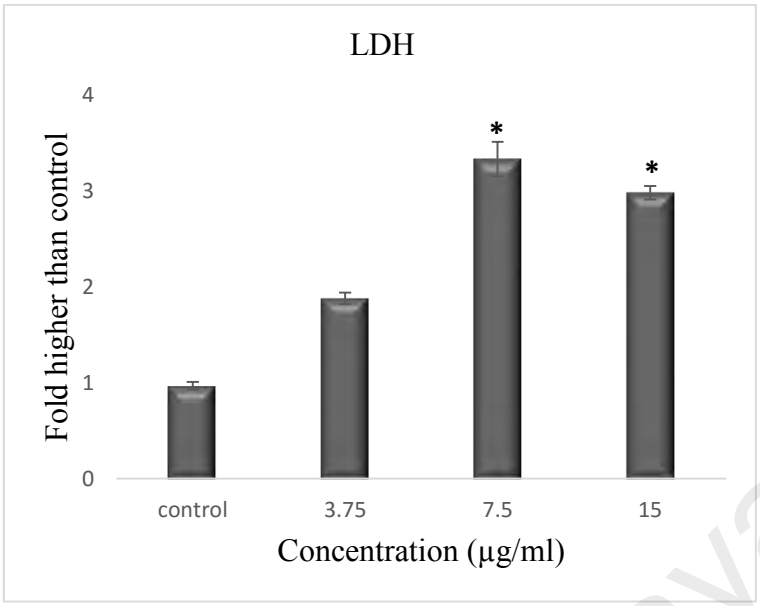


Figure 4. 5: Lactate dehydrogenase (LDH) assay of compound 5. The release of LDH was significant when MCF-7 cells were incubated with quinazolinone based compound at concentrations of 7.5-15µg/mL after 24 h. The data indicate the means ± SD of three independent experiments (significant values: $p<0.05$).

4.1.3 Morphological examination of apoptotic cells using AO/PI double-staining

Morphological changes of treated MCF-7 cell with the quinazoline-based compounds were examined after 24, 48 and 72 h treatment at IC_{50} value. Cell morphological assessment showed the effects of compounds to be more prominent in treated MCF-7 cells when compared to untreated cells. The untreated MCF-7 cells showed green healthy intact nuclei. After 24 h of treatment, cell morphological changes were not significant. However, after 48 h of treatment, chromatin condensation and membrane blebbing (indicated by small protrusions of the membrane) are most significant properties of early apoptotic events. These features were more pronounced at 72 h of treatment, which was due to the presence of orange colour as a result of the PI-positive band of denaturated DNA. Furthermore, the presence of secondary necrosis was also prominent, as the prolonged incubation of treated MCF-7 cells might induce secondary necrosis following the late apoptosis (Figures 4.6, 4.7, 4.8, 4.9 and 4.10). The criteria

for identification are as follows: (A) green intact nucleus, viable cells; (B) dense green areas of chromatin condensation in the nucleus, early apoptosis; (C) dense orange areas of chromatin condensation, late apoptosis; and (D) orange intact nucleus, secondary necrosis.

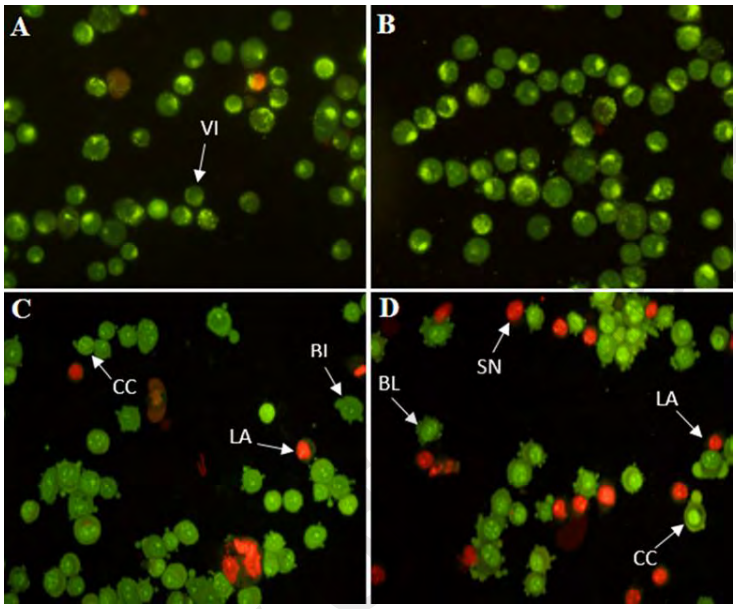


Figure 4. 6: Morphological changes in treated MCF7 cells with compound 1**.** A) Untreated MCF7 cells after 72 h demonstrated normal structure without prominent apoptosis and necrosis. Early apoptosis features (membrane blebbing and chromatin condensation were seen after 24 h (B) and 48h (C). (D) Late apoptosis were noticed in 72 h of treatment (magnification: 200×). VI: Viable cells; BL: Blebbing of cell membrane; CC: Chromatin condensation; LA: Late apoptosis; SN: Secondary necrosis.

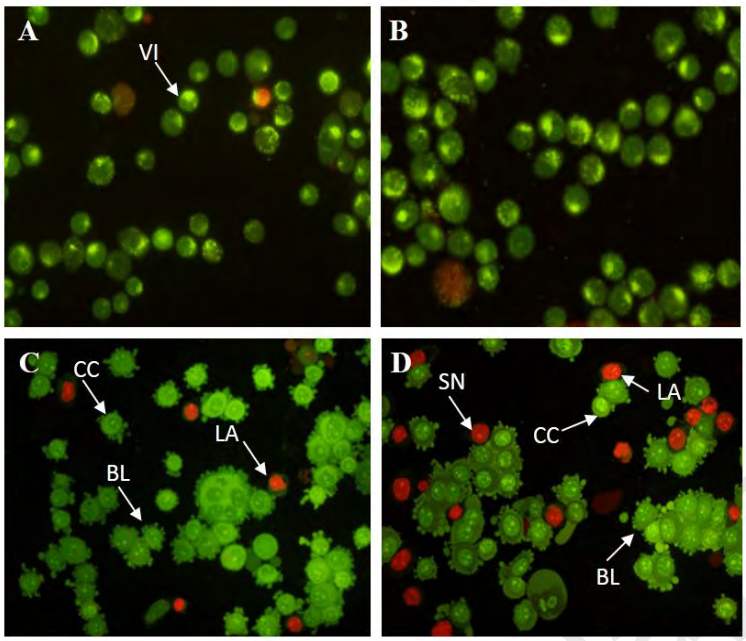


Figure 4. 7: Morphological changes in treated MCF7 cells with compound 2.

A) Untreated MCF7 cells after 72 h demonstrated normal structure without prominent apoptosis and necrosis. Early apoptosis features (membrane blebbing and chromatin condensation were seen after 24 h (B) and 48h (C). (D) Late apoptosis were noticed in 72 h of treatment (magnification: 200×). VI: Viable cells; BL: Blebbing of cell membrane; CC: Chromatin condensation; LA: Late apoptosis; SN: Secondary necrosis.

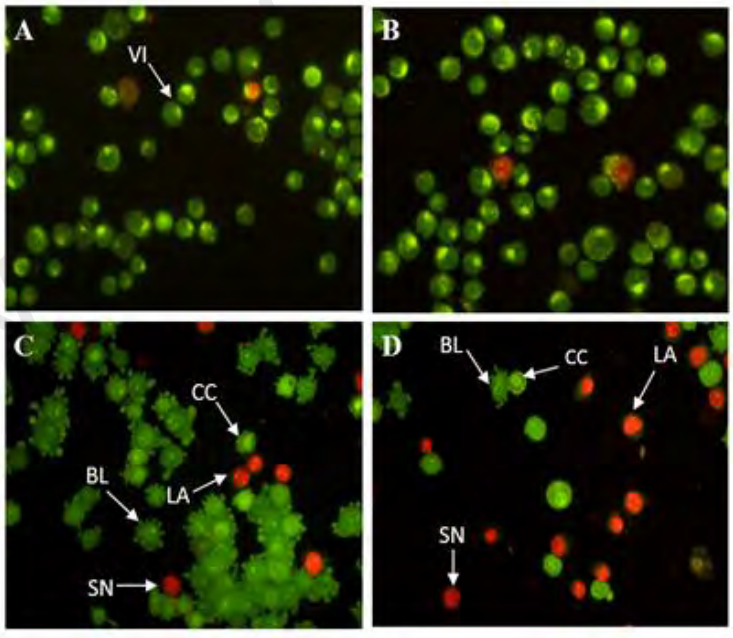


Figure 4. 8: Morphological changes in treated MCF7 cells with compound 3

(A) Untreated MCF7 cells after 72 h demonstrated normal structure without prominent apoptosis and necrosis. Early apoptosis features (membrane blebbing and chromatin condensation were seen after 24 h (B) and 48h (C). (D) Late apoptosis were noticed in 72 h of treatment (magnification: 200×). VI: Viable cells; BL: Blebbing of cell membrane; CC: Chromatin condensation; LA: Late apoptosis; SN: Secondary necro

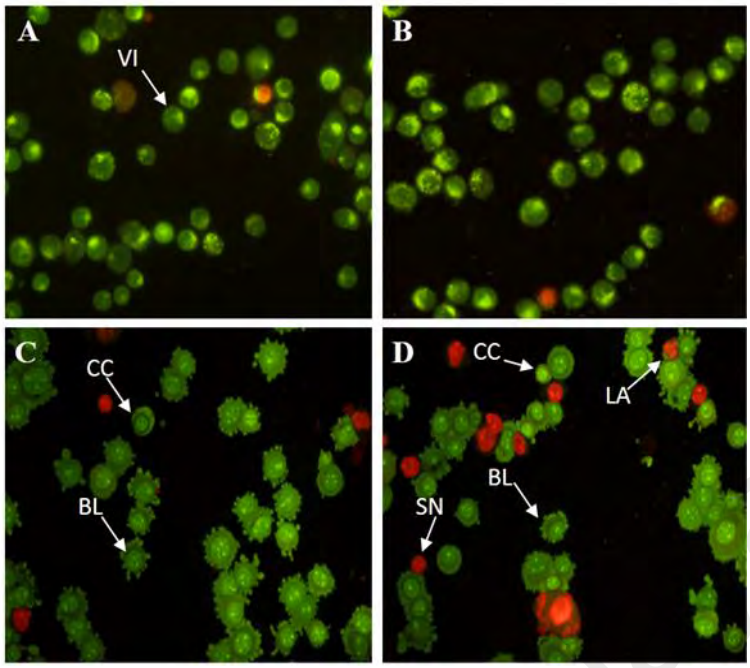


Figure 4. 8: Morphological changes in treated MCF7 cells with compound 4

(A) Untreated MCF7 cells after 72 h demonstrated normal structure without prominent apoptosis and necrosis. Early apoptosis features (membrane blebbing and chromatin condensation were seen after 24 h (B) and 48h (C). (D) Late apoptosis were noticed in 72 h of treatment (magnification: 200×). VI: Viable cells; BL: Blebbing of cell membrane; CC: Chromatin condensation; LA: Late apoptosis; SN: Secondary necrosis.

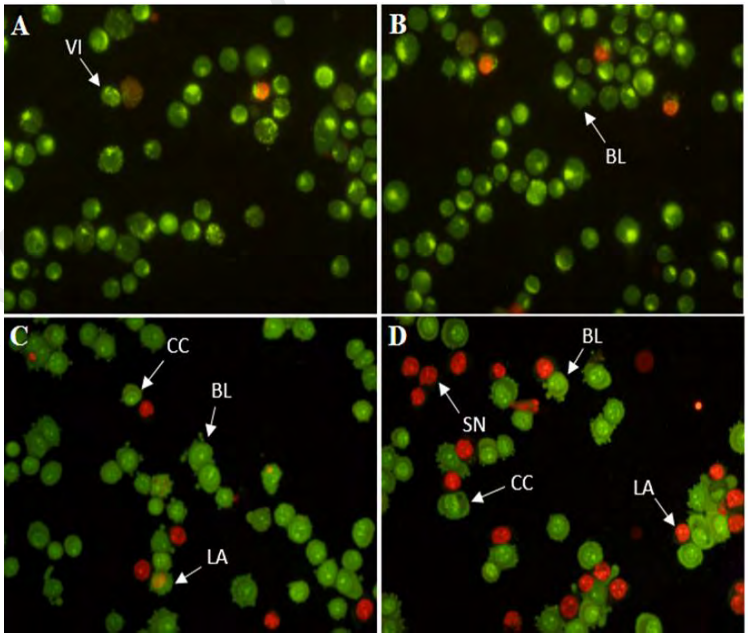


Figure 4. 9: Morphological changes in treated MCF7 cells with compound 5

A) Untreated MCF7 cells after 72 h demonstrated normal structure without prominent apoptosis and necrosis. Early apoptosis features (membrane blebbing and chromatin condensation were seen after 24 h (B) and 48h (C). (D) Late apoptosis were noticed in 72 h of treatment (magnification: 200×). VI: Viable cells; BL: Blebbing of cell membrane; CC: Chromatin condensation; LA: Late apoptosis; SN: Secondary necrosis.

4.1.4 Reactive oxygen species (ROS) generation

Upgrading in the level of ROS can trigger mitochondrial-initiated events leading to cell apoptosis. Generation of ROS was increased in the treated MCF-7 cells with different concentrations of compounds (4-15 µg/ml). The accumulating data from this experiment showed the oxidation of dihydroethidium (DHE) to ethidium in the exposure to the quinazolinone-based compounds in the treated-MCF-7 cells after 24 hours (Figure 4.11, 4.12, 4.13, 4.14 and 4.15).

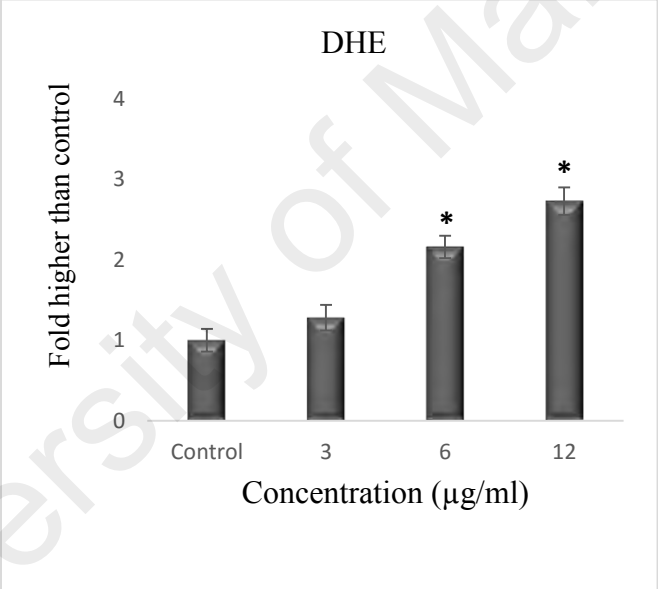


Figure 4. 10: Effect of compound 1 on the generation of ROS. The level of ROS significantly elevated at concenterations of 6 -12 µg/mL after 24h. The data indicate the means ± SD of three independent experiments (significant values: $p<0.05$).

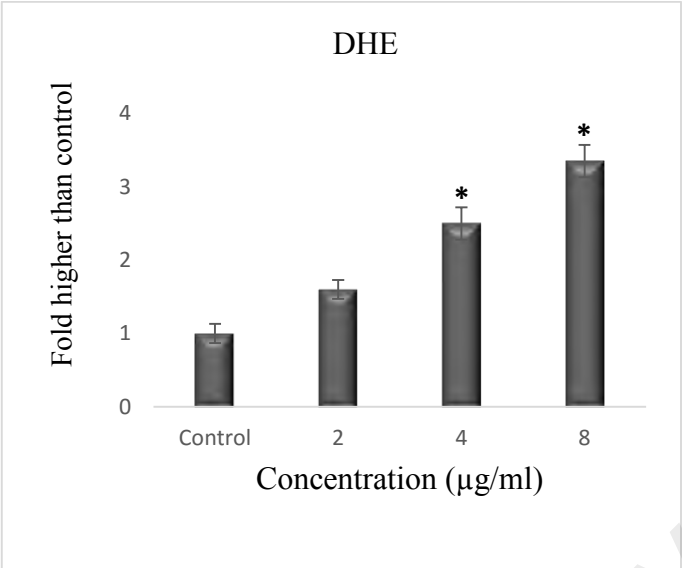


Figure 4. 11: Effect of compound 2 on the generation of ROS. The level of ROS significantly elevated at concenterations of 4 -8 µg/mL after 24h. The data indicate the means ± SD of three independent experiments (significant values: $p<0.05$).

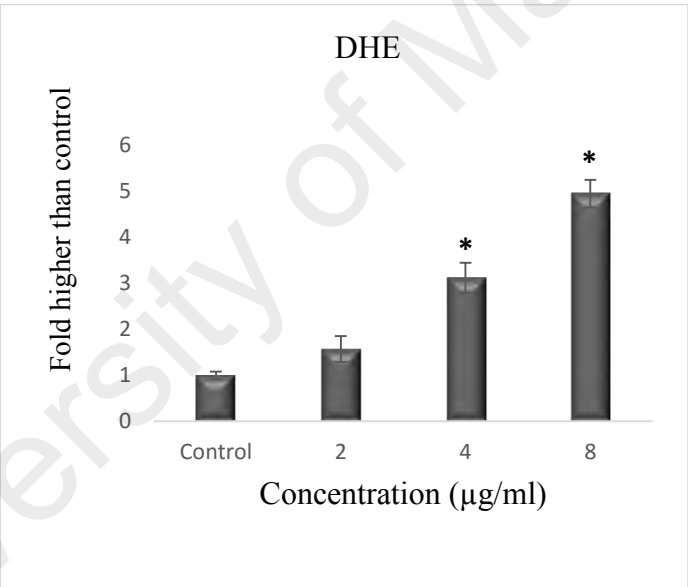


Figure 4. 12: Effect of compound 3 on the generation of ROS. The level of ROS significantly elevated at concenterations of 4-8 µg/mL after 24h. The data indicate the means ± SD of three independent experiments (significant values: $p<0.05$).

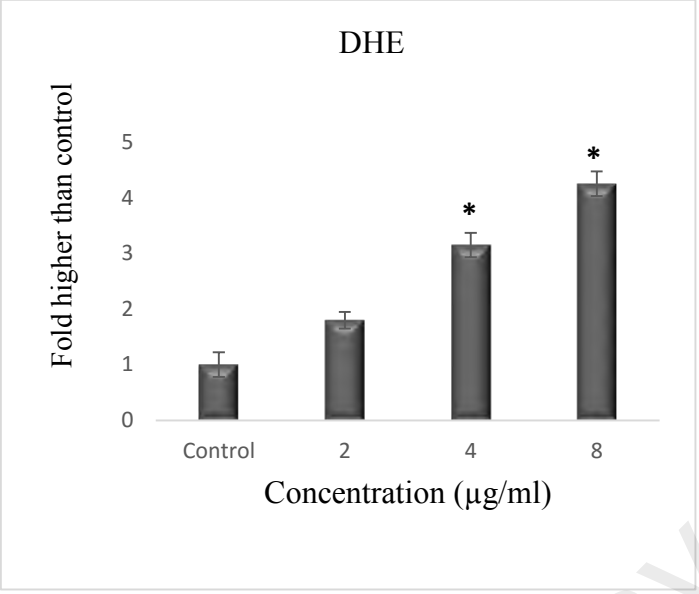


Figure 4. 13: Effect of compound 4 on the generation of ROS. The level of ROS significantly elevated at concenterations of 4-8 µg/mL after 24h. The data indicate the means ± SD of three independent experiments (significant values: $p<0.05$).

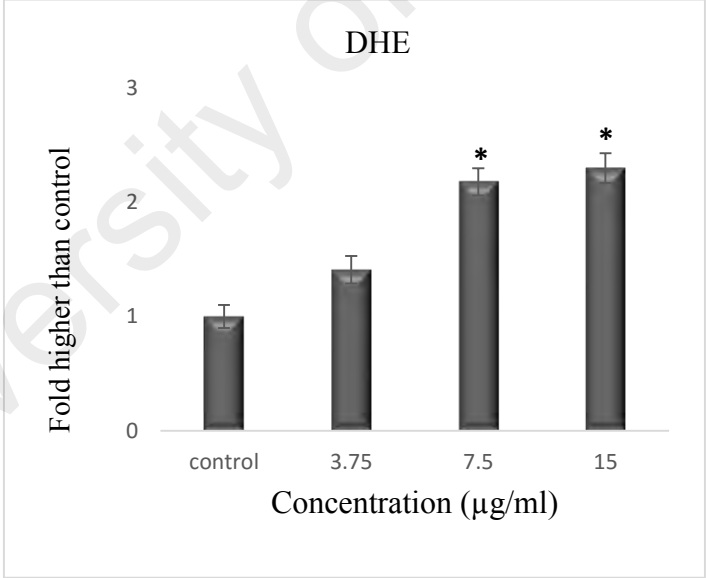


Figure 4. 14: Effect of compound 5 on the generation of ROS. The level of ROS significantly elevated at concenterations of 7.5-15µg/mL after 24h. The data indicate the means ± SD of three independent experiments (significant values: $p<0.05$).

4.1.5 Effects of quinazolinone-based compounds membrane permeability, mitochondrial membrane potential (MMP) and Cytochrome c release

To identify the main mechanisms involved in the quinazolinone bases-dependent apoptotic process, mitochondrial membrane potential (MMP) and cytochrome *c* release were analyzed using Cellomic High Content Screening. As shown in figures 4.16, 4.18, 4.20, 4.22 and 4.24, the untreated cells were strongly stained with MMP dye in comparison to the cells treated with different concentrations of compounds for 24 h. The reduction of MMP fluorescence intensity reflects that the MMP is imbalanced in the treated cells. In contrast, a remarkable increase in cell membrane permeability was also observed in the treated cells after 24 h of treatment. Furthermore, compounds activated a significant translocation of cytochrome *c* from the mitochondria into the cytosol (Figures 4.17, 4.19, 4.21, 4.23 and 4.25).

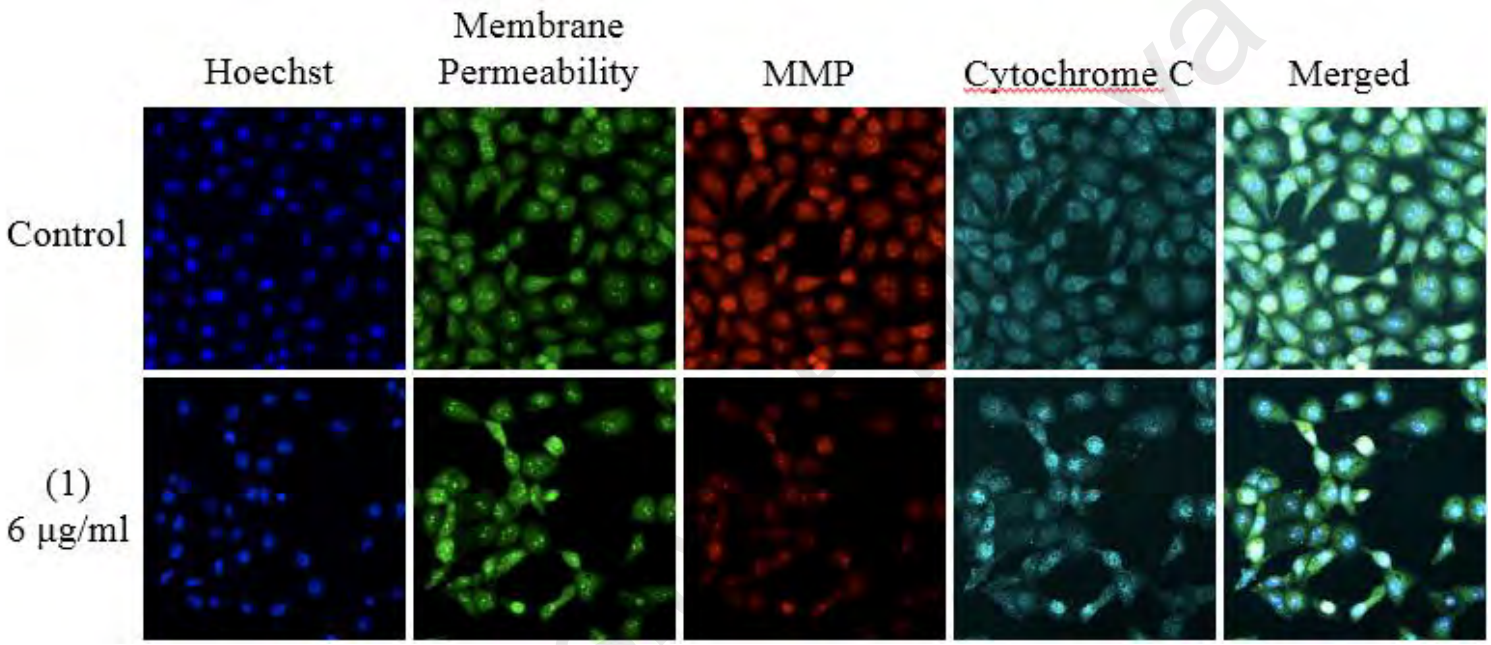


Figure 4. 15: Representative images of immunostaining (Hoechst 33342, cytochrome c and MMP staining) of MCF-7 cells treated with medium alone and compound 1 at concentration of 6 µg/mL. Compound 1 induced a noteworthy elevation in membrane permeability and cytochrome *c* release and a marked reduction in MMP (Magnification: 200×).

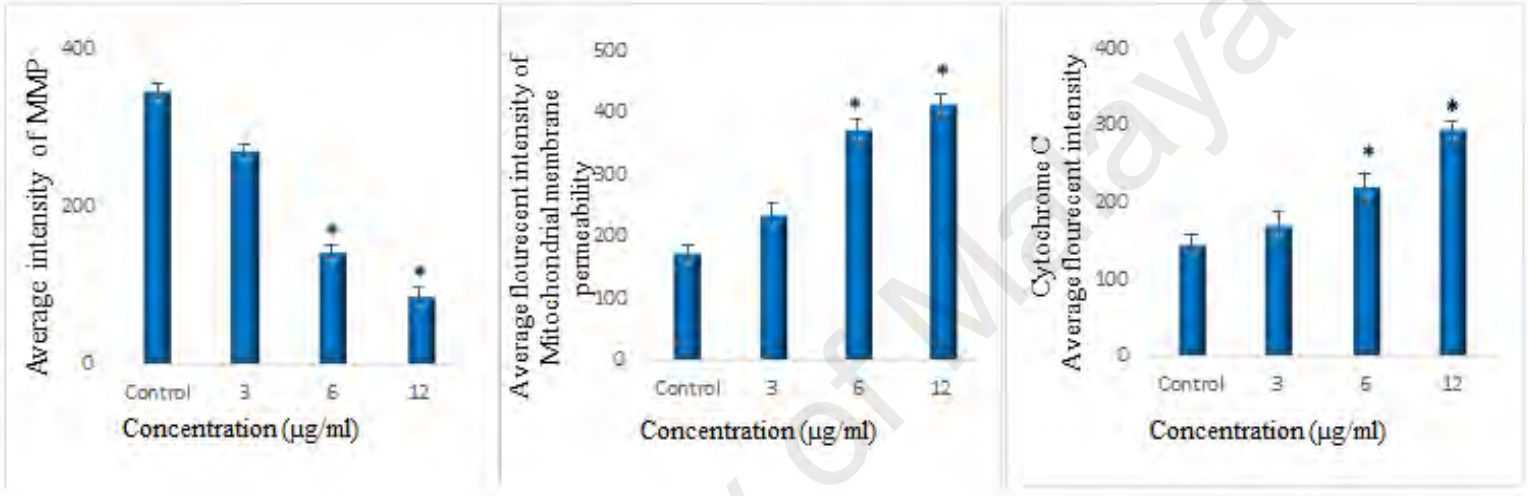


Figure 4. 16: Dose-dependent signal intensities of reduced MMP, increased cell permeability and cytochrome c release of treated cells with compound 1. Compound 1 induced the reduction in MMP, the increased cell permeability, and the cytochrome *c* release in treated MCF-7 cells. All values are mean ± SD for three experiments (significant values: $p<0.05$).

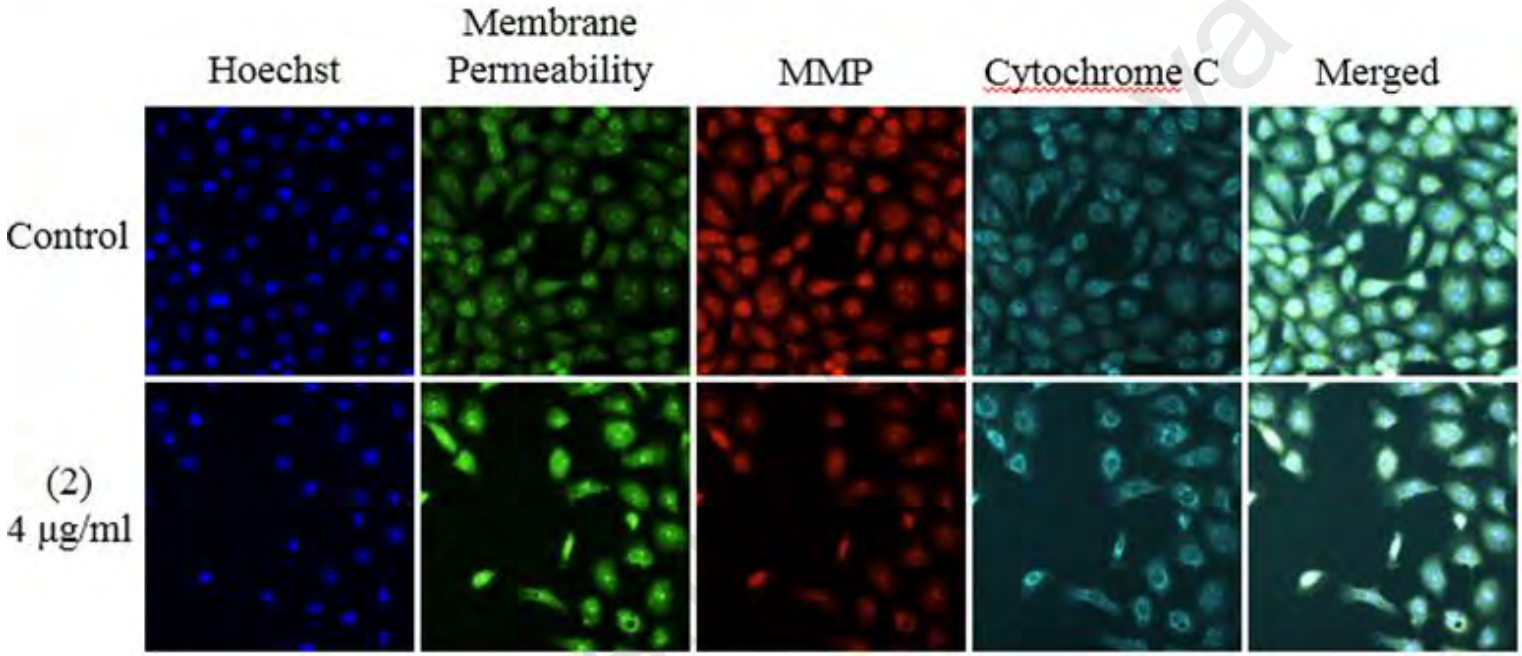


Figure 4. 17: Representative images of immunostaining (Hoechst 33342, cytochrome c and MMP staining) of MCF-7 cells treated with medium alone and compound 2 at concentration of 4 µg/mL. Compound 2 induced a noteworthy elevation in membrane permeability and cytochrome *c* release and a marked reduction in MMP (Magnification: 200×).

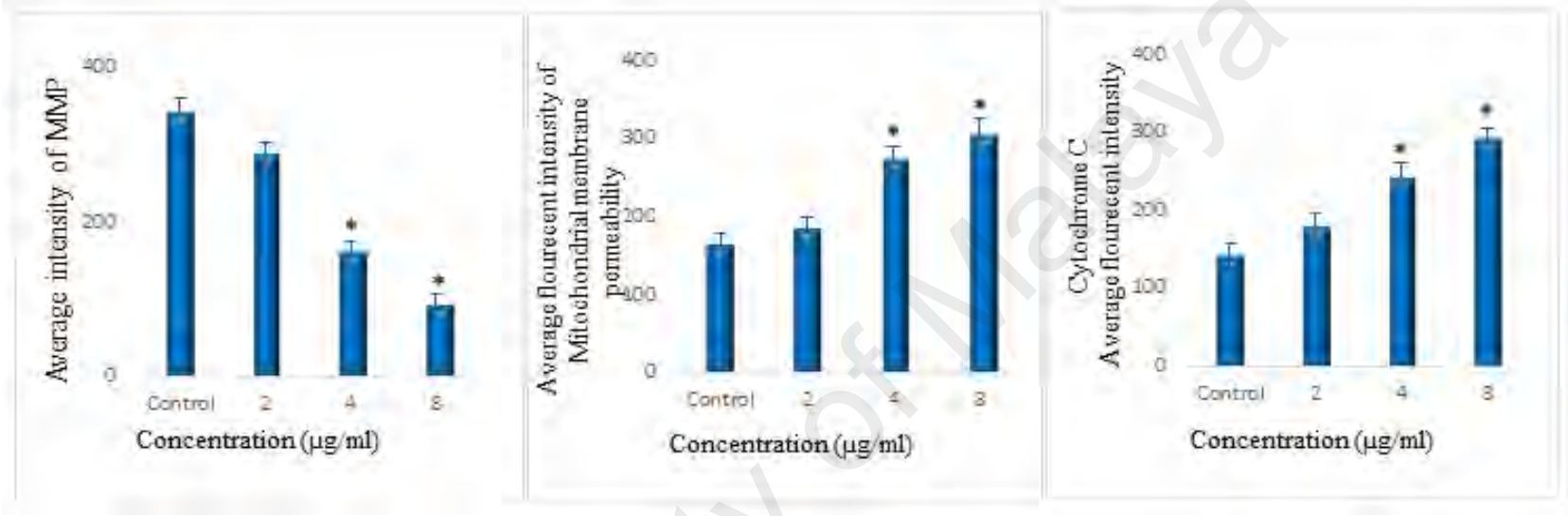


Figure 4. 18: Dose-dependent signal intensities of reduced MMP, increased cell permeability and cytochrome *c* release of treated cells with compound 2. Compound 2 induced the reduction in MMP, the increased cell permeability, and the cytochrome *c* release in treated MCF-7 cells. All values are mean ± SD for three experiments (significant values: $p<0.05$).

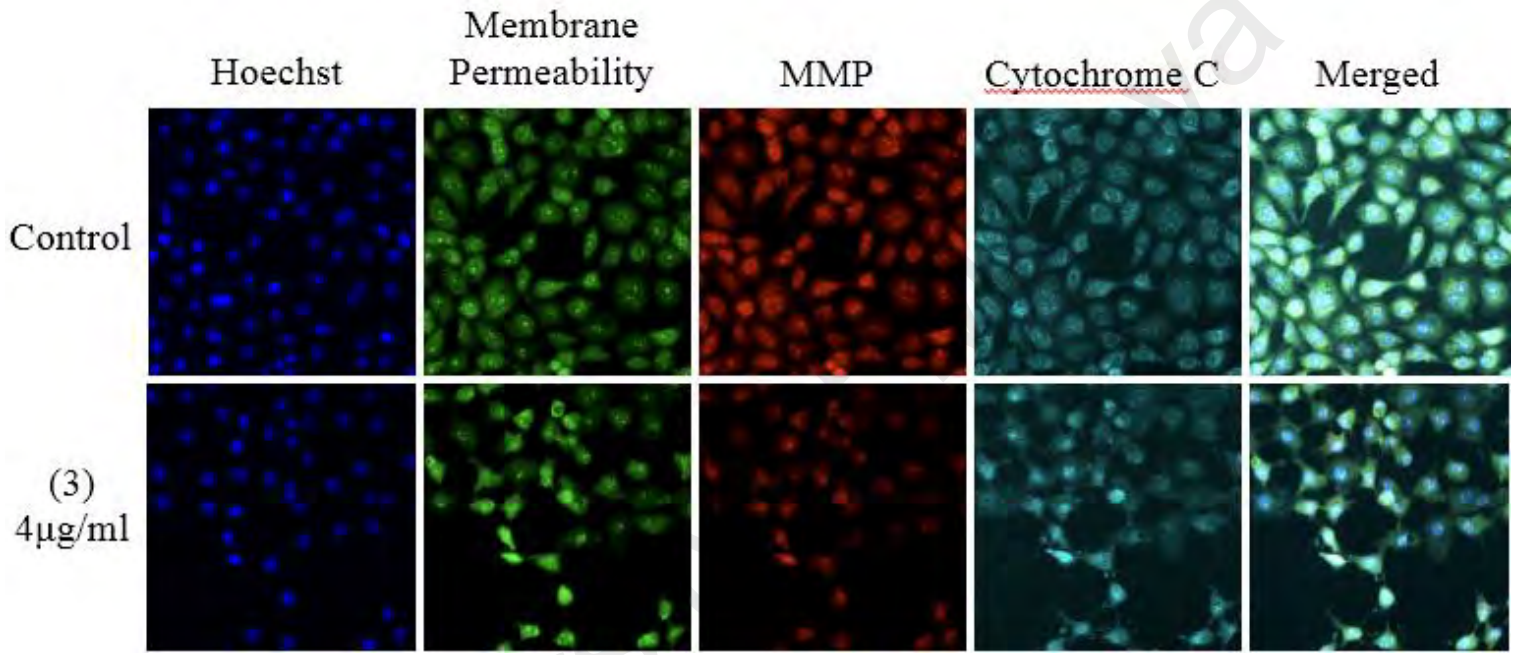


Figure 4. 19: Representative images of immunostaining (Hoechst 33342, cytochrome c and MMP staining) of MCF-7 cells treated with medium alone and compound 3 at concentration of 4 µg/mL. Compound 3 induced a noteworthy elevation in membrane permeability and cytochrome *c* release and a marked reduction in MMP (Magnification: 200).

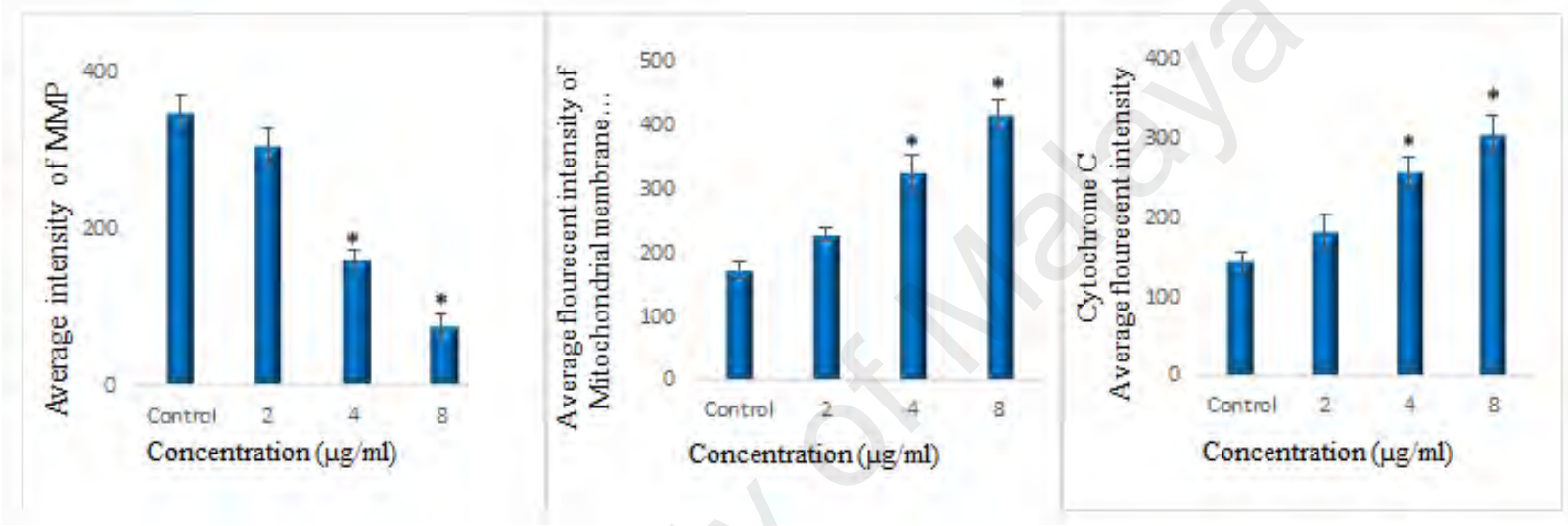


Figure 4. 20: Dose-dependent signal intensities of reduced MMP, increased cell permeability and cytochrome *c* release of treated cells with compound 3. Compound 2 induced the reduction in MMP, the increased cell permeability, and the cytochrome *c* release in treated MCF-7 cells. All values are mean ± SD for three experiments (significant values: $p<0.05$).

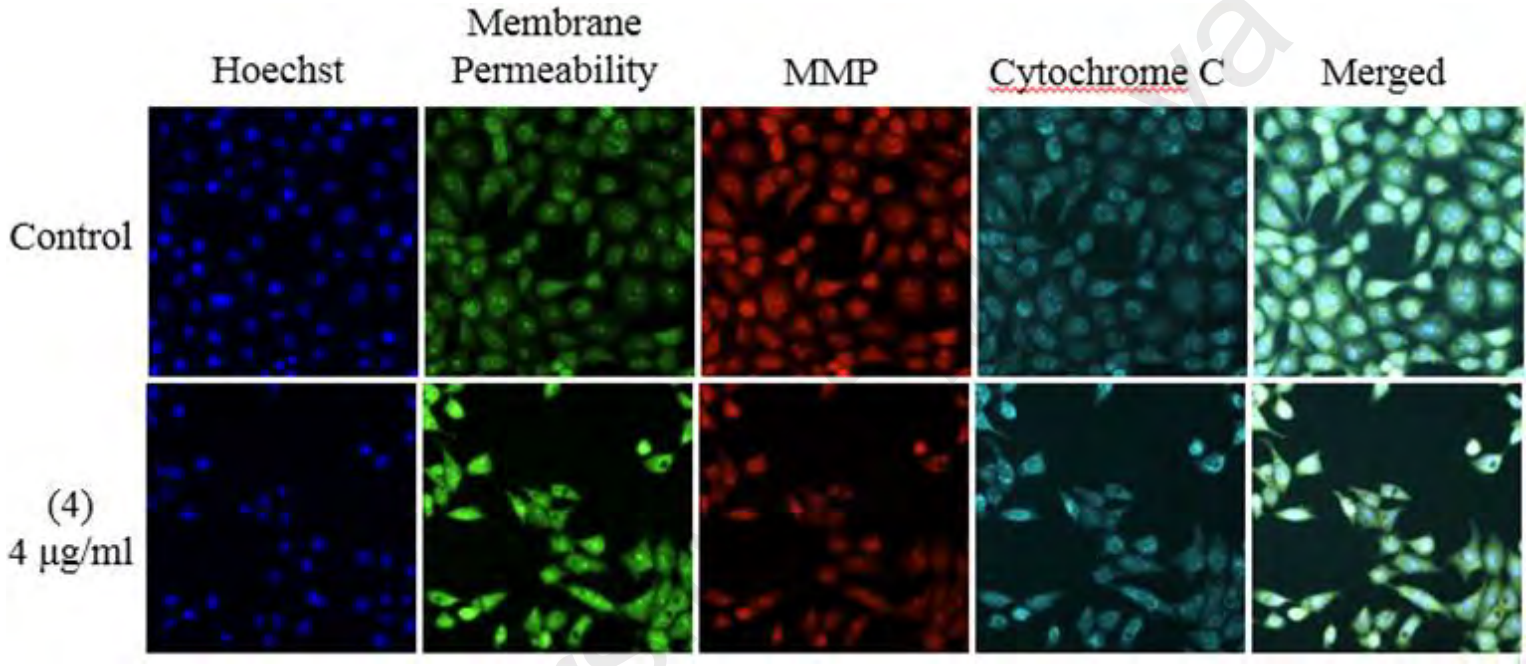


Figure 4. 21: Representative images of immunostaining (Hoechst 33342, cytochrome c and MMP staining) of MCF-7 cells treated with medium alone and compound 4 at concentration of 4 µg/mL. Compound 4 induced a noteworthy elevation in membrane permeability and cytochrome *c* release and a marked reduction in MMP (Magnification: 200×)

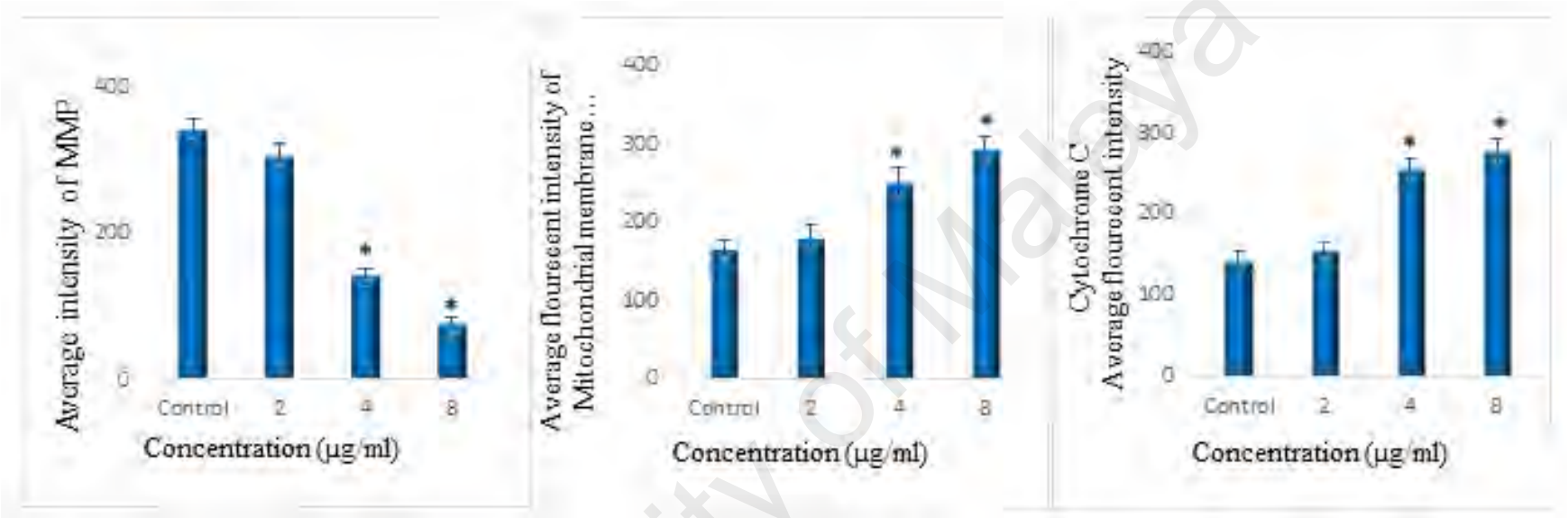


Figure 4. 22: Dose-dependent signal intensities of reduced MMP, increased cell permeability and cytochrome *c* release of treated cells with compound 4. Compound 4 induced the reduction in MMP, the increased cell permeability, and the cytochrome *c* release in treated MCF-7 cells. All values are mean ± SD for three experiments (significant values: $p<0.05$).

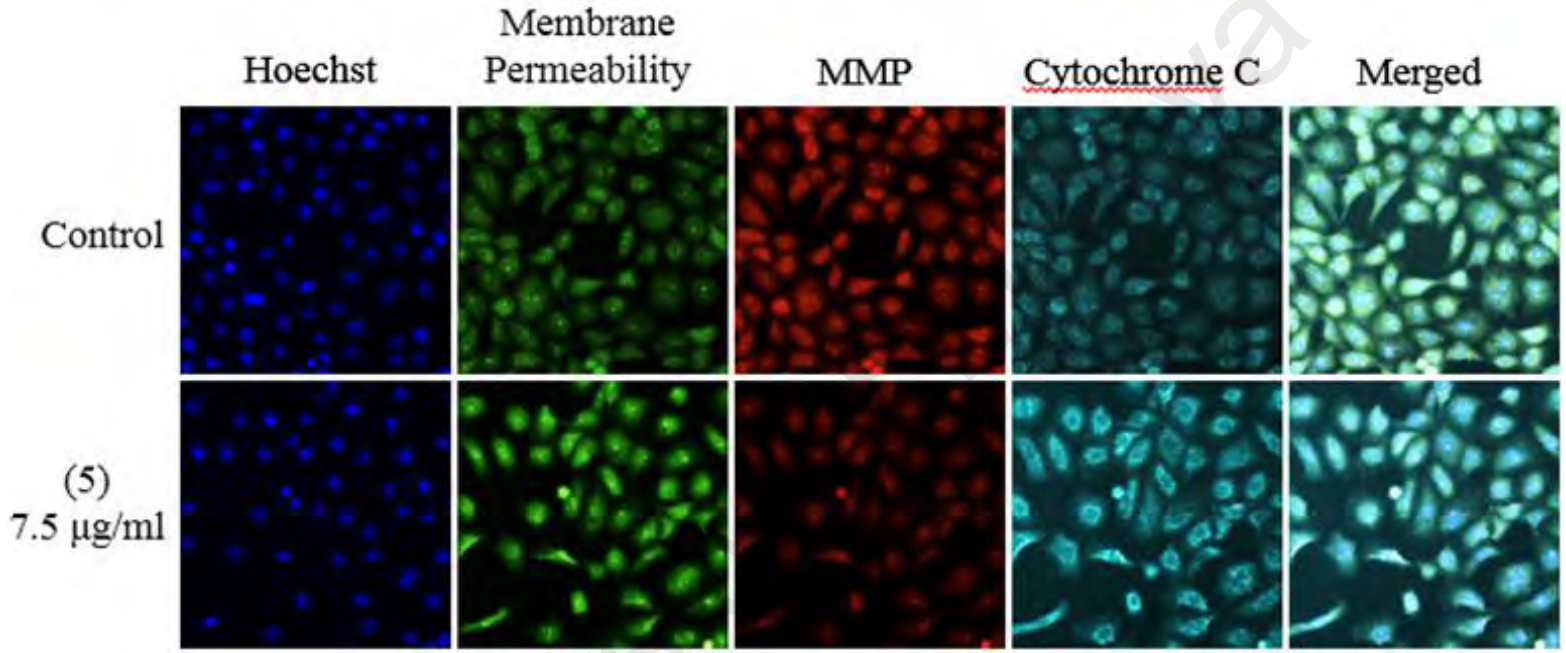


Figure 4. 23: Representative images of immunostaining (Hoechst 33342, cytochrome c and MMP staining) of MCF-7 cells treated with medium alone and compound 5 at concentration of 7.5 µg/mL. Compound 5 induced a noteworthy elevation in membrane permeability and cytochrome *c* release and a marked reduction in MMP (Magnification: 200×).

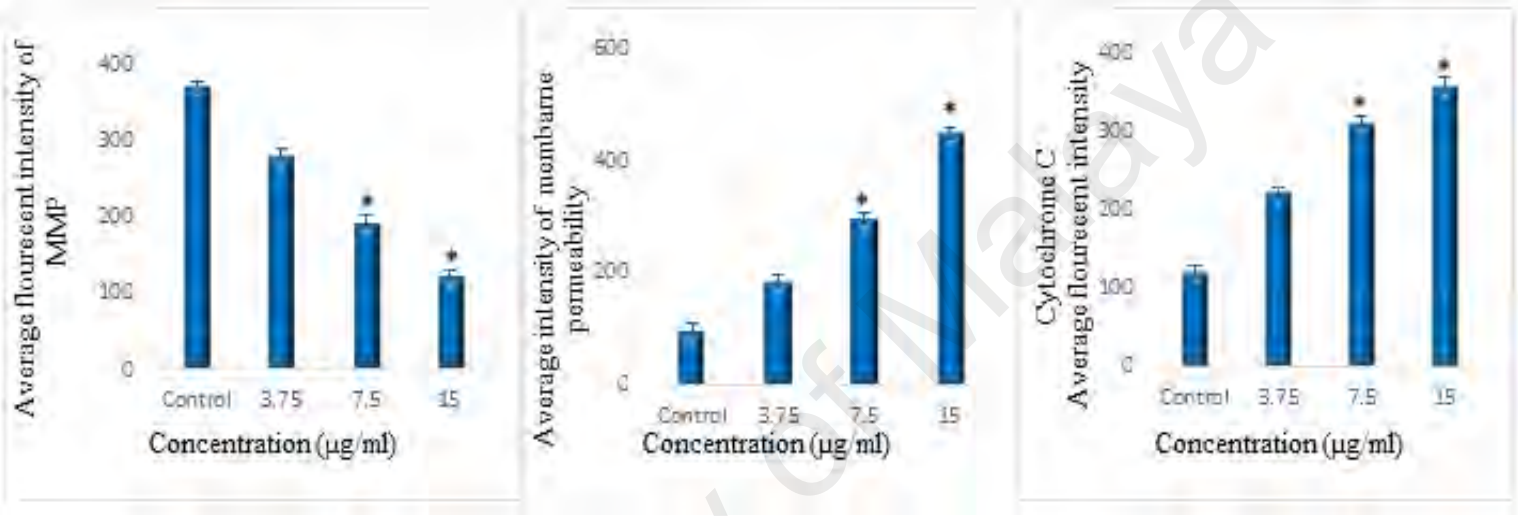


Figure 4. 24: Dose-dependent signal intensities of reduced MMP, increased cell permeability and cytochrome *c* release of treated cells with compound 5. Compound 5 induced the reduction in MMP, the increased cell permeability, and the cytochrome *c* release in treated MCF-7 cells. All values are mean ± SD for three experiments (significant values: $p<0.05$).

4.1.6 Activation of caspase -3/7, -8, -9

The florescence intensities of caspase-3/7, -8, -9 activities quantified in MCF-7 cells after treatment of all five compounds at their IC_{50} value of 48 hours in time dependent- manner. As shown in figures 4.26, 4.27 and 4.28, significant elevation in the activity of caspase -3/7, -8 and -9 were observed after 12 h of treatment with compound 1, 2 and 3, respectively. However, no changes of activation caspase-8 were detected in treated cell with compound 4 and 5 (Figure 4.29 and 4.30).

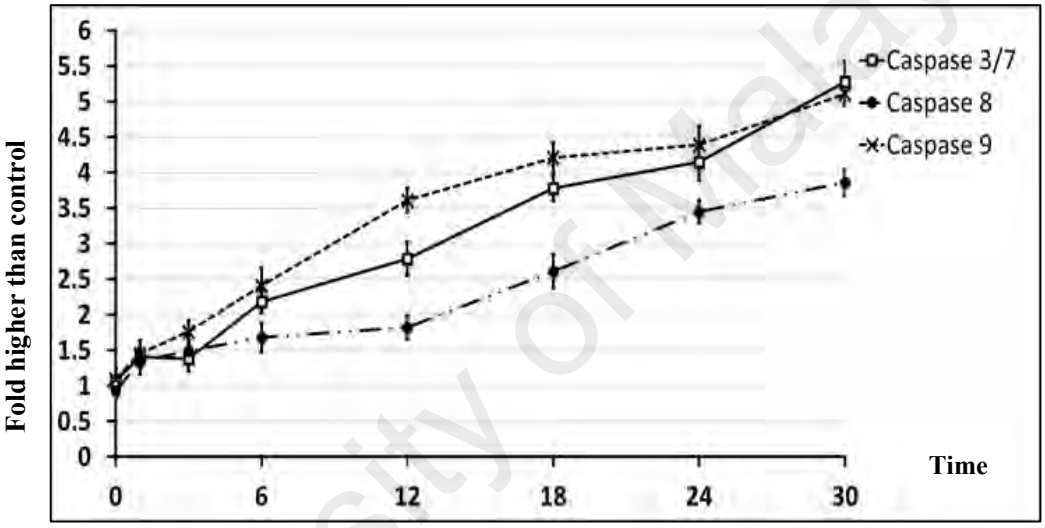


Figure 4. 25: Caspase cascade events during compound 1-induced apoptosis. Compound 1 exerted cytotoxic effect on treated MCF-7 in a time-dependent manner as represented by the up- regulation of caspase 3/7, -8 and -9. All values are mean ± SD for three experiments (significant values: $p<0.05$).

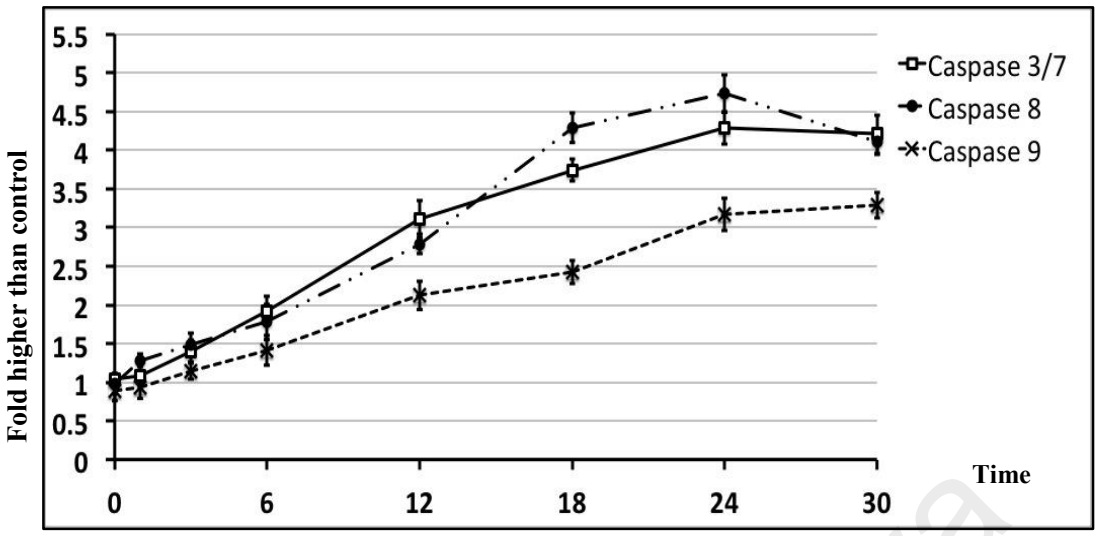


Figure 4. 26: Caspase cascade events during compound 2-induced apoptosis. Compound 2 exerted cytotoxic effect on treated MCF-7 in a time-dependent manner as represented by the up- regulation of caspase 3/7, -8 and -9. All values are mean ± SD for three experiments (significant values: $p<0.05$).

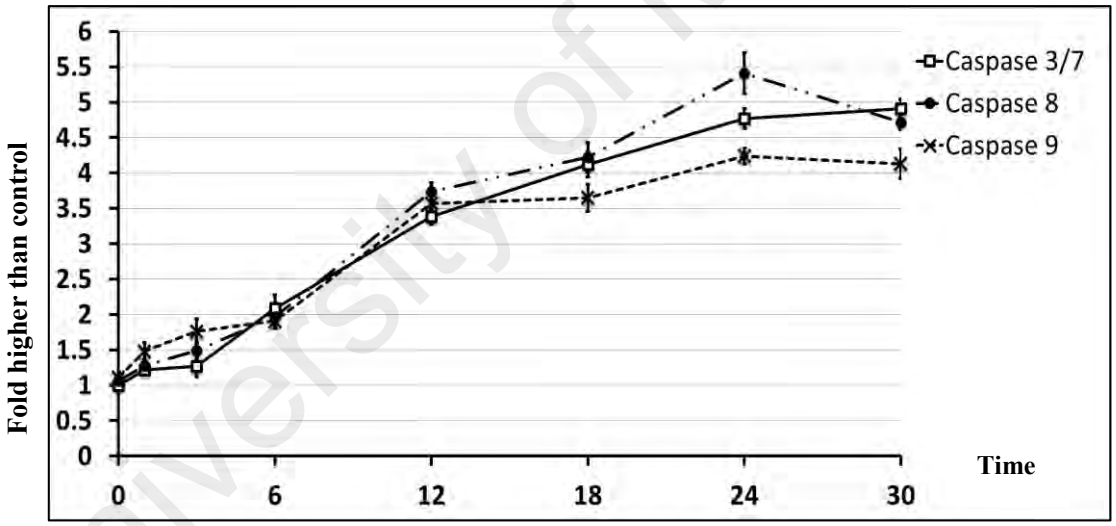


Figure 4. 27: Caspase cascade events during compound 3-induced apoptosis. Compound 3 exerted cytotoxic effect on treated MCF-7 in a time-dependent manner as represented by the up- regulation of caspase 3/7, -8 and -9. All values are mean ± SD for three experiments (significant values: $p<0.05$).

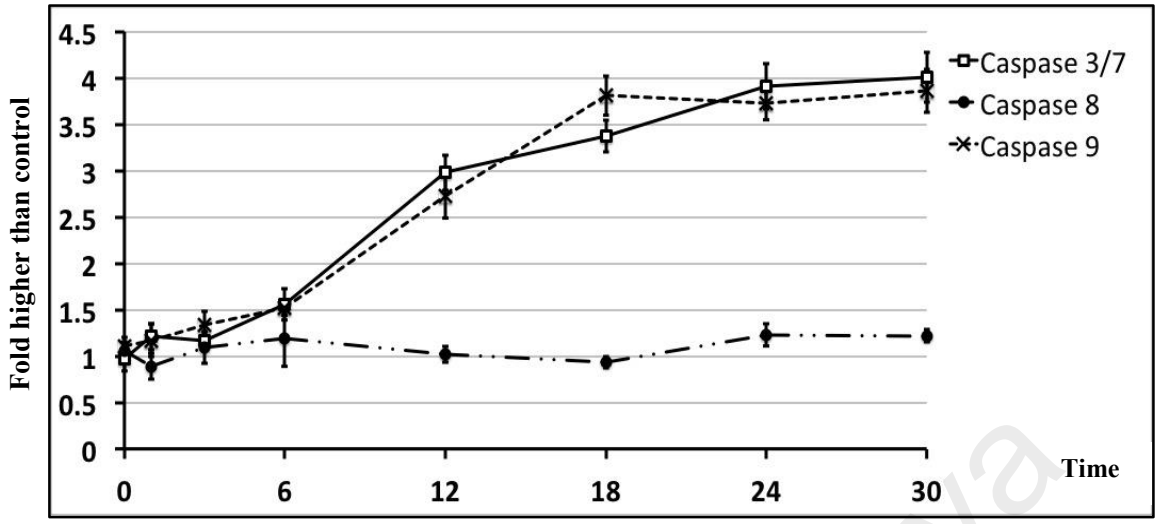


Figure 4. 28: Caspase cascade events during compound 4-induced apoptosis. Compound 4 exerted cytotoxic effect on treated MCF-7 in a time-dependent manner as represented by the up-regulation of caspase 3/7 and -9. In contrast, the expression of caspase 8 was fluctuating and was not significantly higher than control. All values are mean ± SD for three experiments (significant values: $p<0.05$).

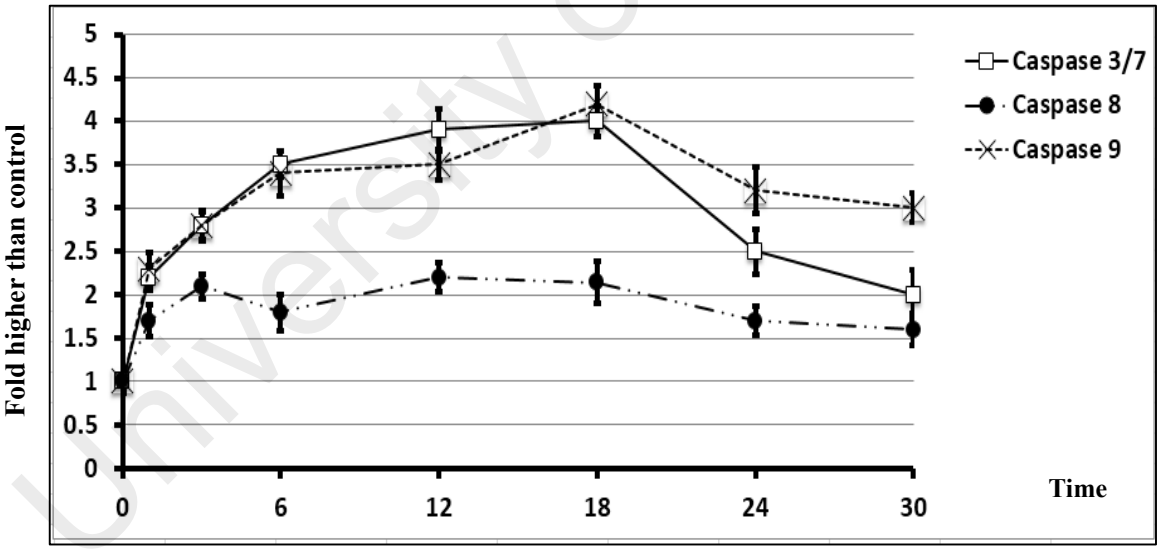


Figure 4. 29: Caspase cascade events during compound 5-induced apoptosis. Compound 5 exerted cytotoxic effect on treated MCF-7 in a time-dependent manner as represented by the up-regulation of caspase 3/7 and -9. In contrast, the expression of caspase 8 was fluctuating and was not significantly higher than control. All values are mean ± SD for three experiments (significant values: $p<0.05$).

4.1.7 NF-κB Translocation

NF-κB activation elicits a crucial role in cell proliferation and could be activated by caspase 8. Inflammatory cytokines, such as Tumour Necrosis Factor- -α (TNF-α), stimulate activation and translocation of NF- κB to the nucleus, thus mediate specific gene expression via DNA binding sites. Therefore, we examined the role of the quinazolinone-based compounds in the inhibition of activated NF-*κ*B was investigated. The result of images and intensities indicated that compound 1, 2 and 3 revealed a significant inhibition on the TNF-*α*-activated NF-*κ*B; however, 4 and 5 showed no difference with TNF-*α* positive control (Figures 4.31,4.32, 4.33, 4.34 and 4.35).

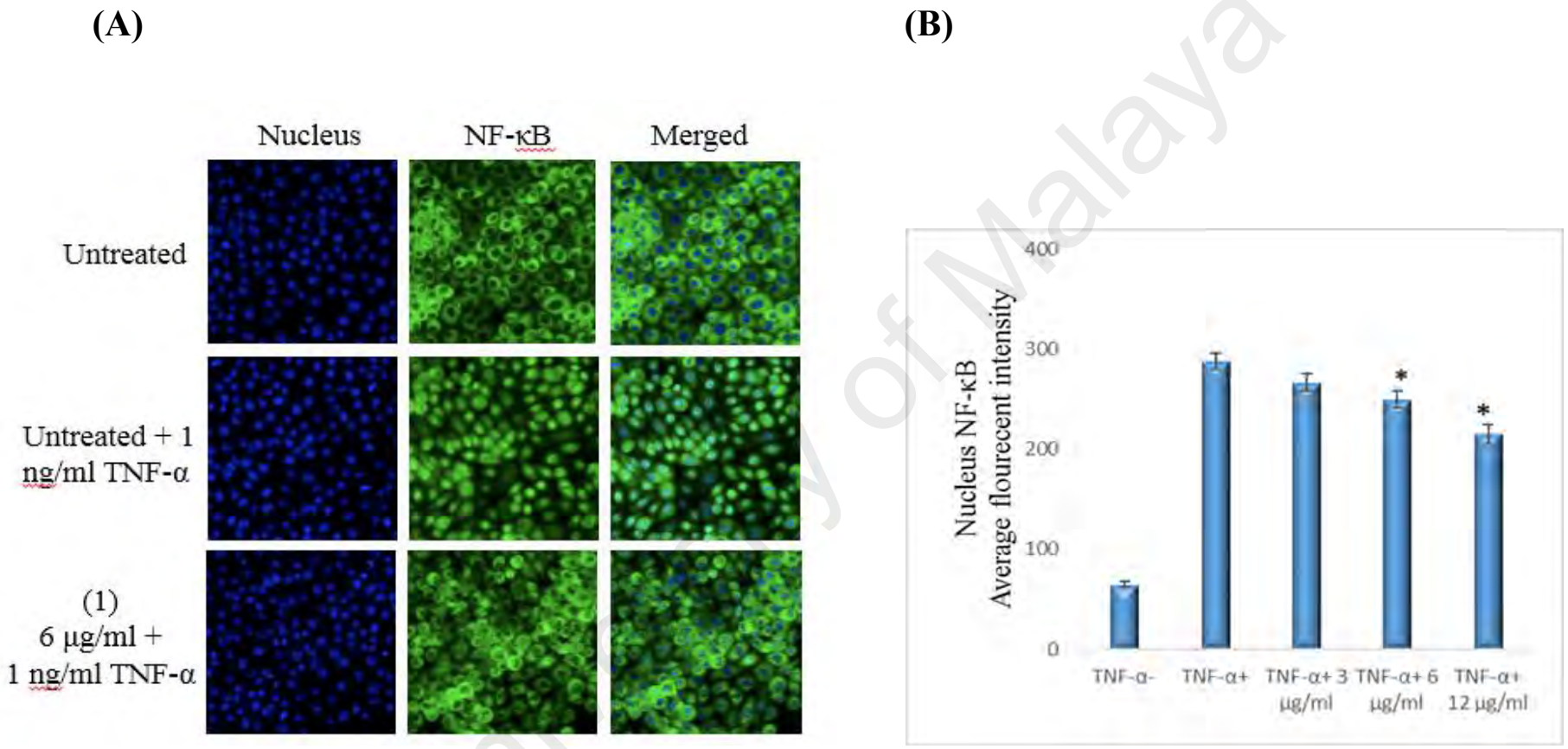
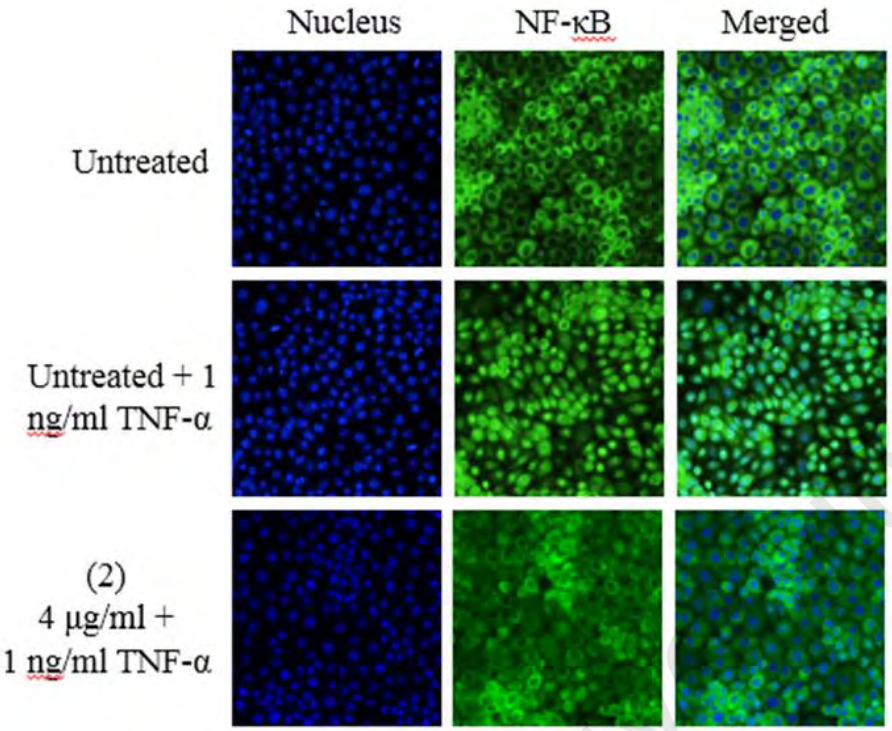


Figure 4. 30: The effect of compound 1 on the intracellular translocation of NF- κB A) Microscopy examination the stained MCF-7 cells for detection of NF-κB at concentration of 6µg/mL. B) Representative bar chart of TNF-α treated and compound 1-treated showing that a significant inhibition of activated NF-κB in treated MCF-7 cells. All values are mean ± SD for three experiments (significant values: $p<0.05$).

(A)



(B)

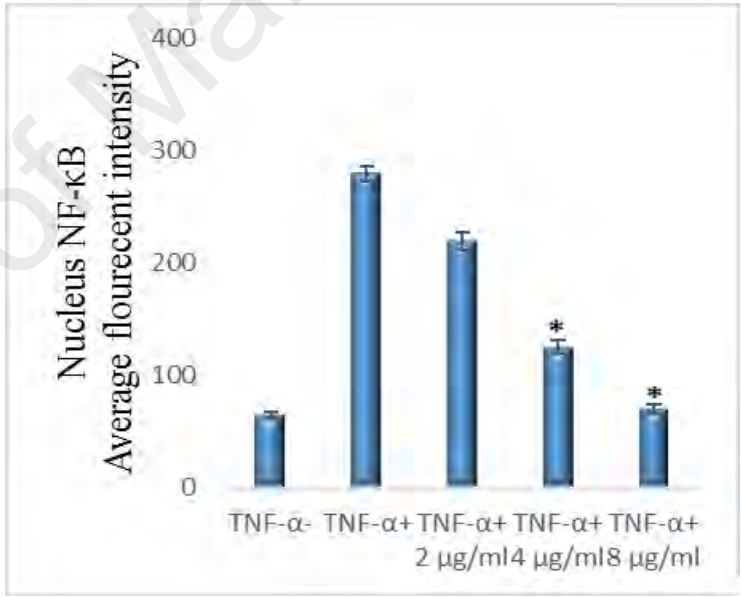
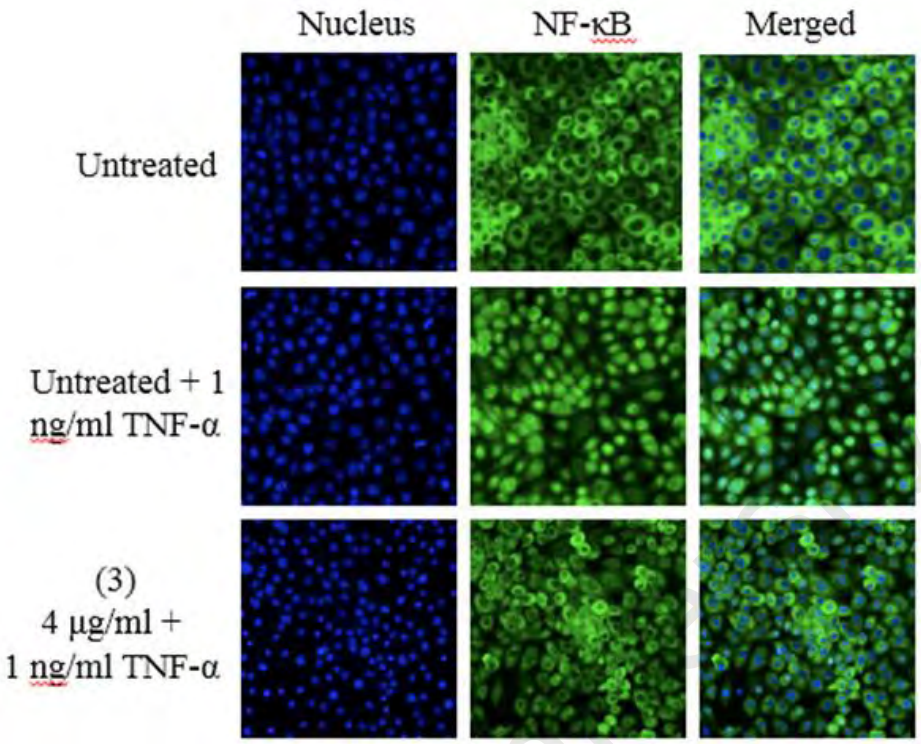


Figure 4. 31: The effect of compound 2 on the intracellular translocation of NF- κB A) Microscopy examination the stained MCF-7 cells for detection of NF-κB at concentration of 6µg/mL. B) Representative bar chart of TNF-α treated and compound 1-treated showing that a significant inhibition of activated NF-κB in treated MCF-7 cells. All values are mean ± SD for three experiments (significant values: $p<0.05$).

(A)



(B)

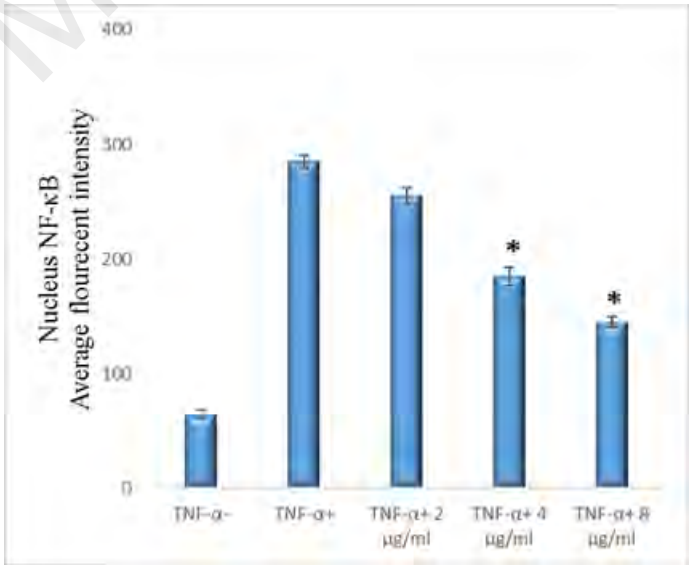


Figure 4. 32: The effect of compound 3 on the intracellular translocation of NF- κB. A) Microscopy examination the stained MCF-7 cells for detection of NF-κB at concentration of 4µg/mL. B) Representative bar chart of TNF-α treated and compound 3-treated showing that a significant inhibition of activated NF-κB in treated MCF-7 cells. All values are mean ± SD for three experiments (significant values: $p<0.05$).

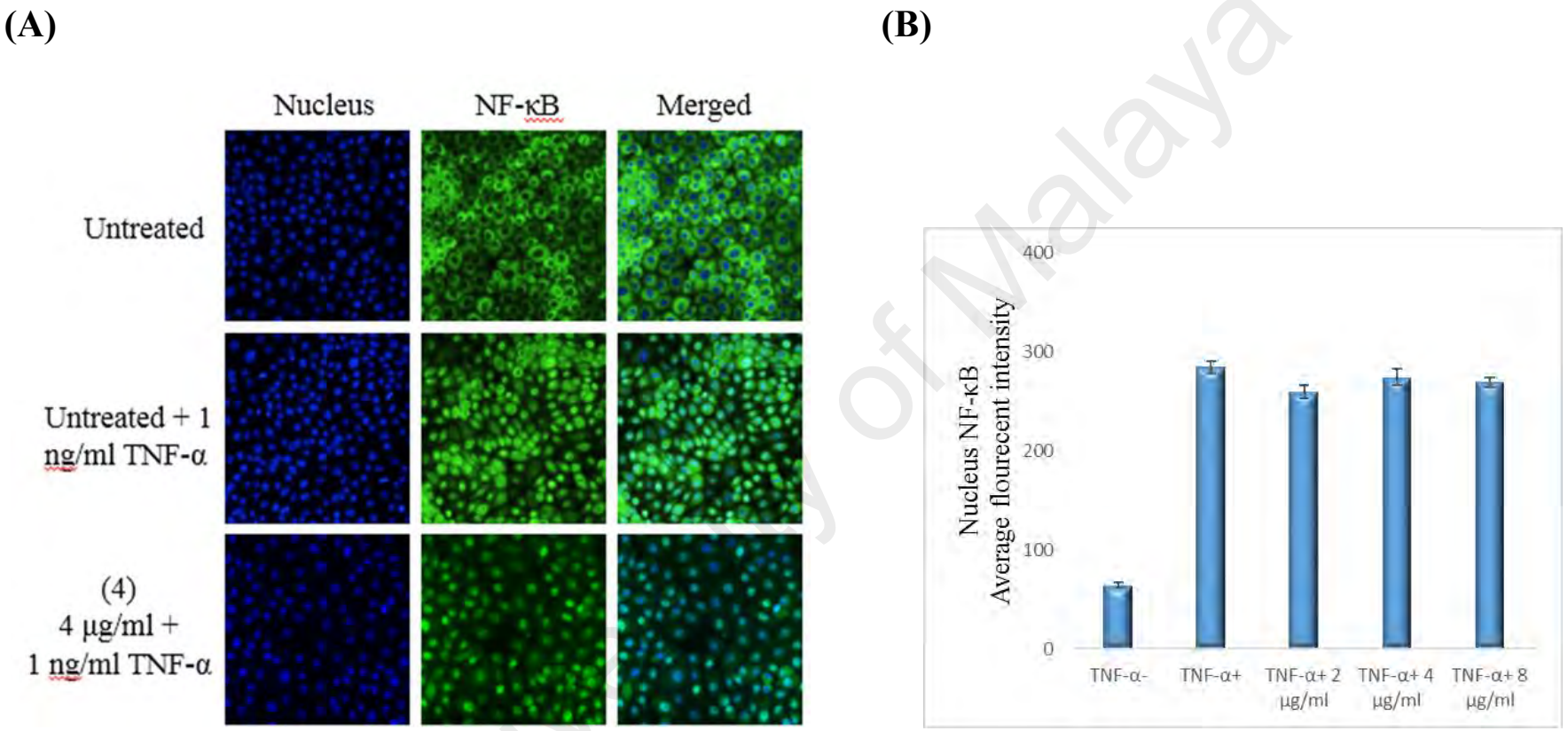
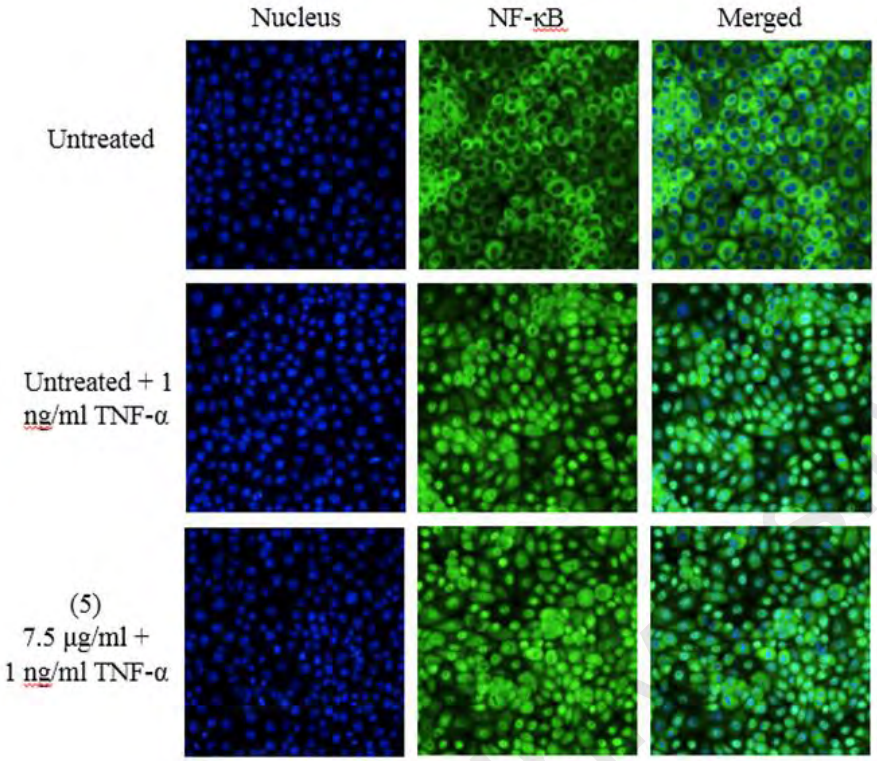


Figure 4. 33: The effect of compound 4 on the intracellular translocation of NF- κB. A) Microscopy examination the stained MCF-7 cells for detection of NF-κB at concentration of 4µg/mL. B) Representative bar chart of TNF-α treated and compound 4-treated showing that even with the highest concentration of compound 4 (8 µg/mL) there was no inhibition of TNF- α induced NF-κB activation in treated MCF-7 cells. All values are mean ± SD for three experiments (significant values: $p<0.05$)

(A)



(B)

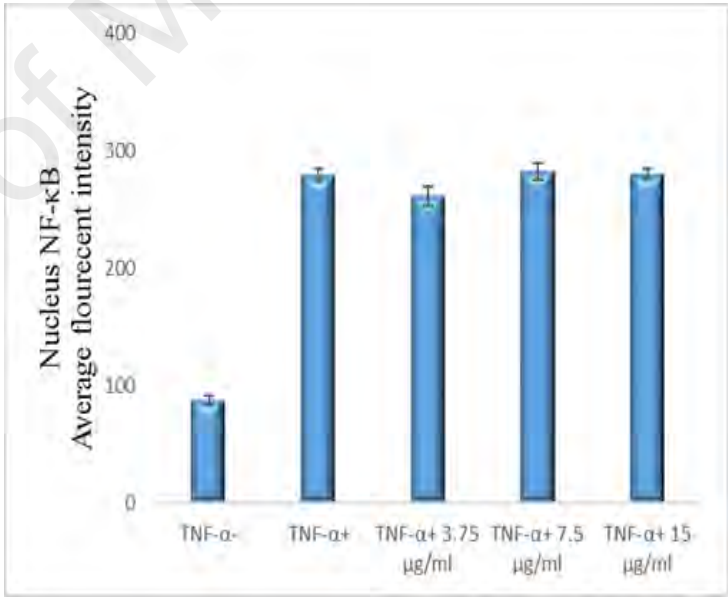


Figure 4. 34: The effect of compound 5 on the intracellular translocation of NF- κB. A) Microscopy examination the stained MCF-7 cells for detection of NF-κB at concentration of 7.5µg/mL. B) Representative bar chart of TNF-α treated and compound 4-treated showing that even with the highest concentration of compound 4 (8 µg/mL) there was no inhibition of TNF- α induced NF-κB activation in treated MCF-7 cells. All values are mean ± SD for three experiments (significant values: $p<0.05$)

4.2 *In vivo* results:

4.2.1 Acute toxicity study

For the acute toxicity study, thirty female mice were treated with all five quinazoline-based compounds at single dosage of 250 mg/kg (n=6 mice). After 15 days of experiment, all animals survived the treatment period. All compounds did not result in any mortality in the treated mice throughout the 14 days' study period. No physical or abnormal changes were observed in the mice's skin, fur, eyes, mucus membranes, tremors, salivation, behavior patterns, and sleep. The body weight, clinical observations and biochemical measurements reflected normal status of the kidney and liver functions, and histopathological evaluations of these organs all together revealed that there were no significant differences between the control and the experimental groups, as shown by the data in clinical observations and mortality (Table 4.2), body weight (Table 4.3), liver and kidney function analysis and (Table 4.4 and 4.5), and histopathology evaluation in figures 4.36, 4.37, 4.38, 4.39 and 4.40. The result of the administration of the compounds to mice provided sufficient evidence to conclude that the orally administered compounds were safe and presented no related toxicity at the selected dose (250mg/kg).

Table 4.2: Effects of the quinazoline-based compounds on mice mortality. There was no mortality observed.

Occurrence of mortality	10min	30 min	3 h	24h	48 h
Vehicle (5%Tween 20)	0/6	0/6	0/6	0/6	0/6
(1) 250 mg/kg	0/6	0/6	0/6	0/6	0/6
(2) 250 mg/kg	0/6	0/6	0/6	0/6	0/6
(3) 250 mg/kg	0/6	0/6	0/6	0/6	0/6
(4) 250 mg/kg	0/6	0/6	0/6	0/6	0/6
(5) 250 mg/kg	0/6	0/6	0/6	0/6	0/6

Table 4.3: Effects of the quinazoline-based compounds on Female ICR mice body weight. Values expressed as mean ± SD. There are no statistically significant differences between the measurements in different groups. The significant value was set at $P < 0.05$.

Compounds	BW(g) Day 0	BW(g) Day 7	BW(g) Day15
Vehicle (5%Tween 20)	24.4 ±1.86	25.8±1.5	28±1.9
(1) 250 mg/kg	25.5±2.39	27.4±2.5	30±2.57
(2) 250mg/kg	28±2.53	31±1.33	33±2.53
(3) 250mg/kg	25.8±1.44	29.5±1.88	32±3.15
(4) 250mg/kg	30.5±1.37	32±2.23	35±2.65
(5) 250mg/kg	29±2.31	32.4± 2.76	34±2.52

Table 4.4: Effects of the quinazoline-based compounds at single dose (250 mg/kg) on liver function test. The administration of the recommended toxic dose showed no statistically significant differences between the measurements of different groups. Values are expressed as the means ± SD (Significant values $P < 0.05$).

Groups	Total protein (g/L)	Albumin (g/L)	Globulin (g/L)	ALT (IU/L)	AST (IU/L)	GGT (IU/L)
Vehicle	62±1.5	12.4±0.65	51.4±1.1	58.3±3.5	251±8.46	3.5±0.1
(1) 250 mg/kg	59±1.0	11.2±0.43	50.6 ±1.42	60.0±4.45	244±5.94	3.0±0.05
(2) 250 mg/kg	65±1.33	12.9±0.59	53.1±1.85	67.7±4.5	245±7.58	3.3±0.07
(3) 250 mg/kg	55±1.58	10.5±0.62	51.3±1.23	62.2±4.34	265±6.52	3.1±0.08
(4) 250 mg/kg	60±1.0	11.8±0.3	55.6 ±1.2	56.1±4.27	264±8.33	3.7±0.09
(5) 250 mg/kg	63±1.34	10.5±0.66	54 ±2.0	60±3.0	255.6±6.32	3.5±0.15

Table 4.5: Effects of the quinazoline-based compounds at single dose (250 mg/kg) on renal function test. The administration of the recommended toxic dose showed no statistically significant differences between the measurements of different groups. Values are expressed as the means ± S.D (Significant values $P < 0.05$).

Groups	Sodium (mmo/L)	Potassium (mmol/L)	Chloride (mmol/L)	CO_2 (mmol/L)	Anion gap (mmol/L)	Urea (mmol/L)
Vehicle	145.6±0.91	8.7±0.15	109.4±0.32	20.5±0.34	27.0±0.67	6.5±0.73
Compound (1)	152.9±0.66	8.2±0.1	113.8±0.38	18.2±0.28	23.2±0.44	7.9±0.37
Compound (2)	151.5±0.58	9.5±0.18	110 .8±0.27	22.8±0.68	26.6±0.95	8.4±0.55
Compound (3)	148.3±0.68	9.0±0.12	108 .5±0.36	23.5±0.78	25.9±0.73	6.8±0.49
Compound (4)	153.3±0.76	9.4±0.2	114.3±0.33	19.2±0.21	22.7±0.56	7.7±0.86
Compound (5)	140±0.55	8.5±0.22	97.6±0.22	18.6±0.37	29.4±0.38	7.0 ±0.35

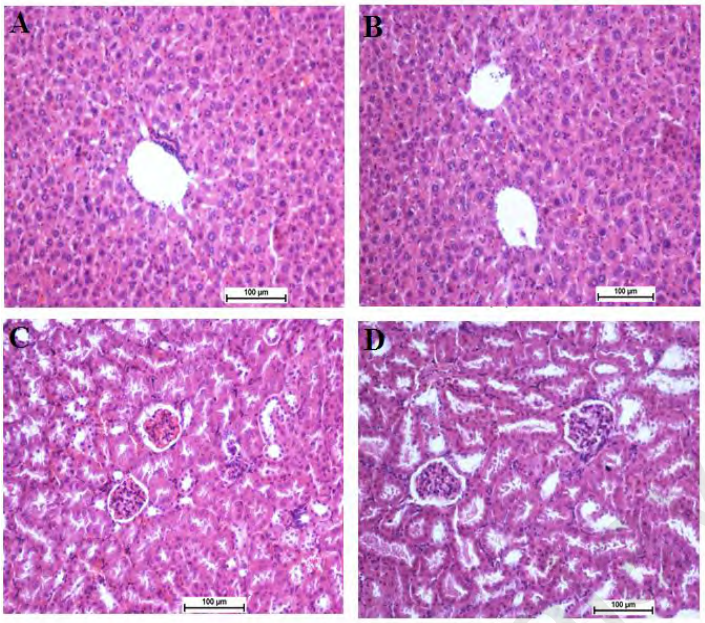


Figure 4. 35: H and E staining histological sections of the liver and kidney from compound 1-treated and 5% Tween-20-treated specimen after 15 days in vivo. Histological sections of liver (first row) and kidney (second row). Untreated mice (control group) received 5 mL/kg vehicle (5% Tween 20) (A) and (C). Animals treated with 250 mg/kg of compound 1 (B) and (D). There were no significant differences in the structures of the liver or kidneys between the treated and untreated group (Magnification: 100x).

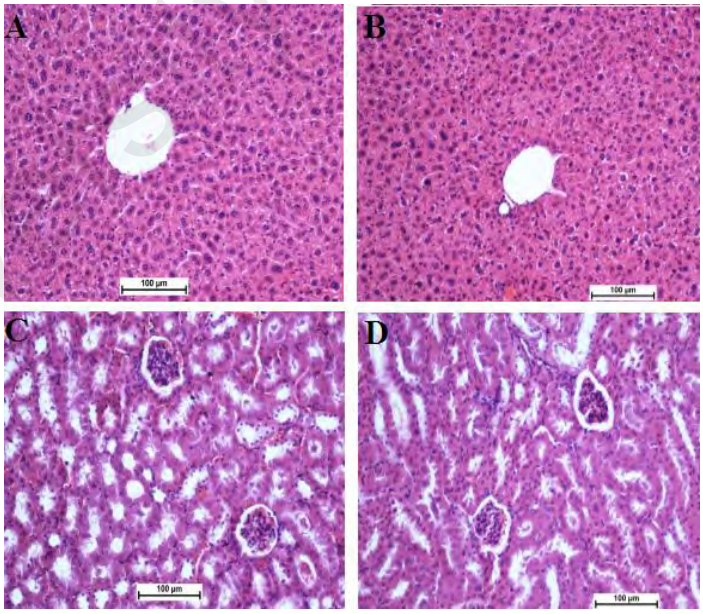


Figure 4. 36: H and E staining histological sections of the liver and kidney from compound 2-treated and 5% Tween-20-treated specimen after 15 days in vivo. Histological sections of liver (first row) and kidney (second row). Untreated mice (control group) received 5 mL/kg vehicle (5% Tween 20) (A) and (C). Animals treated with 250 mg/kg of compound 2 (B) and (D). There were no significant differences in the structures of the liver or kidneys between the treated and untreated group (Magnification: 100x).

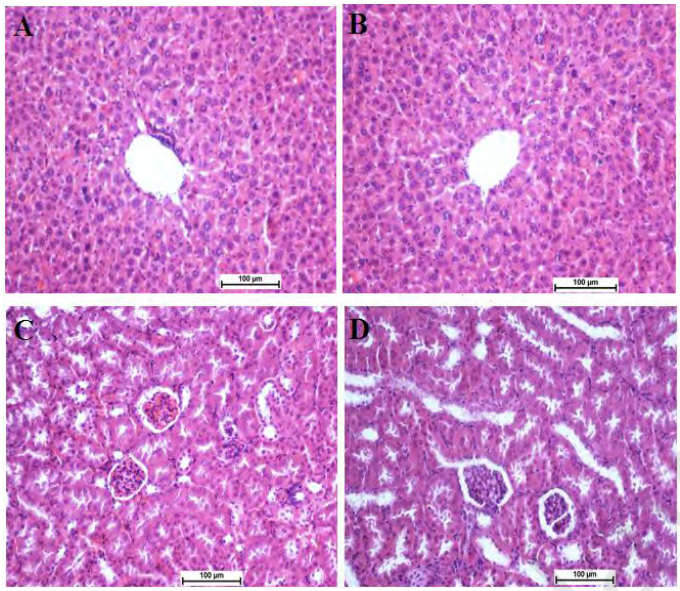


Figure 4. 37: H and E staining histological sections of the liver and kidney from compound 3-treated and 5% Tween-20-treated specimen after 15 days in vivo. Histological sections of liver (first row) and kidney (second row). Untreated mice (control group) received 5 mL/kg vehicle (5% Tween 20) (A) and (C). Animals treated with 250 mg/kg of compound 3 (B) and (D). There were no significant differences in the structures of the liver or kidneys between the treated and untreated group (Magnification: 100x).

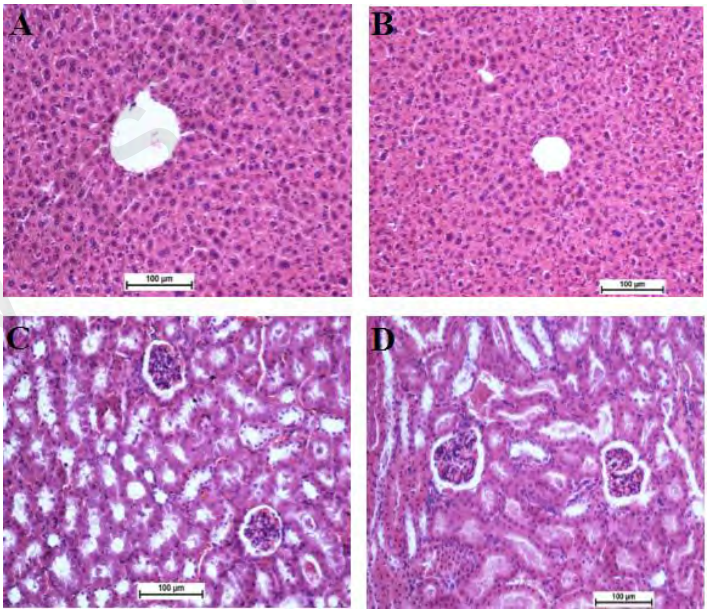


Figure 4. 38: H and E staining histological sections of the liver and kidney from compound 4-treated and 5% Tween-20-treated specimen after 15 days in vivo. Histological sections of liver (first row) and kidney (second row). Untreated mice (control group) received 5 mL/kg vehicle (5% Tween 20) (A) and (C). Animals treated with 250 mg/kg of compound 4 (B) and (D). There were no significant differences in the structures of the liver or kidneys between the treated and untreated group (Magnification: 100x).

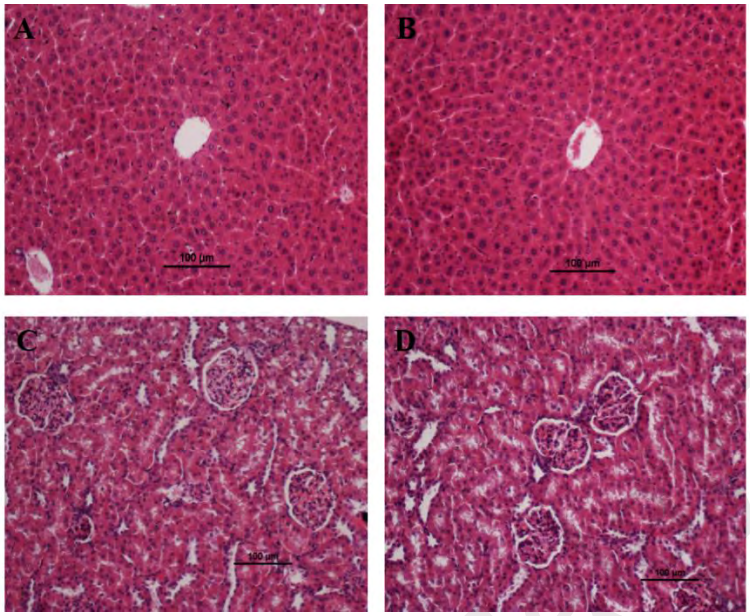


Figure 4. 39: H and E staining histological sections of the liver and kidney from compound 5-treated and 5% Tween-20-treated specimen after 15 days in vivo. Histological sections of liver (first row) and kidney (second row). Untreated mice (control group) received 5 mL/kg vehicle (5% Tween 20) (A) and (C). Animals treated with 250 mg/kg of compound 5 (B) and (D). There were no significant differences in the structures of the liver or kidneys between the treated and untreated group (Magnification: 100x).

4.2.2 Breast cancer chemoprevention results

4.2.2.1 Examination of body weight and tumour size

Measurements of the body weight, tumour volume and the percentage of reduction of tumour volume (%) from control and experimental animals are present in Table 4.6. The body weight was found to be significantly ($p < 0.05$) decreased in the group II of animals when compared with normal control animals (NC) group I. Conversely, the groups treated with 25 mg/kg and 50 mg/kg of 2 and 4 as well as 10 mg/kg of Tamoxifen showed a noteworthy ($p < 0.05$) increase in their body weights when compared to the group II of animals. The tumour growth was measured using a calliper. The tumours in the group II grew rapidly, reaching an average volume of 1537± 232mm3 by day 28. Meanwhile, treated groups (III. IV, V and VI) and Tamoxifen group (VII) displayed a significant ($p < 0.05$) reduction in their tumour volume when compared with the group II.

Table 4.6: Effects of compounds 2 and 4 on rat's body weight and tumour size (mm^3) in experimental breast cancer in rats. The significant value was set at $P < 0.05$. Comparison a: groups II, III, IV, V, VI and VII with group I; b: groups III, IV, V, VI and VII with group II.

Group	Body weight (g) Day 28	Tumor volume (mm^3)	Reduction of tumour (%)
I. NC	253 ± 3.8	0	0
II. TC	211 ± 4.2[a]	1537± 232	0
III. T+2-LD	220 ± 8.4[a,b]	607± 55.7[b]	60.6
IV. T+2-HD	231 ± 5.0[a,b]	395± 43.5[b]	74.4
V. T+4-LD	220 ± 3.3[a,b]	564± 73.2[b]	64.3
VI. T+4-HD	228 ± 2.5[a,b]	388± 58.5[b]	74.8
VII. T+S-TAM	193 ± 3.6[a,b]	284±45.4[b]	81.52

4.2.2.2 Antioxidant activity

In vivo antioxidant studies of compounds 2 and 4 associated with variations in the free radical scavenger enzymes were recorded in the breast supernatants (Table 4.7). The results of this study demonstrated that the levels of SOD, CAT in breast homogenized samples treated with both compounds and Tamoxifen significantly increased compared with cancer control group (TC). In contrast, MDA level of the breast homogenized tissue in treated groups was significantly decreased in comparison with cancer control group (TC).

Table 4.7: The effect of treatment with compounds 2 and 4 at 25 mg/kg, 50 mg/kg and TAM on antioxidant enzymes of breast in experimental breast cancer in rats
Comparison a: groups II, III, IV, V, VI and VII with group I; b: groups III, IV, V, VI and VII with group II.

Groups	SOD U/mg protein	CAT nM/min/mL	MDA μM/mg protein
I. NC	11.52 ± 1.1	150.42 ± 1.35	4.32 ± 1.2
II. TC	7.16 ± 1.23[a]	73.51 ± 2.56[a]	7.82 ± 2.15[a]
III. T+2-LD	8.45 ± 1.5[a,b]	84.5 ± 3.0[a,b]	6.2 ± 2.1[a,b]
IV. T+2-HD	9.5 ± 1.8[a,b]	90.44 ± 3.8[a,b]	5.34 ± 1.0[a,b]
V. T+4-LD	9.22 ± 2.0[a,b]	92.41 ± 2.20[a,b]	5.67 ± 1.62[a,b]
VI. T+4-HD	9.75 ± 1.27[a,b]	97.26 ± 2.61[a,b]	5.29 ± 0.87[a,b]
VII. T+S-TAM	10.5 ± 1.23[a,b]	143.25 ± 2.50[a,b]	4.8 ± 1.16[a,b]

4.2.2.3 Histopathology

This research revealed that the tumour that had developed was of invasive adenocarcinoma subtype with no tubular structures. The tumour also displayed a marked nuclear variation in size and shape, high mitotic index, and necrosis consistency with adenocarcinomas. Our results showed that treatment of the cancer with compounds 2 and 4 in two dosages (25 mg/kg, 50 mg/kg) and 10 mg/kg Tamoxifen prominently decreased ($p < 0.05$) the mitotic events and facilitated tissue re-organization in comparison with the tumours of the untreated animals ($p < 0.05$) (Figure 4.41 and 4.42).

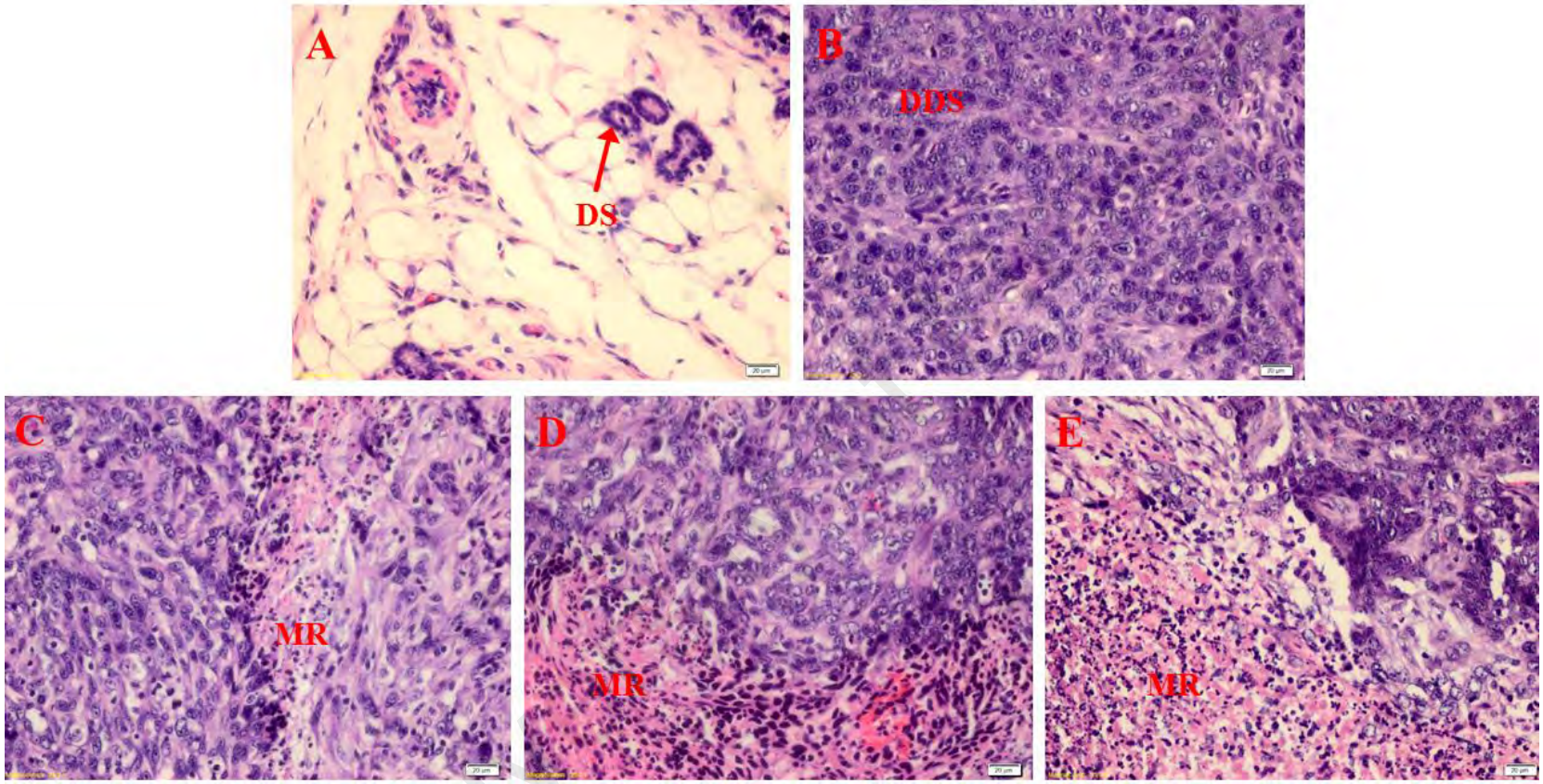


Figure 4. 40: H and E histological study of normal and treated breast cancer tissues with 2 and Tamoxifen. (A) Normal mammary gland showing the normal ductal structure (DS). (B) Mammary gland after experimental tumor induction, disrupted ductal structures with variation in cellular nuclear sizes (DDS). (C) Cancerous mammary gland treated with low dose of compound 2, showing reorganization of mammary structure (MR), (D) Cancerous mammary gland treated with high dose of compound 2, indicating reorganization of mammary structure (MR) (E) Cancerous mammary gland treated with Tamoxifen, showing the most significant reorganization of mammary tissue compared to compound 2- treated groups (Magnification 40x)

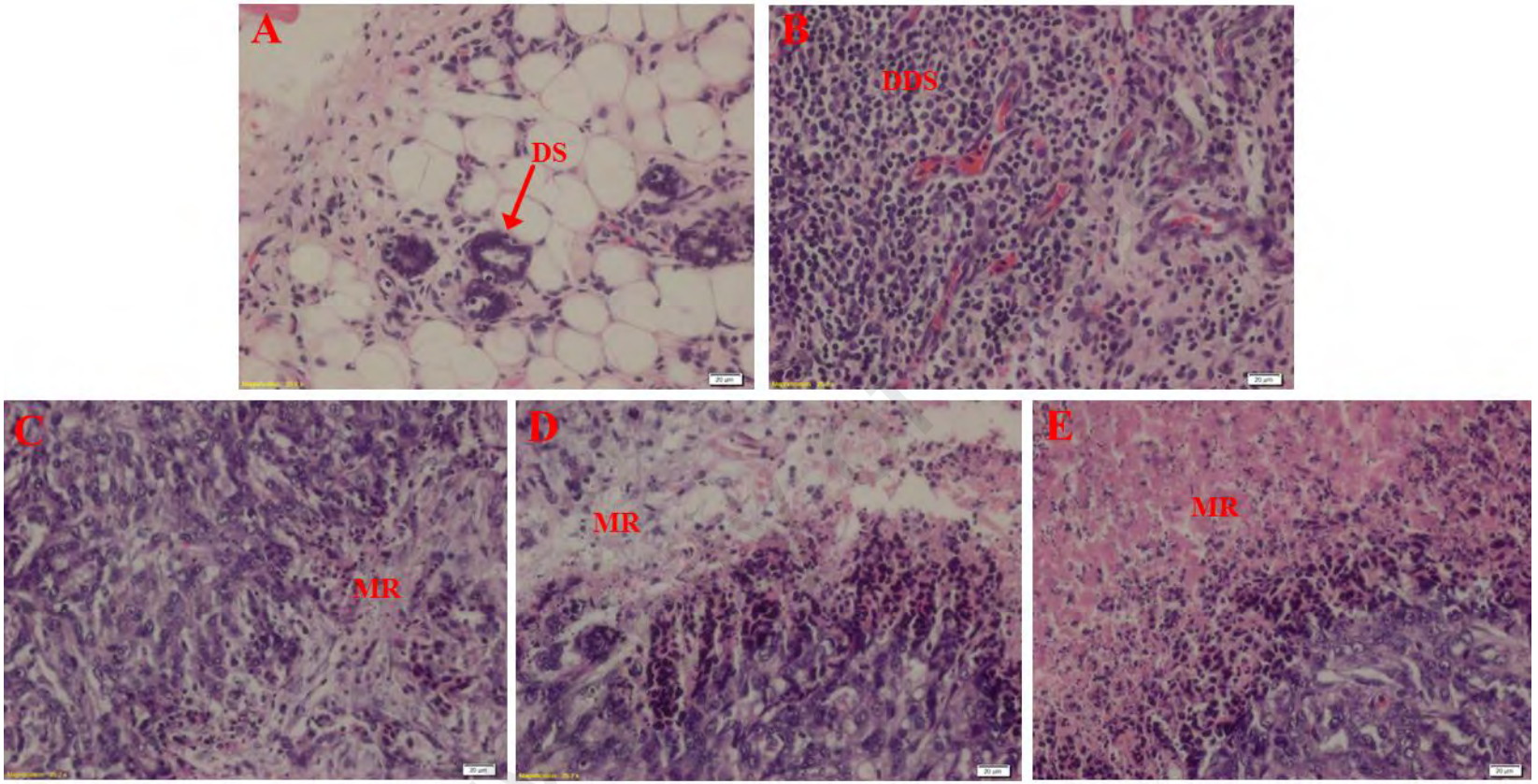


Figure 4. 41: H and E histological study of normal and treated breast cancer tissues with 4 and Tamoxifen. (A) Normal mammary gland showing the normal ductal structure (DS). (B) Mammary gland after experimental tumor induction, disrupted ductal structures with variation in cellular nuclear sizes (DDS). (C) Cancerous mammary gland treated with low dose of compound 4, showing reorganization of mammary structure (MR), (D) Cancerous mammary gland treated with high dose of compound 4, indicating reorganization of mammary structure (MR) (E) Cancerous mammary gland treated with Tamoxifen, showing the most significant reorganization of mammary tissue compared to compound 4-treated groups (Magnification 40x).

4.2.2.4 Immunohistochemistry

Oral administration of 2 and 4 compounds at two dosages (25 mg/kg and 50 mg/kg) to cancer-bearing rats considerably up regulated the expressions of BAX when compared with cancer control rats (Figure 4.43). In contrast, rats treated with compounds down regulated the expressions of BCL2 (Figure 4.44). Furthermore, the immunoexpression of stained cells of P53 and PCNA in both the cancer control and experimental rats in each group were also examined. Our results showed that 2 and 4 significantly up regulated the expressions of p53 protein (Figures 4.45) and down regulated the expressions of PCNA (Figures 4.46). The intensities of positives cells were also determined (Figure 4.47).

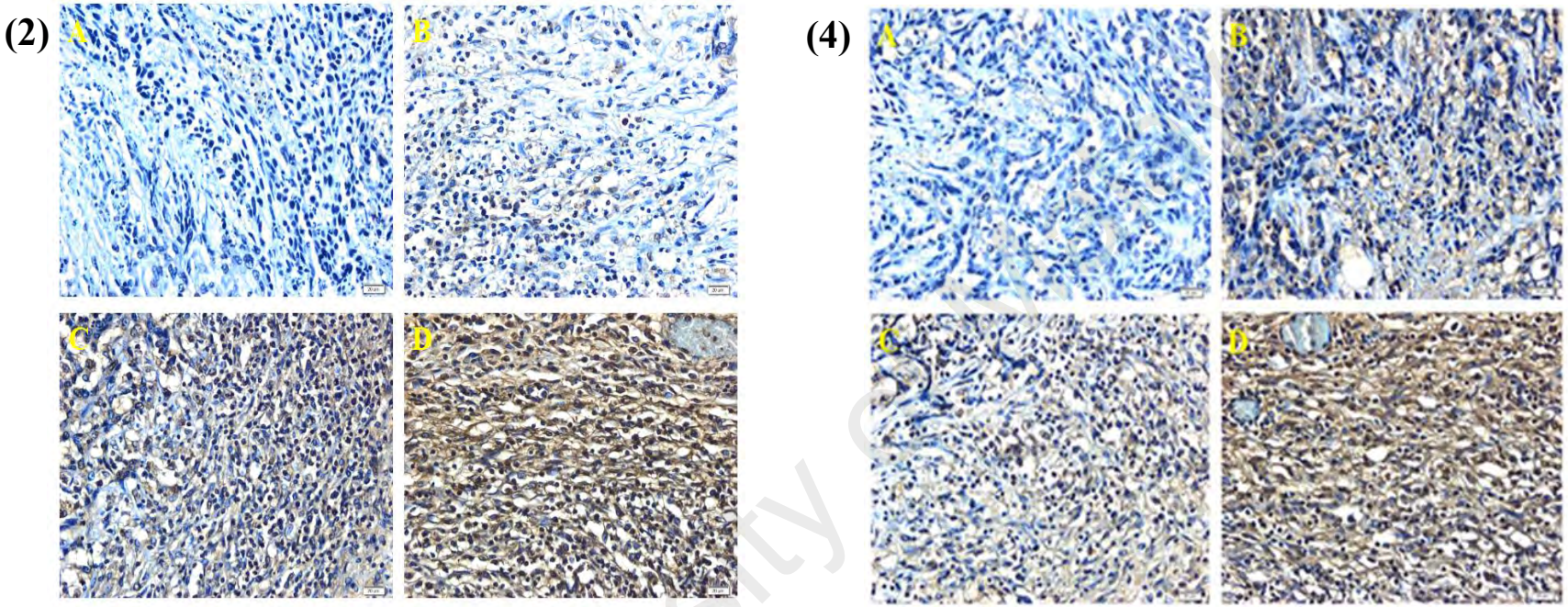


Figure 4. 42: Immunohistochemical analyses of the expression of BAX in the breast tissues treated with 2 and 4. (A) Untreated cancer control rats (TC), (B) Treated with low dose of 2 and 4 showing the significant up regulation, (C)Treated with high dose of 2 and 4 showing the more significant up regulation compared to low dose group, (D)Treated with Tamoxifen (T+S-TAM) showing the most significant up regulation of BAX. Brown staining indicates positive cells (Magnification 40x).

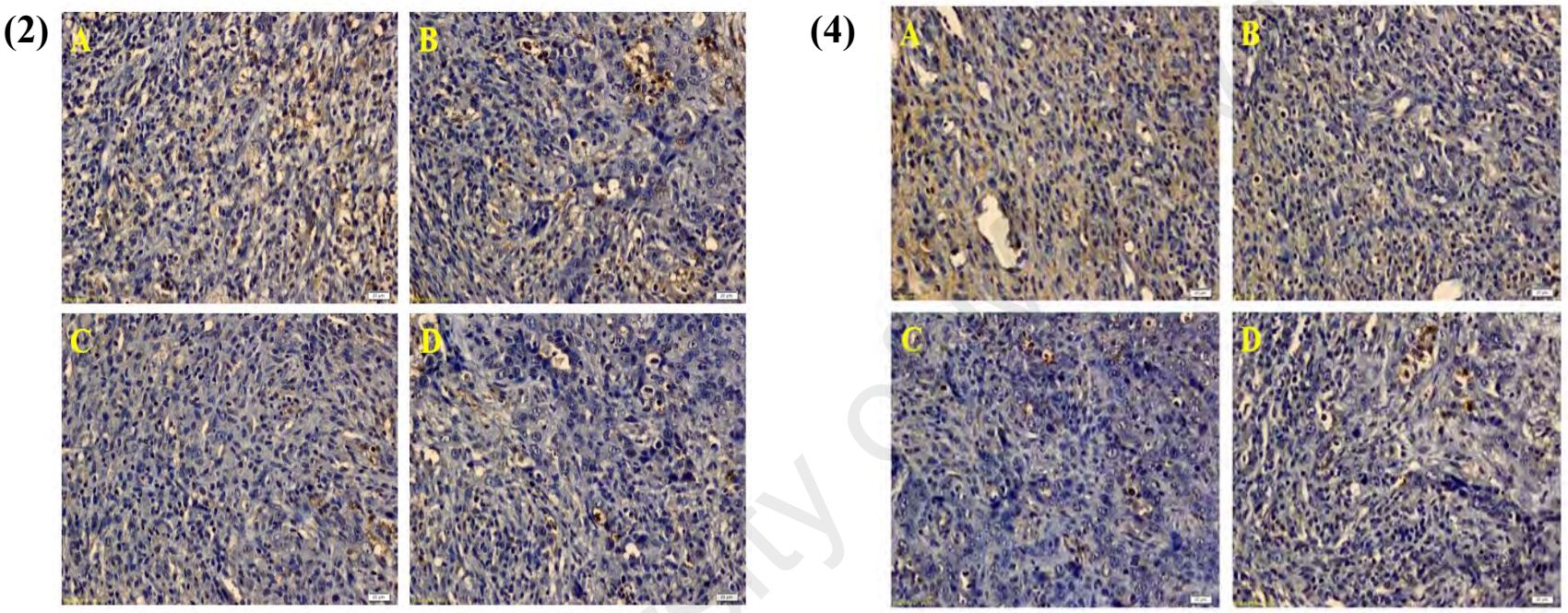


Figure 4. 44: Immunohistochemical analyses of the expression of BCL2 in the breast tissues treated with 2 and 4. (A) Untreated cancer control rats (TC), (B) Treated with low dose of 2 and 4 showing the significant down regulation, (C)Treated with high dose of 2 and 4 showing the more significant down regulation compared to low dose group, (D)Treated with Tamoxifen (T+S-TAM) showing the most significant down regulation of BCL2. Brown staining indicates positive cells (Magnification 40x).

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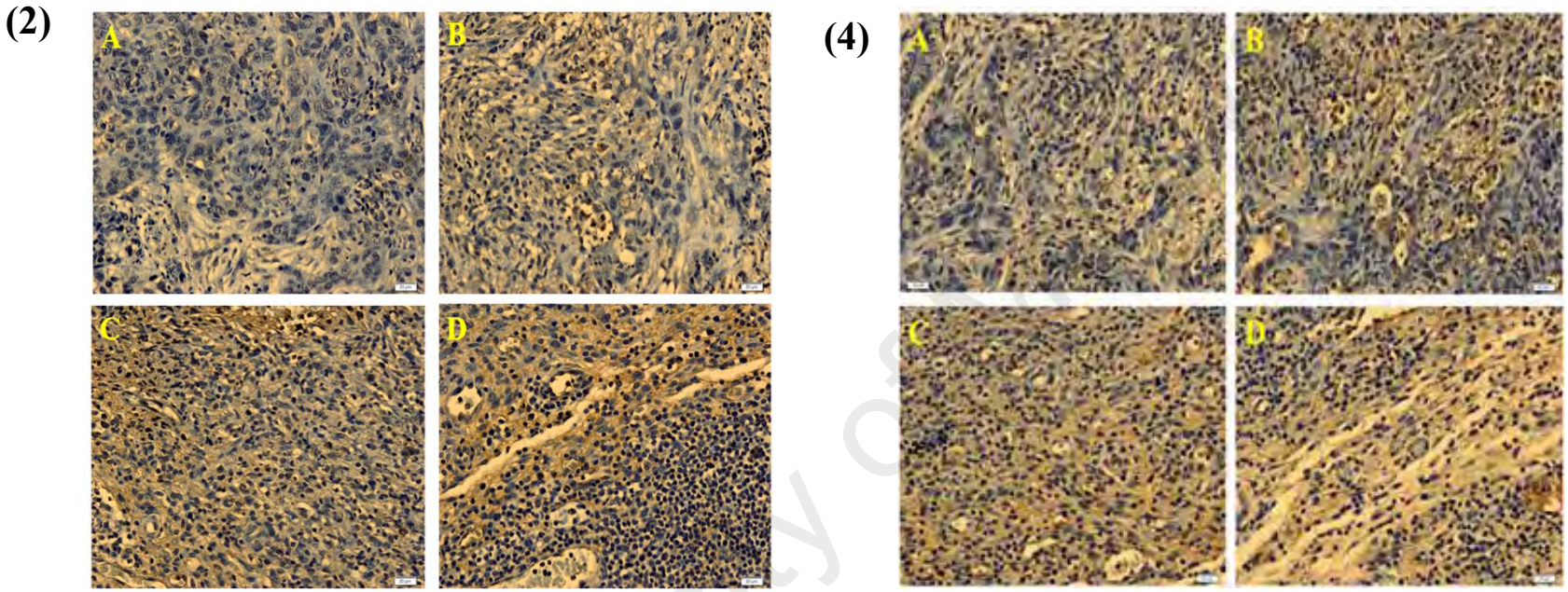

Figure 4. 45: Immunohistochemical analyses of the expression of P53 in the breast tissues treated with 2 and 4. (A) Untreated cancer control rats (TC), (B) Treated with low dose of 2 and 4 showing the significant up regulation, (C)Treated with high dose of 2 and 4 showing the more significant up regulation compared to low dose group, (D)Treated with Tamoxifen (T+S-TAM) showing the most significant up regulation of P53. Brown staining indicates positive cells (Magnification 40x).

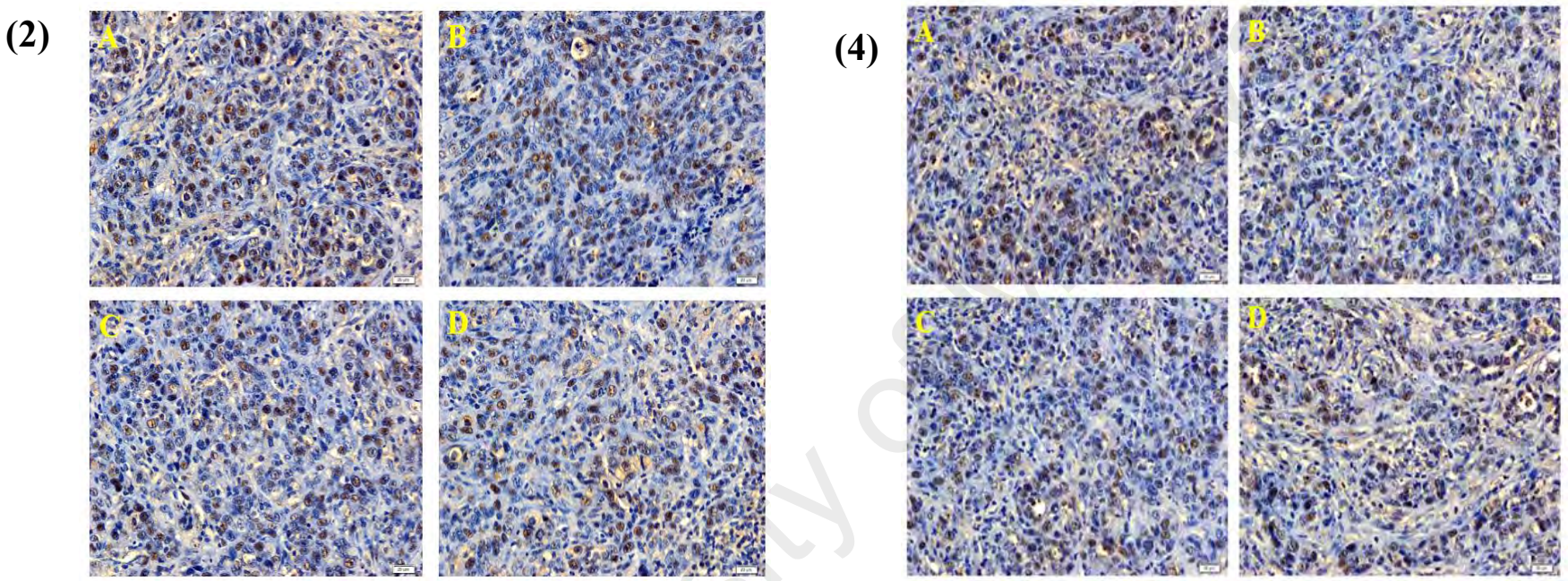

Figure 4. 46: Immunohistochemical analyses of the expression of PCNA in the breast tissues treated with 2 and 4. (A) Untreated cancer control rats (TC), (B) Treated with low dose of 2 and 4 (down regulated), (C) Treated with high dose of 2 and 4 (down regulated), (D)Treated with Tamoxifen (T+S-TAM) (down regulated). Brown staining indicates positive cells (Magnification 40x).

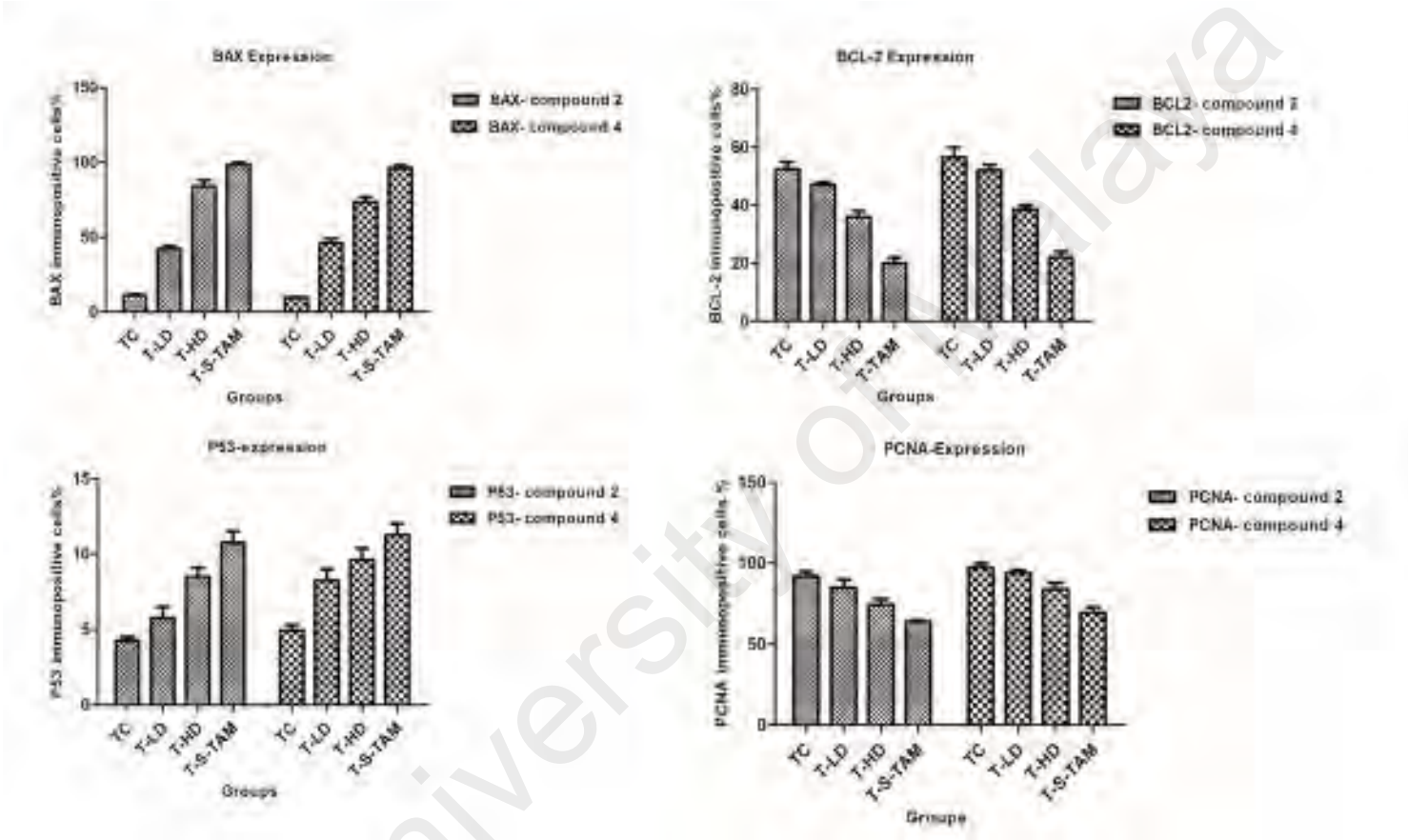

Figure 4.47: Immunohistochemical analyses of the expression of BAX, BCL2, P53 and PCNA in the breast tissues treated with 2 and 4.

4.2.2.5 TANEL assay

The sections of the mammary gland from the rats injected with LA7-induced mammary carcinoma after dosing with 25 and 50 mg/kg of compounds 2 and 4 as well as 10 mg/kg of Tamoxifen displayed considerably ($p < 0.05$) more apoptotic cells compared to the mammary tumour control rats. As shown in figures 4.48, apoptotic cells displayed most significant green stain in section of animals treated with 10 mg/kg of Tamoxifen while high dose groups of 2- and 4- treated sections showed more numerous apoptotic cell compare to low dose groups.

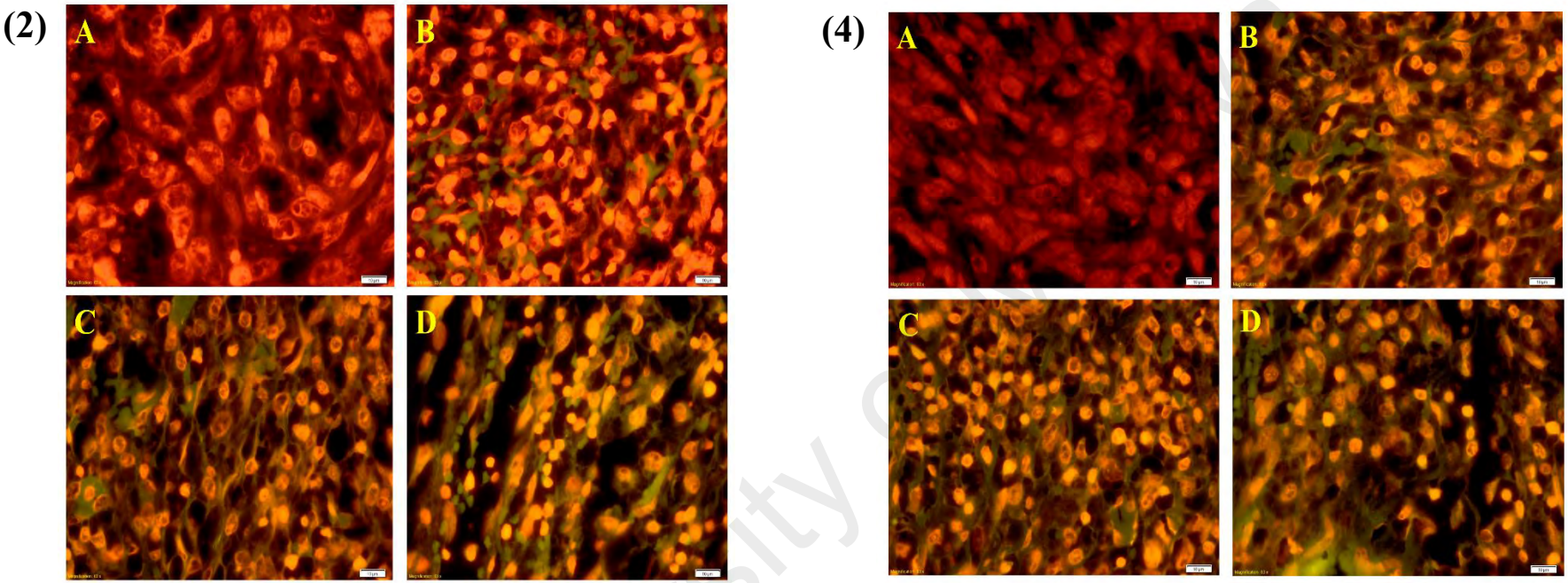

Figure 4.48: In situ TdT-mediated dUTP nick-end labeling (TUNEL assay) in breast tissue of rats treated with 2 and 4. (A) TC group section. (B) Cancerous section treated with 25 mg/kg of 2 and 4. (C) Cancerous section treated with 50 mg/kg of 2 and 4. (D) Cancerous section treated with 10 mg/kg of TAM. Green staining indicates positive cells (Magnification 40x).

CHAPTHER 5: DISCUSSION

This study is relied on the statement that newly synthesized quinazolinone-Schiff bases reduce the incidence of breast cancer, thus suggesting that the compounds can inhibit the growth of breast cancer cells. The present investigation was limited to study the effect of quinazolinone- schiff based compounds on human breast cancer cell lines and in animal study to find out the possible mechanism of action induced by Schiff based compounds in breast cancer inhibition.

Although there are a wide variety of signals and stimuli that can trigger apoptosis, chemotherapy still offers the most effective approach to treat cancer by inducing apoptosis in cells with fewer side effects and higher efficiency. Current anti-cancer drugs are designed to initiate programmed cell death. Programmed cell death, commonly known as apoptosis, involved a series of cellular and nuclear events that regulate the cellular morphological features and homeostasis (Elmore, 2007) .Use of either natural or synthetic compounds capable of inducing biological mechanisms are considered as the main approaches in cancer chemoprevention which is necessary to preserve genomic fidelity (Pan & Ho, 2008). An effective chemo-preventive agent requires to be able to prevent, inhibit, or reverse carcinogenesis before the spread of the invasive disease.

5.1 *In vitro* evaluation of quinazolinone- based compounds

The lack of cellular homeostasis between cell death and cell proliferation leads to deficient in mechanisms of apoptosis and consequently to cancer incidence (Elmore, 2007; Lee et al., 2012) and agents that can trigger apoptosis are known to have potential anticancer properties (Chakraborty et al., 2010; Mohan et al, 2012). Apoptosis pathways are effective targets for cancer therapy as well as chemoprevention. To date, many chemo-preventive drugs have been identified to regulate key actions or molecules in

apoptosis-inducing signal pathways (Suzuki et al., 2006). The accumulating body of data correlates the incidence of cancer to the malfunction of the programmed cell death cascade. Morphological alterations, death signal initiation, and the activation of caspases are associated with this malfunction. The extended consequence of the caspases activation is the promotion of intrinsic and extrinsic mitochondrial pathways (Fulda & Debatin, 2006). Extrinsic mitochondrial pathway requires production of caspase-2 and caspase-8 using siRNA, translocation of the pro-apoptotic protein Bax prior to DNA damage. In addition, free radicals and cytotoxic stress are predisposition factors that can trigger DNA damage, permeabilization of the mitochondria and subsequent formation of the apoptosome, *i.e*. cytochrome c, Apaf-1 and caspase-9 via the intrinsic pathway (Tan et al., 2013).

It has previously been shown that quinazolinones suppress the action of DNA-repairing enzymes, tubulin polymerization, DNA repair enzymes Poly (ADP-ribose) polymerase (PARP), mixed lineage kinases, mammalian aspartate transcarbamylase, HIV reverse transcriptase, some proteins and enzymes such as PgP (Pglycoprotein), MRP (multidrug resistance associated protein), polymerization of tubulin, decreasing midbrain dopamine secretion (Rhee et al., 2011) and as such quinazolinone supplementation improve stress, inflammation, immunodeficiency and promote cancer recovery both *in vitro* and *in vivo* (El-Azab et al., 2012). Apart from breast cancer, previous studies also showed that quinazolinone analogues have powerful effects on human leukemia cells (Danilov et al., 2013).

In this study, the cytotoxicity effect of five quinazolinone-based compounds were investigated *in vitro* on human normal and cancer breast cell lines (MCF-7, MDA-MB-231 and MCF-10A), using MTT assay. MTT cell viability assay is a reproducible, quantitative, and sensitive in vitro assay for determining cell proliferation (Mosmann,

1983). According to the data collected from this experiment, The IC_{50} of all five compounds on MCF-7 cells line were shown to be a range between 3.23 and 5.85 μg/mL after 72 h treatment. However, they all five compounds did not possess noteworthy cytotoxic effect on MDA-MB-231 cell line (IC_{50} >50 μg/mL) compared to MCF-7 cell line. Despite both cell lines are breast cancer cell lines, MDA-MB-231 is triple negative (ER, PR and HER2 negative) breast cancer cell line and MCF-7 is ER and PR positive. Therefore, these differences will affect in drug sensitivity (Pozo-Guisado & Alvare, 2002; Conzen, 2008; Finn et al., 2009).

The compounds with the potential in inhibition of cancerous cell growth, without significantly affecting the viability of normal cells, represent useful anticancer agents (Johnstone et al., 2002). In this research, all five compounds did not induce significant inhibition on the MCF-10A normal human breast cell line (IC_{50} >25 μg/mL) compared to treated MCF-7 cell line. Doxorubicin and Tamoxifen as standard drugs showed the most significant effect on MCF-7 cell line. The cytotoxic effect of the compounds was also confirmed by measuring the level of LDH release from treated cells. Significantly elevated LDH release exhibited that the cytotoxicity of the compounds probably occurred via the loss of membrane integrity, whether through activation of apoptosis or the necrosis pathway (Choi et al., 2012).

MCF-7 Cell morphological assessment of early apoptosis and late apoptosis by fluorescent microscopy analysis using AO/PI double-staining following treatment with the compounds showed morphological changes associated with apoptosis, including chromatin condensation, membrane blebbing, DNA fragmentation, and apoptotic body formation, as evidenced by the bright green colour of the AO and the orange for the PI stained cells when using fluorescence microscopy (Anasamy et al., 2013; Ng et al., 2013). This assay showed that the number of cells with early apoptosis features was

higher at 48 h after treatment. However, when treatment time passed to 72 h, late apoptosis or necrosis characterizations were dominant among treated MCF-7 cells. Simultaneous detection of late apoptosis or necrosis is scientifically possible because treated MCF-7 cells undertaking apoptosis may have progressed into necrosis due to the prolonged incubation with the quinazolinone- based compounds.

To elucidate the mechanisms underlying the observed anti-proliferative effect of the compounds on breast cancer cells, the generation of ROS on treated MCF-7 was analyzed as a key parameter that occurs following the induction of apoptosis (Liu et al., 2012; Mohan et al., 2012). As the main source of cellular ROS and adenosine triphosphate (ATP), mitochondria are the key regulators of mechanisms controlling the survival or death of cells. According to our collected data, after exposing the compounds to MCF-7 cells and analyzing the levels of ROS, it was demonstrated that the level of ROS in treated MCF-7 cells was significantly raised at different concentration of compounds.

The intrinsic or mitochondrial-dependent signaling pathway comprises different factors of non-receptor-mediated stimuli that induce intracellular signals. These signals, mostly through the p53 protein, act on the mitochondrial-initiated events. Increased level of ROS is a signal that can lead to the failure of suppression of anti-apoptotic parameters, thus triggering apoptosis. Hence, mitochondrial membrane potential (MMP) fluorescent probes has been used to examine the effect of elevated ROS production on the function of mitochondria in treated MCF-7 cells. Accumulating data indicated that changes in membrane permeability after treatment with all five compounds led to the release of cytochrome *c* into the cytosol and reduction of its levels in the mitochondria have been shown to occur as a result of changes in MMP (Mohan et al., 2012; Schneider et al., 2013). Moreover, the result highlighted that the breast cancer cell population respond

to the synthetic quinazolinone- based compounds also led to an increase in the level of cytochrome *c* in the cytosol compared to the control untreated cells.

The excessive production of ROS from mitochondria and the failure of MMP can stimulate the downstream caspase molecules and consequently lead to apoptotic cell death. After the binding of cytochrome *c* to apoptotic activating factor-1, caspase-9 is activated via apoptosome generation, which results in activation of the effector caspase (caspase-3/7) (Li et al., 2001; Mohan et al., 2013). On the other hand, in the extrinsic pathway, apoptosis is mediated by death receptors. As an example, FAS ligand interacts with the FAS receptor, leading to the activation of caspase-8 (El-Ghany et al., 2009). Caspase-8 activation cleaves and triggers downstream executioner caspases such as caspase-3/7 (Hyer et al., 2008; Qi et al., 2012)Elmore, 2007; Qi et al., 2012). In our study, all five compounds induced significant elevation in the caspase-3/7 and -9 activities. Meanwhile, three out of five compounds (compounds 1, 2 and 3) induce the activation of caspase-8, suggesting that the apoptosis induced in MCF-7 cells were mediated via both the intrinsic mitochondrial and extrinsic signaling pathways. However, compound 4 and 5 were not able to activate the caspase-8, suggesting the involvement of the intrinsic signalling pathway in apoptosis events (Chou et al., 2010). This study also provided evidence that compounds 1, 2 and 3 could play an anticancer role against MCF-7 cells by inhibition of the activation of the NF-κB signalling pathways, while 4 and 5 didn't have any effect on NF-κB translocation. Numerous studies have revealed the crucial regulatory role of NF-κB signalling in different cancer cells. Some studies have previously confirmed the role of this signalling pathway in the resistance of tumours cells against anticancer drugs. Cell proliferation is inhibited in response to the activation of an inhibitor of NF-κB that prevents its binding to DNA. Consequently, agents that can regulate the NF-κB signalling pathway might be

prominent chemo-therapeutic targets in cancer therapy (Mohan et al., 2012; Shakibaei et al., 2013).

5.2 *In vivo* evaluation of quinazolinone-based compounds

5.2.1 Acute toxicity evaluation

Acute toxicity study is the initial step towards toxicological examination of chemical or pharmaceutical substrates (Andersen & Krewski, 2009). Using rodents as models in safety evaluations is currently required in both the pharmaceutical and chemical international guidelines (Danneman et al., 2012). The Food and Drug Administration Guidelines for Toxicological Principles for the Safety Assessment of Food Ingredients elucidated that short-term toxicity studies could be performed using both rats and mice (Redbook, 2000). Thus, healthy male and female mice that have not undergone previous experimental procedures should be utilized in acute toxicity studies.

The results of the current study show a scientific evidence for determining the safety dosage of quinazolinone- based compounds through an acute toxicity study using mice. Acute toxicity is known as the toxicity caused by a pharmaceutical when administered by means of one or more doses and need to be monitored for 15 days following pharmaceutical administration. All the clinical signs, mortalities, time of onset, duration, and reversibility of toxicity must be documented within 15 days (Bidlack, 2002). In the present study, female ICR mice were orally gavaged with all five quinazolinone- based compounds suspension at single dose of 250 mg/ kg for 14 days. Throughout the experiment, treatment with compounds did not produce any mortality or toxicity in the mice. No adverse treatment-related effects were noticed in the body weight. The effect of compounds on the kidney and liver function was carried out to evaluate the nephrotoxicity and hepatotoxicity of this compounds on female mice which the results showed no noteworthy changes in the liver and renal serum biochemical

parameters (Danneman et al., 2012). In addition, histological examination of the hematoxylin and eosin-stained sections also demonstrated that there were no nephrocellular or hepatocellular injuries in mice gavaged with 250 mg/kg of compounds. In addition, several studies have been conducted to evaluate the pharmacokinetics and toxicity of new quinazoline-based compounds in different animal model to prove the safe nature of the synthesized compounds (Uckun et al., 1999; Trieu et al., 2000).

5.2.2 Chemopreventive effects of quinazolinone- based compounds 2 and 4 against LA7 induced mammary in rats

Quinazolinone- based compounds have a proven record of impeding various cancers (Kumar et al., 2011; Al-Omary et al., 2012; Antypenko et al., 2013). In the present study, both low (25 mg/kg b.w.) and high (50 mg/kg b.w.) dosages of compounds 2 and 4 exhibited chemopreventive activities against LA7 cell-induced mammary tumours in rats. This study showed the body weight of all groups was decreased statistically compared to normal control group (p <0.05) while body weight of all treated rats slightly raised compared to cancer control group. Treated group with high dose of 2 and 4 (50 mg/kg) showed strong chemotherapeutic activity, a decreased tumour volume by an average of 74.4 % and 74.8%, respectively. Moreover, treatment with low dose of 2 and 4 (25 mg/kg) significantly reduced the tumour volume, respectively, by an average of 60.6 % and 64.3%, whilst, on average, TAM (10 mg/kg) treated groups decreased the mammary tumour by 81.52%.

Antioxidants act as the primary line of defence against ROS and suggest their usefulness in estimating the risk of oxidative damage induced during carcinogenesis. SOD and CAT are endogenous oxidative enzymes involved in the defense system against reactive oxygen species (Formigari et al., 2007). Previous studies have proven

the role of the increased activities of antioxidant enzymes SOD and CAT as well as reduced level of MDA which are induced by antitumour agents, in the mechanism of chemopreventive therapy (Ray et al., 2000; Hajrezaie et al., 2014). The *in vivo* evaluation of antioxidants performed in this study demonstrated significant elevations in the SOD and CAT activities and considerable decrease in MDA levels in the groups treated with both compounds compare to cancer control group.

Histopathological examination is able to detect adenocarcinomas as a feature of mammary gland tumours (Hampe & Misdorp, 1979). The LA7-induced tumour in rats treated with (25 mg/kg b.w) AM and (50 mg/kg b.w) compounds 2 and 4 showed dramatic improvement in the overall mammary gland tissue structure, with a lower number of mitotic figures compared to the untreated cancer control. To further investigate the chemopreventive activity of both compounds in breast cancer, the expressions of Bax (pro-apoptotic protein), Bcl-2 (anti-apoptotic protein), p53 and PCNA in the different groups were analyzed using immunohistochemistry examination (Tjalma et al., 2001; Karmian et al., 2015). The balance of the expressions of Bcl-2 and Bax is a vital event in the process of cell death (Butt et al., 2000) which might prove helpful in cancer therapy. Bax protein has a role in the release of a factor that promotes apoptosis into the cytoplasm. In contrast, Bcl-2 plays an important role in controlling the process of cell death by blocking various apoptosis signals (Khare et al., 2008; Jaaskelainen et al., 2010). Our results revealed that both compounds increased the expression of the BAX protein, whereas down-regulated the expression of Bcl-2 protein, and this pattern of expression changes prominently suggests that apoptosis has been induced via the mitochondrial pathway (Ma et al., 2012). Furthermore, the p53 tumour suppressor gene is one of the main factors in regulating cell proliferation, growth and transformation. It is strongly associated in animal and human carcinogenesis and it is a significant regulator of apoptosis (Elmore, 2007). The p53 tumour suppressor

gene has been found to be mutated in over 50% of human cancers (Sigal & Rotter, 2000). Cancer-associated mutant forms of p53 have a long half-life that promotes tumorigenesis and tumour aggressiveness (Sirvent et al., 2004). In this study, the up-regulation of p53 recorded in the animals pre-treated with the quinazolinone derivatives recommended the tested compound as chemopreventive against breast cancer. Moreover, Proliferating Cell Nuclear Antigen (PCNA) has been identified as a useful marker for assessing the proliferation and progression of tumour cells (Oashi et al., 2013; Hajrezaie et al., 2014). Alterations in the gene that regulates the timing of events in the cell cycle contribute to carcinogenesis. Overexpression of PCNA has been reported in various malignancies including breast cancer (Jia et al., 2012). In this study, the expression of PCNA in mammary gland tissues was examined during mammary carcinogenesis with 2 and 4 compounds treatment. They reduced the expression of this proliferative marker, which highlights their antiproliferative efficacy.

Apoptosis includes a cascade of cytoplasmic and nuclear events that cause a series of morphological changes (Elmore, 2007) Such a phenomenon is characterized by the production of multimers of 180–200 base pair DNA fragments through the activities of endogenous endonucleases that result in apoptosis (Bhalla, 2003). The TUNEL assay indicated valuable evidence concerning apoptosis in breast cancer rats treated with 2 and 4 with abundant TUNEL-positive cells compared to untreated cancer control (Gibson et al., 2002; Karimian et al., 2015).

In this study, the effect of all compounds on cancer cells is consistent with the results presented by Abou-Seri and co-authors reporting that quinazolinone α1-adrenoceptor anatagonists induced cytoarchitectural regression in cultured prostate cancer cells (Abou-Seri et al., 2011). All tested compounds and other naturally occurring quinazolinone molecules could generate possible therapeutic option for cancers.

CHAPTER 6: CONCLUSION

On the basis of the data stated in this research, it can be concluded that the treatment of MCF-7 cells with quinazolinone-based compounds stimulated apoptosis with cell death-transducing signals that caused generation of ROS and reduction in MMP, thereby activating cytochrome c release from the mitochondria to the cytosol. The released cytochrome c activated the triggering of caspase-9 along with the executioner caspase 3/7. In addition, compounds 1, 2 and 3 caused a significant block to the induced translocation of NF-κB from cytoplasm to the nucleus of cells and increase of caspase-8 that had showed the involvement of extrinsic pathway. The results from this study showed that single oral doses of quinazolinone- based compounds at 250 mg/kg did not give any adverse effect on the kidney and liver tissues of female ICR mice. Moreover, treatment of 2 and 4 compounds to LA7-induced rats ameliorated all the observed biochemical changes thus establishing its chemotherapeutic efficacy against LA7-induced mammary cancer. The present study addressed the beneficial influence of quinazolinone- based compounds on the levels of antioxidants enzymes, induction of pro-apoptotic protein Bax and inhibition of anti-apoptotic protein Bcl-2 during LA7-induced experimental mammary cancer. Possible mechanisms of this chemoprevention include the down-regulation of cell proliferation-promoting proteins in cancer cells (which was demonstrated by PCNA immunohistochemistry). Reductions in the damaged area caused by LA-7 cancerous cells in the treated rats supported the microscopic evidence and demonstrated the significant chemopreventive activity of 2 and 4.

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