Anti-proliferative properties of quercetin-3-*O*-glucoside and its six long chain fatty acid acylated derivatives in human hepatocellular carcinoma HepG2 cells

by

Sudhanshu Sudan

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
August 2013

"Take the first step in faith. You don't have to see the whole staircase, just take the first step"

Dr. Martin Luther King Jr.

Dedicated to:
My parents, family and elder brother

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Six long chain fatty acid esters of quercetin-3-*O*-glucoside (Q3G) acylated enzymatically were used for determining their antiproliferative action in comparison to precursor compounds (quercetin, Q3G and six fatty acids namely, stearic acid, oleic acid, linoleic acid, alpha-linolenic acid, eicosapentaenoic and docosahexanoic acids) using HepG2 cells. Long chain fatty acid esters of Q3G showed significant inhibition of cell proliferation (approximately 85% to 90%) compared to the precursor compounds and two prescribed anticancer-drugs (Sorafenib and Cisplatin) after 6 hrs and 24 hrs by inducing cell cycle arrest, apoptosis and DNA topoisomerase II inhibition. Among the six fatty acid esters of Q3G, oleic acid ester (OA-Q3G) displayed the greatest anti-proliferation action and upon further investigation showed significant regulation of expression of genes involved in cell cycle, growth, survival and apoptosis at gene and protein level. Overall, results of the study suggest strong potential of these novel compounds in treatment of liver cancer.

LIST OF ABBREVIATIONS USED

ATCC- American Type Culture Collection
AFB1- Aflatoxins B1
AKT2-V-akt murine thymoma viral oncogene homolog 2
AMPK- 5' AMP-activated protein kinase
AURKB- Aurora kinase B
BCL2- B-cell CLL/lymphoma 2
Bcl-XL- B- cell lymphoma extra large
Bcl-xS- B- cell lymphoma extra small
BIRC5-Baculoviral IAP repeat containing 5
CDC25A- Cell division cycle 25 homolog A (S. pombe)
CDK1- Cyclin-dependent kinase 1
CDK2- Cyclin-dependent kinase 2
CDK8- Cyclin-dependent kinase 8
CTSB- Cathepsin B
DAPI- 4'-6-Diamidino-2-phenylindole
DHA- Docosahexanoic acids
DMSO- Dimethyl sulfoxide
EASL- European Association for the Study of the Liver
EDTA- Ethylenediaminetetraacetic acid
EGFR- Epidermal Growth Factor Receptor
EGF-R- Epidermal growth factor receptor
EMEM- Eagle's Minimum Essential Growth Medium
EPA- Eicosapentaenoic
ERK- Extracellular regulated kinase
FBS- Fetal Bovine Serum
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase
GFP- Green Fluorescent Protein
GRB2- Growth factor receptor-bound protein 2
GSK3β- Glycogen synthase kinase 3 beta
HBV- Hepatitis B virus
HBx - Hepatitis B virus protein
HCC- Hepatocellular carcinoma
HCV- Hepatitis C virus
HDAC2- Histone deacetylase 2
HDAC6- Histone deacetylase 6
HH- Hereditary hemochromatosis
HIF1A- Hypoxia inducible factor 1, alpha subunit
HPLC- High-performance liquid chromatography
HRAS- V-Ha-ras Harvey rat sarcoma viral oncogene homolog
HSP90AA1- Heat shock protein 90 kDa alpha (cytosolic), class A member 1
IRF5- Interferon regulatory factor 5
LDH- Lactate dehydrogenase
MEK- Mitogen activated protein (MAP) kinase
mTOR- Mammalian target of rapamycin

NAFLD- Non-alcoholic fatty liver disease
NASH- Nonalcoholic steatohepatitis
NFKB1- Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NMR- Nuclear magnetic resonance
p53- Protein 53 or tumor protein 53
PARP1- Poly (ADP-ribose) polymerase 1
PARP4- Poly (ADP-ribose) polymerase family, member 4
PBS- Phosphate buffer saline
PDGFRA-Platelet-derived growth factor receptor, alpha polypeptide
PH - Phloretin
PI- Phosphatidylinositol kinases
PI3K- PI 3-Kinase
PIP- Diphosphoinositide kinase
PIP3- Phosphatidylinositol (3,4,5)-triphosphate
PRKCA- Protein kinase C, alpha
PTX- Paclitaxel
Q-3-G - Quercetin-3-*O*-glucoside
RFA- Radiofrequency ablation
RHOB- Ras homolog gene family, member B
ROS- Reactive oxygen species
SGLT1- Sodium-dependent glucose transporter ()
TAE- Tris base, acetic acid and EDTA
TLC- Thin layer chromatography
TNM- Tumor node metastasis
TOP2A- Topoisomerase (DNA) II alpha 170 kDa
TXN- Thioredoxin
α1-AT- alpha1-antitrypsin

ACKNOWLEDGMENTS

Foremost, I would like to express my sincere and deepest gratitude to my supervisor Dr. Vasantha Rupasinghe for the continuous support of my MSc study and research, for his patience, motivation, constructive criticism, enthusiasm, immense knowledge and providing me with an excellent atmosphere for doing research. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my MSc research study.

Besides my advisor, I would like to pay my special thanks to my thesis committee: Dr. Robin Robinson, Dr. Berney Benkel, for their encouragement, insightful comments, enthusiasm and continuous help in research and thesis. I feel blessed to have gained guidance from such experienced committee for my research.

I would like to express my deepest acknowledgement and thanks to my lab mates, Dr. Nair, Dr. Ziaullah, Indu, Khushwant, Neelika, Sumudu, Ruchira, Satvir, Prasanna and Wasundra for their constant help and support throughout my research in the lab. Additionally, I would like to thank all the administrative and management staff of Dalhousie Agricultural campus for providing help and support in various forms throughout my graduate study.

Finally, I would like to thank my parents who are the only god and religion to me, my elder brother and my girlfriend, Ankita for always supporting me, encouraging me with their best wishes, never losing faith in me and for standing by my side in good times and bad.

CHAPTER 1. INTRODUCTION

Hepatocellular carcinoma (HCC) is a cancer that originates in liver and is the third principle cause of cancer deaths worldwide (Parkin et al., 2001; Parikh et al., 2007). According to a recent survey by the American Cancer Society, more than 700,000 people are diagnosed with this type of cancer each year throughout the world making liver cancer one of the leading causes of death due to cancers worldwide. It is more prevalent in sub-Saharan Africa and Eastern Asia; however, recent data indicates a gradual increase in its incidence rates in Western countries despite the advances in surgical and nonsurgical therapies (Parkin et al., 2001). Additionally, treatment induced cytotoxicity and numerous side effects caused by current cancer drugs limit the efficiency of these drugs in treatment (Robert et al., 2005). This has raised a great demand for developing new drugs having specific action with better management of the disease.

Epidemiology studies have consistently shown an inverse correlation between high consumption of fruits and vegetables which contain high amounts of phytochemicals and incidence of various cancer types (Knekt et al., 2002; Manson et al., 2003). Several different mechanisms have been suggested for the action of phytochemicals. Besides playing the role of strong antioxidants, phytochemicals can alter DNA, modulate gene expression and bring changes in signal transduction pathways (Debolt, 2001; Noe, 2004; Manson, 2003) including pro- and anti-apoptotic proteins, cell cycle proteins and protein kinases (Andreadi et al., 2006 and Aggarwal et al., 2006, Russo et a., 2012). Among phytochemicals, one of the most studied group of antioxidant compounds are flavonoids. Results from cell culture and in vivo studies reveal that flavonoids exert preventive

effects in carcinogenesis by interfering with several cancer processes and inhibit cell proliferation and induce apoptosis in several types of cancer cells (Romagnolo et al., 2012). One of the most ubiquitous and very well studied flavonoid is quercetin which has been shown to possess strong antiproliferative effects via regulation of various signal transduction proteins involved in cell survival, DNA repair and cell cycle (Kuo et al., 2004; Gibellini et al., 2011). Consequently, the proapoptotic effects of quercetin may result from collective interaction of these pathways. Q3G is one of the naturally occurring forms of quercetin and has been shown to exhibit strong antioxidant activity *in-vitro* (Razavi et al., 2009). In a recent study, Q3G was shown to the exhibit strongest antiproliferative activity among its aglycone quercetin and its other glucosides (You et al., 2010). However, the mechanism of action of the inhibitory effect of Q3G on cancer cells has not yet been clearly known.

Although several mechanisms of action of aglycone quercetin have been elucidated, its efficacy and bioavailability needs further understanding. Flavonoids in natural form exist as glycosides with different sugar moieties (Moon et al., 2006) thus making the compound partially hydrophillic and in turn effecting its bioavailability (Arts et al., 2004). Consequently, it is shown that glycosides exhibit less cellular absorption than aglycones (Karawajczyk et al., 2007; Passamonti et al., 2009). Relatively, despite its antiproliferative effect, the low bioavaiability of Q3G limits its use as a therapeutic agent. Therefore, finding different ways of increasing the bioavailability has become an interesting area of recent research.

The number of hydroxyl groups, presence of a methoxy group in the B ring of flavonoids and lipophilicity have been shown to influence the bioavailability of flavonoids (Lin et

al., 2003). One of the effective approaches to enhance the cellular absorption is to increase the lipophilicity of these compounds by acylation (Salem et al., 2011). Essential fatty acids have unique biological activities. In previous studies, omega-3 and omega-6 polyunsaturated acids have been shown to exhibit antiproliferative nature (Mainou-Fowler et al., 2001; Hardman 2002). However, their use is very limited as they are prone to autooxidation. However, combining the essential fatty acids with certain flavonoids with strong antioxidant properties will not only preserve them from oxidation but also improve properties of the flavonoid. Salem (2011) in their recent study has revealed that Q3G esters showed increased antioxidant properties by enhancing lipophilicity.

In this research, with the aim of increasing the lipophilicity of Q3G, a relative strategy of enzymatic acylation with fatty acid was performed. Q3G was acylated with six long chain fatty acid esters (namely, stearic acid, oleic acid, linoleic acid, alpha-linolenic acid, eicosapentaenoic (EPA), docosahexanoic acids (DHA) as previously described (Ziaullah et al., 2012). The research was conducted in three phases. The focus of the first phase was to evaluate the antiproliferative effect of Q3G in HepG2 cells and elucidate the mechanism of action. The second phase of the research focused on evaluating antiproliferative effect of six long chain fatty acid esters of Q3G in HepG2 cells and the mechanism of action. Finally the third phase of the study focussed on elucidating the cell signalling pathway regulations upon treatment with long chain fatty acid esters of Q3G by examining various characterized cell signalling pathway components of apoptosis, cell cycle and cell proliferation.

This was a comparative study which involved the evaluation of the antiproliferative nature of six long chain fatty acid esters of Q3G in comparison to the precursor

compounds (quercetin, Q3G and six fatty acids) and currently prescribed drugs used in chemotherapy for liver cancer (Sorafenib and Cisplatin); treatment induced toxicity of long chain fatty acid esters of Q3G on normal cells using normal primary rat hepatocytes.

CHAPTER 2. OBJECTIVES

2.1 RESEARCH HYPOTHESIS

Structural modification of Q3G by acylation with long chain fatty acids can enhance its antiproliferative properties possibly through better interactions with plasma membrane associated signal transductions.

2.2 SPECIFIC OBJECTIVES

2.2.1 Determine the cytotoxic dosage and antiproliferative activities of Q3G and its six long chain fatty acid esters in hepatocellular carcinoma using Hep G2 cell culture system.

2.2.2 Evaluate the hepatotoxicity caused by the Q3G and its six long chain fatty acid esters using primary normal liver cells.

2.2.3 Elucidate the possible mechanism of action of Q3G and its six fatty acid esters in hepatocellular carcinoma cells, HepG2 by investigating effects on cell cycle, apoptosis and DNA topoisomerase II activity.

2.2.4 Elucidate the possible molecular targets of one selected fatty acid ester of Q3G (the long chain fatty acid ester of Q3G with the most effective anti-proliferative nature selected out of six tested) in hepatocellular carcinoma cells, HepG2 cells by investigating the effect on key molecules involved in cell growth, survival and cell death signaling pathways.

CHAPTER 3. LITERATURE REVIEW

3.1 LIVER AND LIVER CANCER

Liver is the largest organ inside our abdomen. It filters and breaks down harmful wastes from the blood, produces bile for emulsifying and absorbing fatty materials and also produces most of the clotting factors in the body. In addition, the liver is involved in various metabolic activities and production of plasma proteins (Williams, 2009). In general, cancer starts when the cell experiences genetic alterations or mutations that make the cell grow abnormally and uncontrollably (Gibbs et al., 2003). Tumors in the liver can be benign or malignant. Benign tumors are not much of a threat as they can be removed by surgery and they don't grow back. In contrast, malignant tumors are serious threats to life since even after removal they can grow back and can invade other organs as well. By metastasis from the original tumor, cancer cells from the liver can enter the blood stream and lymph streams and can start invading and infecting other organs by forming more tumors (Gibbs et al., 2003).

Hepatocellular carcinoma (HCC) is a cancer that originates in liver and is the third principle cause of cancer deaths worldwide (Parkin et al., 2001; Parikh et al., 2007). According to a recent survey by the American Cancer Society, more than 700,000 people are diagnosed with this type of cancer each year throughout the world making liver cancer one of the leading causes of death due to cancers worldwide. Certain risk factors have been defined which increase the probability and hence cases of occurring of liver cancer. With the recent surveys and analysis by National Cancer Institute (NIH, USA) and World Health Organisation (WHO), the occurrence and deaths with liver cancer is

continuously increasing worldwide despite the advances in surgical and nonsurgical therapies.

The classical classification of HCC has been done into four different groups based on growth pattern and tumour differentiation; well, moderately, poorly differentiated and undifferentiated tumours, respectively. HCC starts with a well differentiated cancer and continues with a stepwise dedifferentiation process. In the initial stages of any type of liver disease, the liver becomes inflamed. If left untreated, the liver tissue starts to scar and slowly covers up the original healthy tissue which is known as fibrosis. Delay in treatment leads to cirrhosis (Raynard et al., 2003). This is the stage where liver tissue is completely damaged and from here it leads to serious complications like liver cancer (Bartolomeo et al., 2011). As explained above, each stage is significantly affected by different risk factors. Once, cancer forms, treatment becomes necessary keeping in mind the limited options. Treatment includes surgery, cryosurgery, ablation, chemotherapy and radiation therapy (Olsen et al., 2010).

3.1.1Epidemiology and Risk factors

HCC is the fifth most common malignancy worldwide and the third leading cause of cancer-related death, exceeded only by cancers of the lung and stomach. Important differences have been noted between countries. It has been shown that HCC exhibits various epidemiologic features which are dynamically based on marked variations among geographic regions, racial and ethnic groups, and between men and women; and various risk factors which are again linked to the geographical trends (Serag et al., 2007). East Asia and sub-Saharan Africa have a very high incidence, whereas Italy, Spain, and Latin American countries are at intermediate risk. A relatively low but increasing incidence is

found in Western Europe, the United States, Canada, and Scandinavia. Cirrhosis is considered one of the major risk factors. Most of the geographic variation etiologic agents for liver cirrhosis; a large group of environmental factors may cause cirrhosis. The other major known risk factors for HCC are the hepatitis viruses B (HBV) and C (HCV), toxins such as alcohol and aflatoxins, metabolic diseases such as diabetes and obesity, hereditary hemochromatosis, and immune-related chronic liver diseases such as primary biliary cirrhosis and autoimmune hepatitis (Serag et al., 2007).

Interestingly, it has been seen that in almost all populations, males exhibit higher liver cancer rates than females (Serag et al., 2007). It was previously believed that the higher rates of liver cancer in males might be related to sex-specific differences in exposure to risk factors as men are more likely to be infected with HBV and HCV, consume alcohol, and smoke cigarettes. Later a study depicted that there was a 2-8 fold increase in liver cancer development in male mice (Serag et al., 2007) which suggested that male specific hormones might play an equal role in development of HCC in males rather than just sex-specific risk factor exposure. Age is another significant distribution factor for HCC development. In almost all populations the highest age-specific rates occur among persons aged 75 and older in developed countries and around 60 to 70 in high risk populations like African regions (Serag et al., 2007). The variations in the age specific patterns again correlate with the existence and distribution of above stated risk factors in high and low risk poplulations.

Risk factors are the certain known factors which can explain the cause and increase the probability of the disease occurrence in individuals. Major risk factors for HCC vary by region. The prime factors are explained as follows:

(i) Infection with *Hepatitis B virus* (HBV) and *Hepatitis C virus* (HCV): HBV and HCV infections have been established as the prime most risk factors for the development of HCC (Chuang et al., 2009). Case-control studies have shown that chronic HBV carriers have a 5- to 15-fold increased risk of HCC compared with the general population. Similarly, markers of HCV infection are found in a variable proportion of HCC patients (Serag et al., 2007). HBV and HCV increase HCC risk by promoting fibrosis and eventually cirrhosis. Once cirrhosis is established, HCC develops at an annual rate of 1% to 4% (Serag et al., 2007). Additionally, patients with dual HBV and HCV infection have more severe liver disease, and are at an increased risk for progression to HCC (Liu et al., 2006).

(ii) Liver cirrhosis: It is a situation where liver cells and tissue are seriously damaged mainly due to HBV and HCV infection, some drug intake, and heavy alcohol consumption. Alcohol drinking and tobacco consumption have been associated with an increased risk of liver cancer (Chuang et al., 2009). The most probable mechanism of alcohol and smoking-related liver cancer is through the development and promotion of liver cirrhosis. Studies showed that there was almost 8-fold risk increase in the patients with alcoholism and cirrhosis than patients with alcoholism alone (Kuper et al., 2001) and even higher risk in the patients with the combination of alcoholism, cirrhosis and hepatitis infection, suggesting cirrhosis as a prerequisite intermediate for the development of HCC.

(iii) Inherited metabolic failure diseases:

Liver is a very significant organ in terms of functioning in mammals. It maintains body's homeostasis. It maintains storage of iron and other vitamins and minerals and detoxifies all kinds of chemicals and toxins from the bloodstream. Inherited metabolic diseases are linked to various functions of the liver. There are various inherited metabolic diseases that have a pathologic impact on the liver. There are three genetically determined diseases in which the liver may be the principal target organ. These are hereditary hemochromatosis (HH), a major disorder of iron overload, Wilson's disease, a genetic disorder of copper overload, and alpha1-antitrypsin (α1-AT) deficiency, a disorder in which the normal processing of a liver-produced protein is disturbed within the liver cell (Serag et al., 2007; Zang et al., 2007). HH is characterized by dysregulation of iron absorption leading to increased intestinal absorption of iron. Excessive iron deposits in various organs results in cirrhosis, HCC, diabetes, cardiomyopathy, arthropathy, skin hyperpigmentation, and hypogonadism (Zang et al., 2007). It has been suggested that many cryptogenic cirrhosis and HCC cases represent more severe forms of non-alcoholic fatty liver disease (NAFLD), namely nonalcoholic steatohepatitis (NASH). Interestingly, it has been seen in studies that about 30-40% of patients of chronic liver disease do not show signs of major risk factors like HCV, HBV, heavy alcohol intake or smoking (Serag et al., 2007). Obesity and diabetes have emerged as two major NASH conditions as risk factors of HCC. In a large cohort study in the United States, liver cancer mortality rates were found five times greater in the individuals with greatest body weight than the normal individuals (Calle et al., 2003). Additionally, in two another population-based studies it was seen that obese men and women are at high risk of developing HCC than

normal body mass index (Moller et al., 1994; Wolk et al., 2001). Several studies have examined the association between HCC and diabetes (Suh et al., 2011). Earlier studies reported no relationship between diabetes and HCC, while more recent studies have identified diabetes as a risk factor for HCC (El-Serag et al., 2004). In addition, a recent study suggested that diabetes and overweight alone are not adequate to increase the risk of HCC in the absence of parallel viral hepatitis or liver disease which is in contradiction to previous studies where the investigators have found no hepatitis or liver disease or any major risk factor in chronic HCC patients in almost 50% cases (Davila et al., 2010).

(iv) Aflatoxins (AFB_1) exposure: AFB_1 is a toxic naturally occurring mycotoxin produced by the *Aspergillus fungus*, that grows on food items and when ingested poisons the body (Chuang, 2009; Lagiou, 2000; Marryann, 2001). Once ingested, AFB_1 gets metabolized to an active intermediate, AFB_1-exo-8,9-epoxide, which can bind to DNA and cause damage, including producing a characteristic mutation in the p53 tumor-suppressor gene (p53 249ser) (Stern et al., 2001; Serag et al., 2007). This mutation has been observed in 30%– 60% of HCC tumors in aflatoxin-endemic areas (Stern et al., 2001; Serag et al., 2007).

3.1.2 Progression

The pathogenesis for progression of HCC is complex. One of the most accepted hypothesis is described by Thorgeirsson et al., (2002). Prolonged infection with chronic hepatitis viruses and cirrhosis, genomic structure of the liver cells is shown to get altered. The review explains that, due to the gradual conversion of hepatitis virus infection to chronic infection, the damaged liver tissue sections contain phenotypically altered hepatocytes which gradually with time give rise to dysplastic hepatocytes. With the

abnormalities in hepatocytes, genomic alterations occur ultimately leading to the onset of hepatocarcinoma. It basically describes a step-by-step process through which external factors mainly risk factors and epigenetic factors induce genetic alterations in mature hepatocytes leading to cell death and cellular proliferation. Chronic inflammation progresses towards fibrosis and cirrhosis in association with the hyperactivation of survival cell replicating pathways leading to the production of monoclonal populations. With the result of constant altered gene expression and chromosome abnormalities these populations lead to dysplastic hepatocytes. This process may last 10–30 years (Thorgeirsson et al., 2002).

3.1.3 Clinical features and screening of liver cancer

HCC is a heterogeneous condition with multiple variables that vary from region to region, complicating diagnosis, prognosis, and treatment recommendations. The typical clinical symptoms of HCC are right upper quadrant abdominal pain, early satiety, and weight loss (Parikh et al., 2007). Physical findings in patients with HCC generally reflect the severity of the underlying chronic liver disease and cirrhosis. The inflammation of liver hence, enlarged abdomen, easy bruising and bleeding and yellow skin are some other manifestations of liver cancer.

The Tumor Node Metastasis (TNM) staging is one of the most used staging system for HCC classification. It provides an assessment of solid tumors based on size and extent of invasion. This is measured according to the size of the primary tumor, presence of tumor in the regional lymph nodes, and presence of metastatic spread beyond the lymph nodes (Marrero et al., 2010).

Although there is no definite evidence that screening in HCC improves survival, many physicians screen patients in high-risk groups with either serum alphafetoprotein or ultrasound of the liver or both. The American Association for the Study of Liver Diseases and EASL recommend ultrasound of the liver every 6 months for high-risk patients (Bruix et al., 2005).

3.1.4 Molecular pathogenesis of liver cancer

HCC is one of the major causes of death among cirrhotic patients. The introduction of highly sophisticated genomic technologies has led to extensive research on the molecular pathogenesis of this disastrous disease. The molecular pathogenesis of HCC is important to understand the mechanism of tumor development as well as the high-recurrence behavior of HCC as every step of progression could be a target.

As explained above, rapid proliferation of hepatocytes and development of monoclonal hepatocyte populations occur in all preneoplastic conditions and altered cell populations, and these changes continue throughout the onset of HCCs (Thorgeirsson et al., 2002). Additionally, it is believed that epigenetic changes are not enough to cause the onset of HCC. However, these epigenetic alterations during the rapid proliferation act indirectly by creating conditions that alter expression of cellular pathways responsible for proliferation and immortality leading to the onset of HCC (Thorgeirsson et al., 2002). With the advancement in genomic technologies, it is now easier to understand different pathways and factors that are responsible in the activation of HCC.

Oxidative stress has been shown to be one of the major contributing factors in hepatocarcinoma (Wang et al., 2002). Oxidative stress occurs due to the overloading of iron or copper ions in liver particularly in patients suffering from hemochromatosis and

Wilson disease respectively (Wang et al., 2002). Hussain et al., (2000) showed that oxidative stress combined with reactive aldehydes causes mutations and alterations in p53 tumor suppressor gene which in turn effect DNA repair, cell cycle and apoptosis. Another significant molecular mechanism mediated by Hepatitis B virus protein, HBx has also been shown operational in hepatocellular carcinoma. HBx gets incorporated with liver cells DNA upon infection with the hepatitis virus and has been shown by various studies to directly interact with the tumor suppressor gene p53 and modulate apoptosis (Kew et al., 2011; Wang et al., 2002). In addition, another major factor that has been shown to be prevailing in molecular pathogenesis in hepatocarcinoma is role of microRNAs. These are a class of RNA which have a short nucleotide sequence that bind to complementary sequences on mRNAs and regulate the gene expression post transcriptionally mainly by gene silencing. Huang et al., (2011), in their recent review has clearly summarised that specific microRNAs are associated with hepatocarcinoma that play a substantial role in regulating apoptosis, cell proliferation and metastasis by targeting crucial molecules in these pathways.

There are various signalling pathways active inside the cell that take charge of cell cycle regulation, cell survival, repair, and apoptosis. The transformed cells keep multiplying immortally and develop tumours. It is therefore understood that to achieve immortality, cell survival pathways take charge over the cell cycle regulation and apoptotic pathways. Major pathways involved in the development and progression of liver cancer are mainly cell survival pathways which include PI3K/AKT/mTOR and Ras/Raf/MEK/ERK pathways, cell cycle regulation pathway through p53, Wnt/β-catenin pathway and apoptotic signalling pathway (Tommasi et al., 2007). In addition loss of tumor suppressor

gene p53 has also been shown to be associated with hepatocarcinoma (Iakowa et al., 2012). Upon activation of Akt by PI3K through PIP3, apoptotic signalling is directly regulated by inactivation of pro-apoptotic proteins like Bad, caspase-9 and survival of anti-apoptotic proteins like Bcl-XL and mTOR (Tommasi et al., 2007). In addition FoxO transcription factors which have growth inhibitory functions are inhibited by activation of Akt (Tommasi et al., 2007). Several growth factor signalling pathways have been shown to be deregulated in hepatocarcinoma (Breuhahn et al., 2006).

3.1.6 Treatments and limitations

When liver cancer is diagnosed and staged, the treatment options are followed depending on the stage. However, before treatment, it is advised to have a second opinion from a liver cancer specialist (American Cancer Society, 2010). In choosing the treatment plan, important factors are considered which include the stage (extent) of the cancer and the health of the rest of the liver. Depending on these factors the treatment based on its side effects are designed (American Cancer Society, 2010).

(i) *Surgery*: Partial hepatectomy or liver transplant is the two options that are considered in case of surgery. Partial liver removal is only attempted if the person is healthy enough and all of the tumor can be removed while leaving enough healthy liver behind. In case where there is an availability of liver, a liver transplant may be the best option for some people with small liver cancers. Major risks and side effects involve the body's response to new liver. It is a complex operation involving the removal of part of liver or complete liver and signs of body rejecting the liver are usually high. In addition the drugs used in combination with the surgery have their own side effects causing hepatotoxicity (Ryder et al., 2003; American Cancer Society, 2010).

(ii) *Tumor ablation for liver cancer*: Ablation refers to treatments that destroy liver tumors without removing them. These techniques are often used in patients with few small tumors. This include radiofrequency ablation (RFA), ethanol (alcohol) ablation, microwave thermotherapy, cryosurgery. Side effects include abdominal pain, infection in the liver, and bleeding into the chest cavity or abdomen (Ryder et al., 2003).

(iii) *Embolization therapy for liver cancer*: Embolization is the injection of substances to try to block or reduce the blood flow to cancer cells in the liver (Ryder et al., 2003).

(iv) *Targeted therapy*: With the advancement in knowledge against cancer, the researchers have narrowed down particular major molecular targets because of which they are able to design drugs against those targets. The most common side effects include fatigue, rash, loss of appetite, diarrhea, high blood pressure, and redness, pain, swelling, or blisters on the palms of the hands or soles of the feet (Palumbo et al., 2013).

(v) *Chemotherapy*: Chemotherapy (chemo) is treatment with drugs to destroy cancer cells. Systemic (whole body) chemotherapy uses anti-cancer drugs that are injected into a vein or given by mouth. After reaching the blood stream, these drugs act on the cells which are rapidly growing like cancer cells specially during metastasis. Since these drugs act on the cells which are rapidly dividing, the side effects are commonly related to these cells which include hair loss mouth sores, loss of appetite, nausea and vomiting, diarrhea, increased chance of infections (from low white blood cell counts) and easy bruising or bleeding (from low blood platelet counts) (Palumbo et al., 2013).

3.2 POLYPHENOLS

Fruits and vegetables contain naturally occurring, bioactive compounds or plant secondary metabolites known as phytochemicals which have been shown to reduce the

risk of various chronic diseases like cancer, cardiovascular disease, diabetes and age related diseases such as cancer, cardiovascular disease and diabetes (Bidlack et al., 2000; Liu et al., 2003; Temple et al., 2000). Their major properties include antioxidant nature, antibacterial nature, stimulation of enzymes and hormonal action resulting in modulation of gene expression (Bidlack et al., 2000). Polyphenols are the structural class of phytochemicals and are common constituents of foods of plant origin. The main dietary sources include fruits, fruit extracts, juices, vegetables, tea, coffee and red wine. Research on effects of dietary polyphenols on human health has considerably advanced over a decade. Polyphenols comprise of single or several phenol groups and are hence classified according to the number of phenol rings they contain (D'Archivio et al 2007). The major groups include flavonoids, phenolic acids, stilbenes and lignans (D'Archivio et al 2007).

3.2.1 Health benefits

Current evidences strongly suggest the protective role of naturally occurring polyphenols in the prevention of various chronic diseases including cardiovascular, cancer, neurodegenerative diseases and diabetes (Arts et al., 2005; Scalbert et al., 2005; Khushwant et al., 2013). Polyphenols have been shown to possess a strong antioxidant effect and consequently may protect the cellular DNA from oxidative damage in various degenerative diseases related to oxidative stress (Luqman et al., 2006; Pandey et al., 2009). The cardio-protective effect of polyphenols has been critically reviewed by García-Lafuente et al, (2009). The review summarised the various mechanisms involved in antioxidant and cardio-protective effects of polyphenols in relation to various cardiovascular diseases. Several studies have also shown anti aging effect of fruit polyphenols (Cao et al., 1998; Joseph et al., 2005; Maurya et al., 2008), Importantly,

effect of polyphenols on human cancer cell lines have also been studied vastly. A significant number studies in cell culture, animal models and clinical trials have suggested a protective effect of dietary polyphenols against different types of cancers; however, the mechanism of action varies for different polyphenols (Surh et al., 2003; García-Lafuente et al., 2009).

3.2.2 Polyphenols and Cancer prevention

Carcinogenesis is a multistep process usually recognized with alterations in various molecular signalling mechanisms. Several in vitro, animal and population based studies have indicated the potential ability of polyphenols in reducing the risk of cancers by modulating various cell signal transduction mechanisms (Surh et al., 2003). Polyphenols are the most studied amongst the phytochemicals for their role as a significant nutrient in diet and in reducing the risk of chronic diseases. Besides antioxidant activity, polyphenols have been shown to have antimicrobial, antiviral, anti-inflammatory and vasodilatory actions (Rauha et al., 2000; Parvez et al., 2003; dos Santos et al., 2006). These are further characterized as phenolic acids, flavonoids, stilbenes, coumarins, and tannins. Issa et al., (2006) has critically reviewed the role of phytochemicals in inhibition of cancer and inflammation. The review summarizes some of the molecular mechanisms through which several phytochemicals may inhibit inflammation and cancer which includes the antioxidant effect and protection of DNA damage from oxidative radicals. Additionally several in vivo studies have reported antioxidant effect of polyphenol rich diet; however, it should be noted that the amount of polyphenols used for such studies are in a much larger proportion than normal diet (Duthie et al., 2000). In their review, Duthie et al (2000) summarized various studies both human and animal that strongly suggest the

protective role of polyphenols in various types of cancers. Additionally, these studies also show that some polyphenols may have tumor-specific action (Duthie et al., 2000). Chemoprevention of liver cancer (HCC) by plant polyphenols have also been intensely reviewed by Stagos et al., (2012). Various polyphenols including quercetin have been shown to depict strong antiproliferation effects in HepG2 cells (Stagos et al., 2012). It has also been shown to increase the pro apoptotic effect action of anticancer drugs in Hep3B and HepG2 cells (Stagos et al., 2012). Additionally, various polyphenols including quercetin and polyphenols from green tea have also shown promising results in animal models for HCC as well (Stagos et al., 2012).

3.2.3 Mechanism of action

Several different mechanisms have been suggested for the action of phytochemicals. Besides playing the role of strong antioxidants, phytochemicals can alter DNA, modulate gene expression and bring changes in signal transduction pathways (Noe et al, 2004; Stagos et al., 2012). Several molecular targets of dietary phytochemicals have been identified in different signalling pathways which include, pro- and anti-apoptotic proteins, cell cycle proteins, protein kinases, transcription factors to metastasis (Andreadi et al., 2006; Aggarwal et al., 2006, Russo et al., 2010). Excess production of the oxidants can cause oxidative stress mainly due to free radicals of oxygen which react with proteins and DNA and other important components of the cells resulting in chronic diseases development (Ames et al., 1993; Benz et al., 2008). Nitric-oxide is one of the major free radicals in the body which has been shown to increase tumour growth assisted metastasis (Ng et al., 2007). A recent study by Jayakumar et al., (2010) showed that phytochemicals in fruits scavenge nitric oxide and inhibit nitric-oxide induced proliferation in MCF-7

cells in vitro. Phloretin (PH) is a polyphenolic compound found in apples, apple juice and pears which has been shown to increase antitumor activity of paclitaxel (PTX), an anticancer drug through the induction of apoptosis in human HepG2 cells (Yang et al., 2009). Procyanidin, an apple flavonoid was shown to activate caspase-3 and caspase-9 induced apoptosis in cancer cells through mitochondrial pathway (Miura et al 2007). In recent reviews by Gerhouser et al., (2008) and Rupasinghe et al., (2012), different biological activities associated with apple polyphenols were critically analysed and summarized. Besides antioxidant action, an anti-inflammatory mechanism has also been described. Additionally, role of apple polyphenols in inhibition of various signalling pathways like inhibition of growth-stimulating pathway mediated by the protein kinase family, Wnt signalling through inhibition of kinase GSK3β is critically summarized. Furthermore, apple polyphenols have been shown to exhibit strong antiproliferative effect by inducing apoptosis through regulation of mitochondrial pathway and caspases cascade mechanism (Gerhouser et al., 2008). Overall, major mechanisms underlining antiproliferation effect of polyphenols include antioxidant and pro-oxidant effect, cell cycle arrest, estrogenic/antiestrogenic activity, apoptosis and various modulations in cell signalling processes (García-Lafuente et al., 2009).

3.3 QUERCETIN AND QUERCETIN-3-*O*-GLUCOSIDE

One of the most studied groups of antioxidant compounds in phenolics is flavonoids. They can be further divided into six different classes, namely flavonols, flavones, flavanones, flavanols, isoflavones and anthocyanidins, according to their molecular structure (Bravo et al., 1998). Flavonoids are abundantly found in fruits, vegetables, tea, coffee, medicinal herbs, red wine (Rice-Evans et al., 2001). Quercetin is the most

abundant molecule in the extensive class of flavonoids ubiquitously found in high amounts in apples, onions, berries (Hertog et al., 1992 and 1993) and has been shown to possess a unique ability to act as a strong antioxidant as well as prooxidant depending on its concentration (Vargas et al., 2010). With a strong ability to scavenge free radicals, inhibit lipid peroxidation and chelate metals (Rice Evans et al. 1996), quercetin may protect DNA from oxidative damage (Potenza et al., 2008). Interestingly, over a decade, quercetin has gained importance due to its strong antiproliferative action on several types of cancers (Gibellini et al., 2011: Chirumbolo et al., 2013). Quercetin in natural form exists as glycosides with sugar moeity usually attached at the C-3 or C-4 position of the pyrone ring (Chang et al., 2005) which also affect the bioavailability of these quercetin glycosides depending on sugar moiety (Olthof et al., 2000). Interestingly, quercetin glucosides have been shown to get absorbed to a greater extent than other quercetin glycosides (Hollman et al., 1997 and 1999). Quercetin-3-O-glucoside (Q3G) is one of the most important naturally occurring glucoside of aglycone quercetin and has been shown to possess a strong antioxidant property. (Soundararajan et al., 2008; Razavi et al., 2009). Q3G has been shown to possess neuroprotective (Appleton et al., 2010), blood vessel protection (Vitor et al., 2004), anti-depressant, anti-diabetic and anti-inflammatory activities (Appleton et al., 2010). Interestingly, in another study, bioconversion of rutin to Q3G was shown to exhibit stronger antiproliferative effect than quercetin aglycone (You et al., 2010). However, unlike quercetin aglycone, the mechanism of action and antiproliferative effect of Q3G on cancer cells still remain unresolved.

3.3.1 Cancer prevention and mechanism of action

The research on quercetin as a potent chemotherapy drug has gained tremendous importance due to its ability to modulate various tumour related processes such as oxidative stress, apoptosis, cell cycle arrest, proliferation and its potential tumour-specific action (Lugli et al., 2009). Quercetin has been shown to exhibit antiproliferative effects by various studies and can induce apoptosis in different types of cancer cell lines. Interestingly, several modes of action have been evaluated for antiproliferative nature of quercetin. This section summarizes some of the major mechanisms.

Quercetin exhibits a unique ability to act as both antioxidant and pro-oxidant depending on the concentration as mentioned previously. The antioxidant mechanism may include direct scavenging of reactive oxygen species (ROS), activation of antioxidant enzymes (Nijveldt et al., 2001), metal chelation (Ferrali et al., 1997) and inhibition of oxidases (Cos et al., 1998). On the other hand, under some circumstances, quercetin may behave as a pro-oxidant and induce oxidation of other compounds (Procházková et al., 2011). Additionally, the pro-oxidant nature of quercetin has been suggested to be concentration dependent (Yen et al., 2003). Longer exposures with increasing high concentrations of quercetin (over 125 μM) increased the levels of H_2O_2, superoxides and products of lipid oxidation thereby inducing DNA strand breakage (Yen et al., 2003). This suggests the inability of quercetin to cope with the ROS thereby behaving as a pro-oxidant and damaging various cellular components ultimately causing cell death.

Apart from the anti and pro-oxidant effects, another important mechanism of action of quercetin has been suggested as cell cycle modulation and arrest. Quercetin was shown to inhibit cell proliferation in human breast cancer MDA-MB-453 and MDA-MB-231 cells

by causing cell cycle arrest in G1 phase via induction of p21 and down-regulation of cell cycle progression component cyclin B1 and cyclin-dependent kinase (CDK) 1 (Jeong et al., 2009). Quercetin also down-regulated cyclin B1 and caused G2/M phase arrest in human esophageal squamous cancer cell line (Zang et al., 2009). Furthermore, quercetin-induced cell cycle arrest in G2/M phase was observed in Hela cells (Priyadarsini et al., 2010) through a p53 dependent mechanism. In addition, quercetin also behave as a strong DNA topoisomerase II inhibitor (Bandele et al., 2008 and Lopez-Lazaro et al., 2010) which may consequently lead to cell cycle arrest and apoptosis.

Another significant mechanism of quercetin against cancer cells is the induction of apoptosis. Modulation of several signalling components together result in the pro-apoptotic effect. Quercetin was shown to induce apoptosis by activating caspase-3 cascade in human promyeloleukemic HL-60 cells (Shen et al 2003). In human breast cancer cell lines, quercetin inhibited the growth and induced cytotoxicity by affecting the signal transduction pathway where it down-regulated phosphatidylinositol kinases (PI) and diphosphoinositide kinase (PIP) kinase and phosphatidylinositol (3,4,5)-triphosphate (PIP3) activity which are normally in high amounts in cancer cells (Singhal et al., 1995). Quercetin treatment on A549 lung cancer cell lines showed decrease in cell viability and induction of apoptosis through alteration in B-cell lymphoma (Bcl) family and inactivation of Akt which are responsible for cell growth (Nguyen et al., 2004). Interestingly it was also shown that MEK-ERK activation is also essential for apoptosis in lung cancer cells (Nguyen et al., 2004). Studies have shown that quercetin inhibits growth and proliferation of cancer cells by inhibiting various kinases required for growth and signal transduction (Agullu et al., 1997). This includes PI3-kinase, protein kinase C

(PKC), epidermal growth factor receptor (EGF-R) (Agullu et al., 1997). This shows the ability of quercetin to affect kinase dependent signalling pathway and hence, their downstream dependent cellular responses. Additionally, as mentioned above quercetin has been shown to inhibit DNA topoisomerase II activity strongly *in vitro* leading to cell division malfunction thereby arresting cell proliferation (Lanoue et al., 2010).

Quercetin exhibits its antioxidant, antiproliferative and anticancer actions on human liver cancer HepG2 cells as well. For instance, quercetin exhibited its antioxidant action and protected the HepG2 cells from oxidative stress caused by tert-butyl hydroperoxide by significantly lowering the levels of reactive oxygen species (Alia et al., 2005). This shows the protective effect of quercetin on cell viability. Normal cells tightly regulate growth via programmed cell death known as apoptosis which balances cell division, growth and death. Apoptosis is mediated by a regulated intracellular proteolytic casacade. In the cells undergoing apoptosis, procaspases which are effector proteases are activated by binding to adaptor proteins and inducing cell death via intrinsic or extrinsic pathways of apoptosis. Granado-Serrano et al., (2006) performed another study to see the effect of quercetin on signal transduction pathways in HepG2 cells including caspases in apoptotic pathways and Bcl-xL, Akt/PI-3-kinase, and ERK in survival pathway. In the study, a significant raise in caspase-3 activity was seen followed by caspase-9 (Granado-Serrano et al., 2006). Additionally, quercetin was shown to inhibit the Akt/PI3K and ERK1/2 by down-regulating the level of phosphorylated active Akt and dephosphorylating ERK, respectively (Granado-Serrano et al., 2006). In conclusion, when analysed for Bcl family, quercetin was shown to decrease the concentration of the Bcl-xL and Bcl-xS which have pro-apoptotic functions (Granado-Serrano et al., 2006). Furthermore, tumour suppressor

gene p53 which is known to regulate cell cycle arrest, apoptosis and DNA repair in cells (Hofseth et al., 2004) has been shown to be modulated by quercetin (Kuo et al., 2004 and Tanigawa et al., 2008). On the other hand, as mentioned previously, other than a strong antioxidant effect, not much is known for mechanism of action of Q3G in cancer cell lines.

3.4 Bioavailability of flavonoids and methods of improving bioavailability

Large number of absorption and bioavailability studies of flavonoids including quercetin and its glucosides has been conducted in animals and humans (Morand et al., 2000; Ader et al., 2000; Cermak et al., 2003; Williamson et al., 2005; Rupasinghe et al., 2010). Comparative analysis suggests different mechanisms for aglycone quercetin and its glucosides. It has been shown that quercetin has the lowest bioavailability compared to glucoside Q3G (Appleton et al., 2010). The presence of the glucoside moiety appears to effect the bioavailability (Olthof et al., 2000). Q3G being less lipophilic than quercetin because of the sugar moiety have shown to be better absorbed in both humans and animals (Appleton et al., 2010). Quercetin on the other hand shows a slow increase in absorption showing a sign of passive diffusion from stomach and intestine (Olthof et al., 2000 and Erlund et al., 2000). Furthermore, quercetin glucosides have been shown to be transported into epithelial cells via sodium-dependent glucose transporter (SGLT1) which is proposed to be a reason for an effective absorption of glucosides than aglycone quercetin (Gee et al., 2000).

Despite of the interesting results from cell culture and models, scientists have consistently questioned the ability of these effective flavonoids to show anticancer effects clinically. One of the most important reasons is the high doses and concentrations used in

the studies, which are practically unfeasible to achieve in humans as therapeutics (Gao et al., 2010). Additionally, pro-oxidant effect of flavonoids including quercetin and glucosides in high doses may have a high tendency to exert harmful effects on human body. High dosages of quercetin in cancer patients showed acute renal toxicity (Ferry et al 1996). Furthermore, the difference in the absorption of quercetin and Q3G through the glucose transporter as mentioned above suggests the role of different permeation mechanism through plasma membrane interactions. Therefore, to overcome the problem of low bioavailability at lower effective concentrations researchers are trying to modify the effective flavonoids in such a way that could increase their absorption via better membrane interaction, plasma stability and half-life.

Structural modification of the flavonoids by introducing hydrophobic groups could increase the solubility and absorption and therefore, bioavailability of glycosylated flavonoids. One of the effective approaches to enhance the membrane permeability is to increase the lipophilicity of flavonoid glycosides by acylation (Salem et al., 2011). Previous studies have also shown that enzymatically regulated esterification of flavonoids increases lipophilicity (Mellou et al., 2006). In the same study it was concluded that the flavonoid esters and fatty acids showed enhanced potential as antitumor agents than the parent flavonoid (Mellou et al., 2006). Essential fatty acids have unique biological activities. In previous studies, omega-3 and omega-6 polyunsaturated fatty acids have been shown to exhibit antiproliferative nature (Mainou-Fowler et al., 2001; Hardman 2002). However, their use is very limited as they are prone to autooxidation. However, combining the essential fatty acids with the certain falvonoids with strong antioxidant properties will not only preserve them from oxidation but also improve properties of the

flavonoid. Salem (2011) in their recent study has revealed that Q3G esters showed increased antioxidant properties by enhancing lipophilicity. Compounds for this study were prepared using enzymatic acylation with the idea of increasing the lypophilicity. For this study, Q3G was acylated with six long chain fatty acid esters as previously described (Ziaullah et al., 2012) and was evaluated for their antitumor activity and mechanism of action in human liver cancer HepG2 cells.

3.4.1 Biological activity of acylated flavonoids

To improve the bioavailability and biological properties of natural occurring flavonoids, glycosylation and acylation have received great attention. Where in glycosylation, addition of sugar can increase the hydrophilic nature of some flavonoids, in acylation, addition of fatty acids can increase the lipophilic character of some flavonoids. Interestingly, the regioselective acylation of the flavonoids have been shown to improve some of the biological activities of the flavonoids may be by increasing the ability to better interact with and getting absorbed through cellular membrane (Mellou et al., 2005). Enzymatic acylation was interpreted to be more regioselective and to enhance the solubility and antioxidant properties of flavonoids (Salem et al., 2010). It has been reported that biocatalytic preparation of acylated glycosides enhances their antioxidant and antimicrobial activity (Mellou et al., 2005). In another study, acylated derivatives of isorhamnetin-3-O-glucoside was shown to enhance its antiproliferative activity but decreased its radical scavenging activity (Salem et al., 2011). Furthermore, enzymatic acylation of isoquercitrin with fatty acids was studied. It was reported that isoquercitrin fatty acid esters showed increased antioxidant and enhanced antiproliferative properties

in Caco2 cancer cells (Salem et al., 2010). It was also seen that the enhanced biological properties were a function of increasing chain length (C6-C18) (Salem et al., 2010).

CHAPTER 4. QUERCETIN-3-*O*-GLUCOSIDE INDUCES HUMAN DNA TOPOISOMERASE II INHIBITION, CELL CYCLE ARREST AND APOPTOSIS IN HEPG2 CELLS

4.1 ABSTRACT

Current chemotherapy for hepatocellular carcinoma results in numerous side effects to the patient's body. Flavonoids, such as quercetin which are found abundantly in fruits and vegetables in the form of glucosides have been shown to be associated with reduced risk of various chronic diseases including cancers. Quercetin-3-*O*-glucoside (Q3G) has been shown to be a potent antiproliferative; however, its mechanism of action especially in human liver cancer HepG2 cells has not been studied. Using a dose- and time-dependent study, we evaluated antitumor properties of Q3G and elucidated its mechanism of action in HepG2 cells. Q3G treatment inhibited cell proliferation in a dose- and time-dependent manner in HepG2 cells with the blockade of cell cycle in S-phase. Additionally, Q3G exhibited a strong ability to inhibit DNA topoisomerase II activity. Further, DNA fragmentation and fluorescent microscopy analysis showed that Q3G induced apoptosis in HepG2 cells with the activation of caspase-3. Interestingly, Q3G showed significantly low toxicity to normal liver cells (rat hepatocytes) than Sorafenib ($P<0.05$), a chemotherapy drug for HCC. These results determine Q3G as a potent antitumor agent against liver cancer with possible mechanism of action via cell cycle arrest and apoptosis.

4.2 INTRODUCTION

HCC is the third ranked cause of death by cancer type in the world (Roberts et al, 2005). Current treatments for liver cancer include surgery and chemotherapy; however, use of chemotherapy drugs such as sorafenib is not efficient due to numerous side effects. Therefore, searching and developing safe and efficient antitumor drugs for liver cancer remains a demand in the therapeutic research.

Increased consumption of fruits and vegetables in diet which are rich in phenolics such as flavonoids has been associated with reduced risk of cancer (Yang et al., 2001; Surh et al., 2003). Quercetin is one of the most common flavonoids found in the diet (Yang et al., 2001). Its antiproliferative properties have been reported by various studies (Watson et al., 2000; Bischoff et al., 2008). Quercetin-induced cell death and reduction in cancer cell proliferation has been primarily associated with apoptotic mechanisms in various cancer cell lines including liver cancer cell HepG2 (van Erk et al., 2005; Shen et al., 2003; Singhal et al., 1995; Nguyen et al., 2004; Chi et al., 2001; Ong et a., 2004). Additionally, apoptosis induction by quercetin has also been associated with regulation of cell cycle and various signalling molecules including caspase-3 (Wang et al., 2005; Yoshida et al., 1995; Chang et al., 2005; Vijayababu et al., 2005).

Flavonoids in natural form exist as glycosides with different sugar moieties (Moon et al., 2006). Q3G is a natural occuring form of quercetin and has been shown to exhibit strong antioxidant activity (Razavi et al., 2009). Recent studies showed potential antiproliferative activity of quercetin and its glucosides including Q3G (You et al., 2008). Further, synergistic effect of apple extracts and Q3G has been shown to enhance the antiproliferative activity in human breast cancer MCF-7 cells (Yang et al., 2009).

However, a time and concentration dependent study of Q3G antitumor activity and mechanism of action in human liver cancer HepG2 cells have not yet been reported.

In view of above findings, in this research, I performed a time- and dose-dependent study and characterised the antitumor activity and possible mechanism of action of Q3G in HepG2 cells. Additionally, this study also involved comparative analysis with Sorafenib, a currently prescribed chemotherapy drug for liver cancer.

4.3 MATERIALS AND METHODS

4.3.1 Materials and chemicals

Propidium iodide, DMSO and two-well chambered cover slides were purchased from Sigma-Aldrich (Mississauga, ON). Sorafenib (Nexovar®) was purchased from Cayman Chemical (Ann Arbor, Michigan). All cell culture vessels and plates were purchased from BD Biosciences (San Jose, CA). BCA protein purification kit was purchased from Thermo Scientific (Burlington, ON).

4.3.2 Cell culture and reagents

HepG2 cells (ATCC#8065) were obtained from American Type Culture Collection (ATCC) and maintained according to ATCC's instructions using standard growth environment (37°C and 5% CO_2) in T-75 tissue culture flasks. Sub-culturing was performed every 2 to 3 days when cells reached a confluency of 70-80%. Primary rat hepatocytes (RTCP-10) were purchased from Invitrogen (Burlington, ON) were cultured according to the supplier's instructions. Cells were counted under Nikon Eclipse TS 100 phase contrast microscope (Mississauga, ON) using haemocytometer and then transferred to fresh flasks.

4.3.4 Measurement of cell viability

The assay was performed using Cell Titer 96^{TM} Aqueous One solution cell proliferation (MTS) assay kit (Promega Madison, WI) according to the manufacturer's instructions. Briefly, cells were seeded in 96-well microplate in density of 2 x 10^4 cells per well. The microplates were placed in culture incubator in standard conditions (37°C with 5% CO_2) and cultured for 24 h. After incubation, the cells were treated with six different doses (1, 10, 50, 100, 150 and 200 μM) of Q3G in fresh media in triplicates. The DMSO concentration of test compounds in all the assays was less than 1%. The plates were then incubated for different time intervals (24, 48 and 72 h) in culture incubator (37° C, 5% CO_2, 90% humidity). According to the manual's instructions, MTS reagent was added to each well (5 g/L in PBS) and again incubated for 2 h. Absorbance was recorded directly at 490 nm using Fluostar Optima micro plate reader (BMG Labtech, Ortenberg, Germany).

4.3.5 Measurement of cell cytotoxicity

The assay was performed using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, HepG2 (5000 cells/well) were plated in 96-well microplates. For positive control cells were treated with Triton X-100 to achieve maximum LDH release. The microplates were kept in culture incubator in standard conditions (37° C with 5% CO_2) and cultured for 24 hrs. After incubation, Q3G at a concentration of 100 μM in fresh media was added to each well in triplicates. The plates were incubated for different time intervals (mainly, 12, 24 and 48 hrs) in culture incubator (37° C, 5% CO_2, 90% humidity). After treatment, the 96-well microplate was centrifuged and supernatant was transferred to a fresh 96-well

microplate and subjected to LDH assay. Absorbance was taken at 490 nm using Fluostar Optima micro plate reader (BMG Labtech, Ortenberg, Germany).

4.3.6 DNA fragmentation

The assay was performed using ApoTarget™ Quick Apoptotic DNA Ladder Detection Kit (Invitrogen, Burlington, ON) according to the manufacturer's instructions. Briefly, HepG2 cells (5 x 10^5 cells/well) were grown in 12-well culture plate (75-80%) confluency and then treated with 100 µM Q3G for 24, 48 and 72 h. Cells were collected and total DNA was isolated from each sample. Extracted DNA pellet was dissolved in 30 µl of DNA suspension buffer (provided with the kit) and resolved on 1.2% agarose gel containing GelRed™ stain in 1X TAE buffer (pH 8.5, 20 mM Tris-acetic acid, 2 mM EDTA) in BioRad mini-gel electrophoresis kit. The DNA gel was visualised and photographed by BioRad's Gel Doc™ EZ system (Mississauga, ON).

4.3.7 Measurement of Caspase-3 activity

The assay was performed using Caspase-3/CPP32 Colorimetric Assay Kit (BioVision, Inc. California, USA) according to the manufacturer's instruction. Briefly, HepG2 cells (1 x 10^6 cells/well) were plated in six-well tissue culture plate. After treatment with 100 µM Q3G for 24 and 48 h, the cells were lysed with lysis buffer provided by the manufacturer and centrifuged at 13000 rpm. The supernatant was collected. The protein was quantified using BCA protein quantification kit and 250 µg of protein was used for the assay. After addition of reaction buffer and DEVD-pNA (Asp-Glu-Val-Asp p-nitroanilide) caspase substrate, the microplate was incubated at 37° C for 1-2 h. The absorbance of the samples was read at 405 nm in Fluostar Optima micro plate reader (BMG Labtech, Ortenberg, Germany). Fold-increase in CPP32 activity was determined

by comparing the absorbance levels of Q3G treated lysate with the levels of the uninduced control lysate.

4.3.8 Measurement of DNA topoisomerase II activity

Commercially available topoisomerase II drug screening kit (TopoGEN, Inc., Columbus, OH) was used and the assay was performed as previously described by Patra et al., (2011). Briefly, substrate supercoiled pHot1 DNA (0.25 µg) was incubated with four units (2 µl) of human DNA topoisomerase II, 100 µM Q3G (2 µl) and assay buffer (4 µl) in 37° C for 30 minutes. The reaction was terminated by the addition of 10% sodium dodecyl sulphate (2 µl) followed by digestion with proteinase K (50 µg/µl) at 37° C for 15 minutes. DNA was run on 1% agarose gel in BioRad gel electrophoresis system for 1-2 h and then was stained with GelRed™ stain for two h followed by destaining for 15 minutes with TAE buffer. The gel was imaged via BioRad's Gel Doc™ EZ system. Supercoiled DNA and linear strand DNA were incorporated in the gel as markers for DNA topology and DNA topoisomerase II poison (inhibitor). The inhibitory activity was calculated as relative inhibition of relaxation activity of topoisomerase enzyme in the presence of Q3G in comparison to that in the absence of Q3G.

4.3.9 Fluorescence microscopy

For detecting apoptosis, GFP-Certified™ Apoptosis/Necrosis Detection Kit was purchased from Enzo Life Sciences Inc. (Farmingdale, NY) and used for the assay. Briefly, 2 x 10^5 HepG2 cells were seeded on two-well chambered cover slides followed by treatment with 100 µM Q3G for 24 and 48 h. Staining was performed according to the manufacturer's instructions by dual detection reagent (containing Annexin V coupled with propipium iodide, (PI)). The dual-labeled cells were visualized by fluorescence

microscopy with a Leica DMBL (20x/.040) fluorescent microscope (Houston, TX) incorporated with Nikon Cool Pix 4500 digital camera (Mississauga, Ontario). Cells with bound Annexin-V show green staining in the plasma membrane. Cells that have lost membrane integrity show red staining (PI) throughout the cytoplasm with an impression of green staining on the plasma membrane. Cells with green staining were scored as apoptotic, cells with both green and red staining were scored as late apoptotic, whereas those with only red staining were considered necrotic.

4.3.10 Cell cycle analysis

HepG2 cells were cultured in six well culture plate (1 x 10^6 cells/well) for 24 h at 37º C, 5% CO_2. The cells were then treated with 100 µM Q3G for further 24 h. Cells were trypsinized and centrifuged at 1200 rpm at 4 º C for 10 min followed by PBS wash. The pellet was re-suspended in 0.3 ml of PBS. The cells were fixed by adding 0.7 ml ice cold ethanol for 2 h. The cells were centrifuged again at 1200 rpm at 4 º C for 10 min and cell pellet was re-suspended in 0.25 ml of PBS with the addition of 5 µl of 10 mg/ml Rnase A and incubation at 37°C for 1 h. After incubation, 10 µl of 1 mg/ml PI solution was added to the cell suspension and kept in the dark at 4°C until analysis. The cells were then analysed for cell cycle using flow cytometer FACSCalibur (BD Biosciences, San Jose, CA) with an excitation wavelength at 488 nm and emission at 670 nm. DNA content was determined by MotFit LT™ software, version 4.0 (Topsham, ME), which provided histograms to evaluate cell cycle distribution.

4.3.11Statistical Analysis

Unless otherwise indicated, results were expressed as mean ± SD with at least three independent experiments. Data was analysed using Minitab 16 statistical software. Group

differences were analysed using one way ANOVA. Tukey's test was performed for achieving significant difference between different treatment groups. Differences were considered statistically significant at $P < 0.05$ in all assays.

4.4 RESULTS

4.4.1 Inhibition of HepG2 cell growth

The effect of Q3G treatment on the growth of HepG2 cell lines is shown in Figure 4.1.A. The cell growth decreased in a dose-dependent manner ($P<0.05$) after 24, 48 and 72 h of treatment with Q3G. At higher concentration (200 μM), 24 h treatment showed 50% of inhibition of cell growth, however, lower concentrations (50 and 100 μM) showed over 85% and up to 98% inhibition after 48 and 72 h ($P<0.05$). Consistent results were shown by lactate dehydrogenase (LDH) cytotoxicity assay. After treatment with 100 μM Q3G, cell membrane integrity decreased in a time dependent manner reaching to 93% of cell damage in 48 h of treatment (Fig 4.1.B).

4.4.2 Q3G-induced toxicity in primary normal rat hepatocytes

To evaluate and compare the toxicity caused by Q3G on normal cells, 100 μM Q3G and Sorafenib were incubated with normal rat hepatocytes, RTCP-10 for 24 and 48 h and MTS assay was performed as described earlier. The results showed a significantly higher viability in cell lines in Q3G treated RTCP-10 cells as compared to Sorafenib treated cells at the same concentration. This result showed that Q3G exhibited significantly lower cytotoxicity than chemotherapy drug Sorafenib ($P<0.05$) in normal rat hepatocytes (Figure. 4.2).

4.4.3 Q3G blocked cell cycle in S-phase

To determine the cellular mechanism of growth inhibition of Q3G in HepG2 cells, I investigated cell cycle progression after Q3G treatment. When compared with control, treatment with 100 μM Q3G significantly increased the cell population in the S phase with a corresponding decrease of cells in the G1/G0 phase after 24 h of treatment (Fig 4.3), implying that the DNA synthesis was getting hindered. Whereas, on the other hand, chemo drug sorafenib showed a S/G2-M phase arrest which was consistent with the previous study (Fernando et al., 2012).

4.4.4 Q3G induced apoptotic cell death through caspase-3 activation

Treatment with 100 μM Q3G after 24, 48 and 72 h showed severe changes in the morphology of HepG2 cells as seen through phase contrast microscope (Fig 4.4.A). To examine if the changes were due to apoptosis, basic apoptotic hallmark assays were performed. Treatment with 100 μM Q3G showed clear DNA fragmentation (Fig 4.4.B) and increase in caspase-3 activity (Fig 4.4.C) in HepG2 cells in time-dependent manner. To further understand the differentiation between apoptosis and necrosis, the cells were examined by Annexin V and PI staining after treatment for 24 h with 100 μM Q3G via fluorescent microscopy. Figure 4.4.D shows representative images of Q3G treated and untreated Hep G2 cells. The untreated cells did not show any staining, suggesting that these cells did not undergo significant apoptosis or necrosis. Few Q3G treated cells showed staining for both Annexin V and PI (yellow) which reflects late apoptotic cells and for PI only (red) which denotes necrotic cells. Comparatively more cells showed staining for Annexin V only (green), which signifies apoptotic cells (Fig 4.4.D).

4.5.5 Q3G induced DNA topoisomerase II inhibition

To assess whether the induction of apoptosis was a result of DNA topoisomerase II inhibition, I examined the catalytic activity of DNA topoisomerase II upon treatment with Q3G via gel electrophoresis as described in materials and methods. A representative gel image of the relaxation assay for determining the catalytic inhibition on human DNA topoisomerase II with controls is presented in Figure 4.5. The assay was performed to test whether Q3G acts as a poison and increase the DNA cleavage via topoisomerase II. As shown in Fig 4.5, Q3G did not stabilize topoisomerase II cleavage complexes and failed to exhibit the formation of linear DNA, and increased the supercoiled DNA intensity, whereas, positive control drug VP 16 increased the formation of linear DNA. This result shows that Q3G does not act as human topoisomerase II poison but acts as a catalytic inhibitor by inhibiting the DNA relaxation activity.

4.6 DISCUSSION

In this study, I found that Q3G, a common flavonoid present in fruits can significantly inhibited proliferation in HepG2 cell lines. Proliferation of HepG2 cells was shown to decrease in a dose and time dependent study. To the best of our knowledge, this is the first study to show the time dependent response of Q3G treatment on cell proliferation inhibition and its mechanism of action in HepG2 cells. Fruit extracts containing Q3G have been shown to inhibit cell proliferation against various cancer cell lines (Agarwal et al., 2000; Yoon et al., 2007; Hsu et al., 2012). Synergistic effect of Q3G has also been shown to improve the antiproliferative properties in apple extracts (Yang et al., 2009). Additionally, Q3G has been shown to exhibit stronger antiproliferative action than aglycone quercetin possibly due to the glucose moiety favoring better absorption of Q3G

(You et al., 2008). This makes Q3G an important compound for cancer therapeutics as far as the bioavailability is concerned. Studies have previously shown various side effects of cancer drugs such as Sorafenib which limit the efficiency of these drugs for treatment (Robert et al., 2005). My data demonstrated that Q3G treatment had comparatively less toxicity than sorafenib at the same concentration on primary normal liver cell lines RTCP-10. This may explain specific action of Q3G on cancer cells. Consistent with previous studies my data revealed that Q3G exhibits a strong antiproliferative action on HepG2 cells with the increase in treatment time.

The inhibitory effect of Q3G on the proliferation of HepG2 cells may involve two cellular mechanisms, cell cycle arrest and induction of apoptosis. My investigation of the cell cycle distribution of Q3G-treated HepG2 cells revealed the cell cycle was arrested in the S phase when upon 100 μM Q3G treatment to the cells. Previous studies have shown that quercetin can induce cell cycle arrest in the S phase (Cheong et al., 2004) but also in the G1/S phase (Hosokawa et al., 1990) or G2/M phase (Choi et al., 2001) depending on the cancer cell type. In HepG2 cells, quercetin induced G1 phase arrest (Mu et al., 2007). Relevantly, in our study, Q3G prevented HepG2 cells to enter G2 phase resulting in accumulation of cells in S-phase. This may also explain that the cell cycle regulations are cell type and treatment dependent.

Induction of apoptosis is another possible mechanism by which the antiproliferative activity of Q3G in HepG2 cells may be exerted. In current study, I demonstrated that HepG2 cells articulate an apoptotic reaction upon Q3G treatment. The evidence included Annexin V staining analysis, activation of caspase-3 in the Q3G-treated HepG2 cells and Q3G-induced inhibition of DNA topoisomerase II. Aglycone quercetin has been

previously shown to induce apoptosis via activation of caspases in HepG2 cells (Granado-Serrano et al., 2006). Extract containing Q3G have also shown to increase the caspase-3 activity (Hsu et al., 2012). Consistent with these results, my data showed significant activation of caspase-3 in HepG2 cells upon treatment with Q3G suggesting it as an early event upon Q3G treatment. Apoptosis has been shown to be the most efficient cell death-pathway in tumor cells after topoisomerase II inhibition (El-Awady et al., 2008). Aglycone quercetin has been previously shown to inhibit DNA topoisomerase activity II (Cantero et al., 2006). Additionally extracts containing flavonol quercetin glycosides have been shown to inhibt both topoisomerase I and II in HepG2 cells resulting in cell death (Tselepi et al., 2011). Interestingly, consistent with these studies, my data showed that Q3G strongly inhibited DNA topoisomerase II activity which may be a possible reason for apparent cell death and apoptosis. It should be pointed out that these effects may or may not be specific for the HepG2 cells. Overall, my study suggests that Q3G possess a strong specific antiproliferation activity towards, HepG2 cells and is therefore, potential candidate for liver cancer chemotherapy. However, additional studies need to be performed in order to further understand the specific upstream factors mediating the Q3G induction of cell cycle arrest and apoptosis.

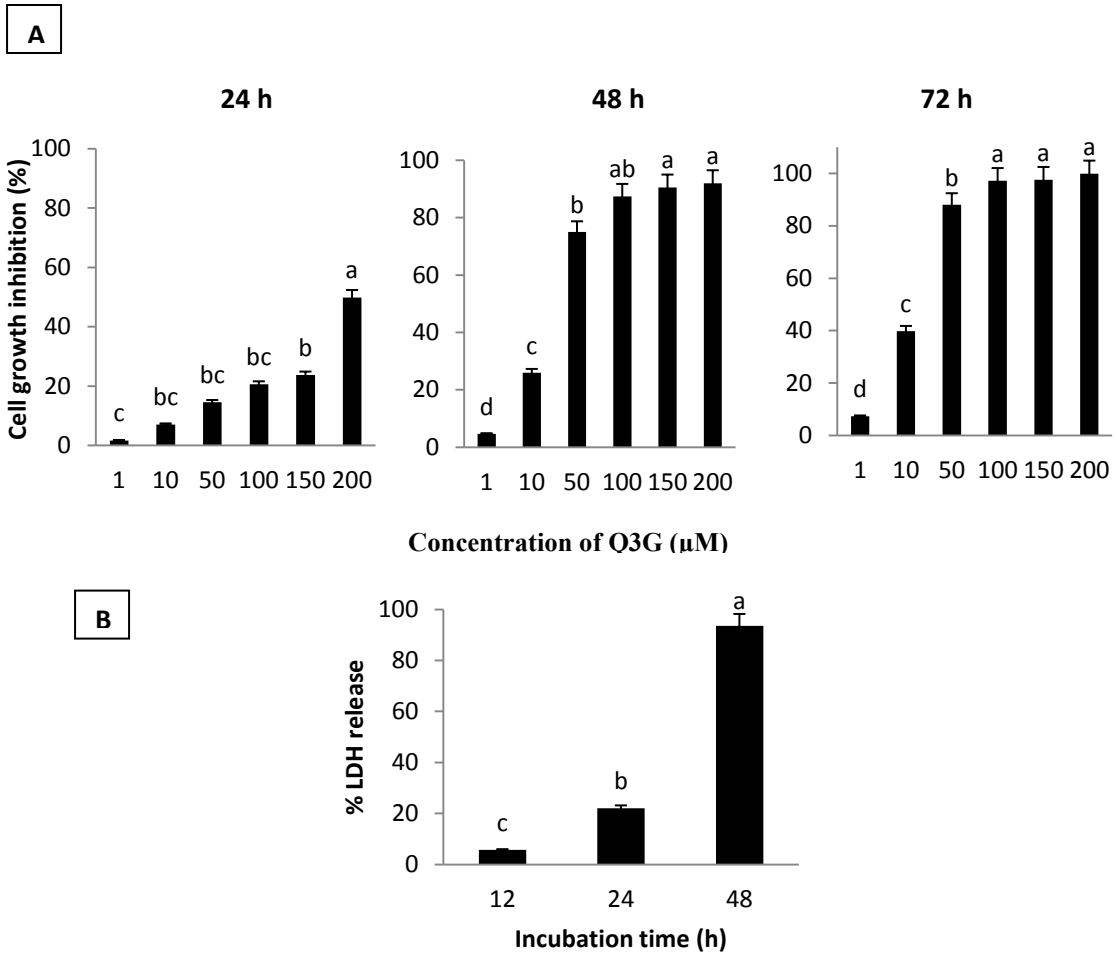


Figure 4.1. The dose- and time-dependent response of HepG2 cells to Q3G

(A) HepG2 cells were treated with increasing concentrations of Q3G as indicated and then incubated at 37°C for 24, 48 and 72 h. Percentage cell death was measured via MTS assay relative to control as described in materials and methods, (B) Percentage LDH release in HepG2 cells was measured via LDH assay upon treatment with 100 µM Q3G for 12, 24 and 48 h as described in materials and methods. Data are the average of three independent experiments conducted in tripicates and are expressed as means ± SD (n=9). Significant differences between the groups were measured by Tukey's test. Values with different letters differ significantly ($P<0.05$).

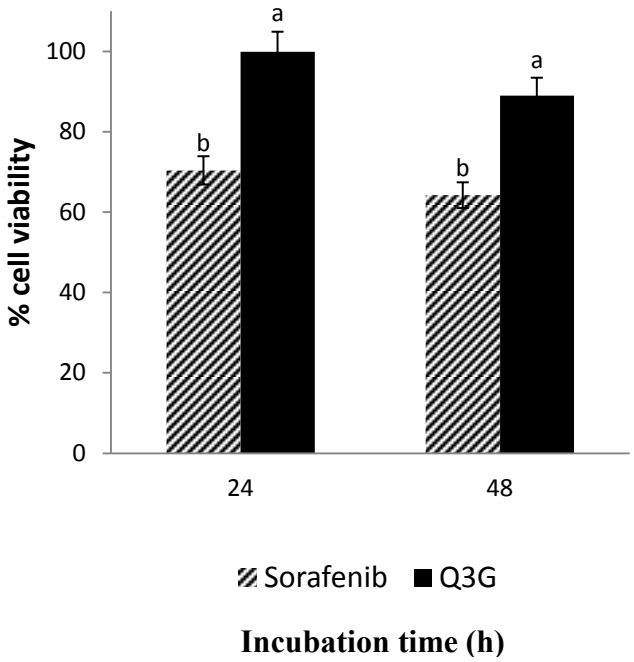


Figure 4.2. Effect of Q3G and sorafenib on viability of primary normal rat hepatocytes cell line (RTCP-10)

The figure describes the percentage of viable primary cells, RTCP-10 after treatment with Q3G and sorafenib. Cells (1 x 10^4 cells per well; 96-well plate) were treated with 100 µM Q3G or sorafenib for 24 and 48 h. After treatment, viable cell percentage was determined by MTS assay as described in materials and methods. Results are expressed relative to the control (24 and 48 h incubation without test compounds). Mean separation between groups was conducted using Tukey's test (n=6). Values with different letters differ significantly ($P<0.05$).

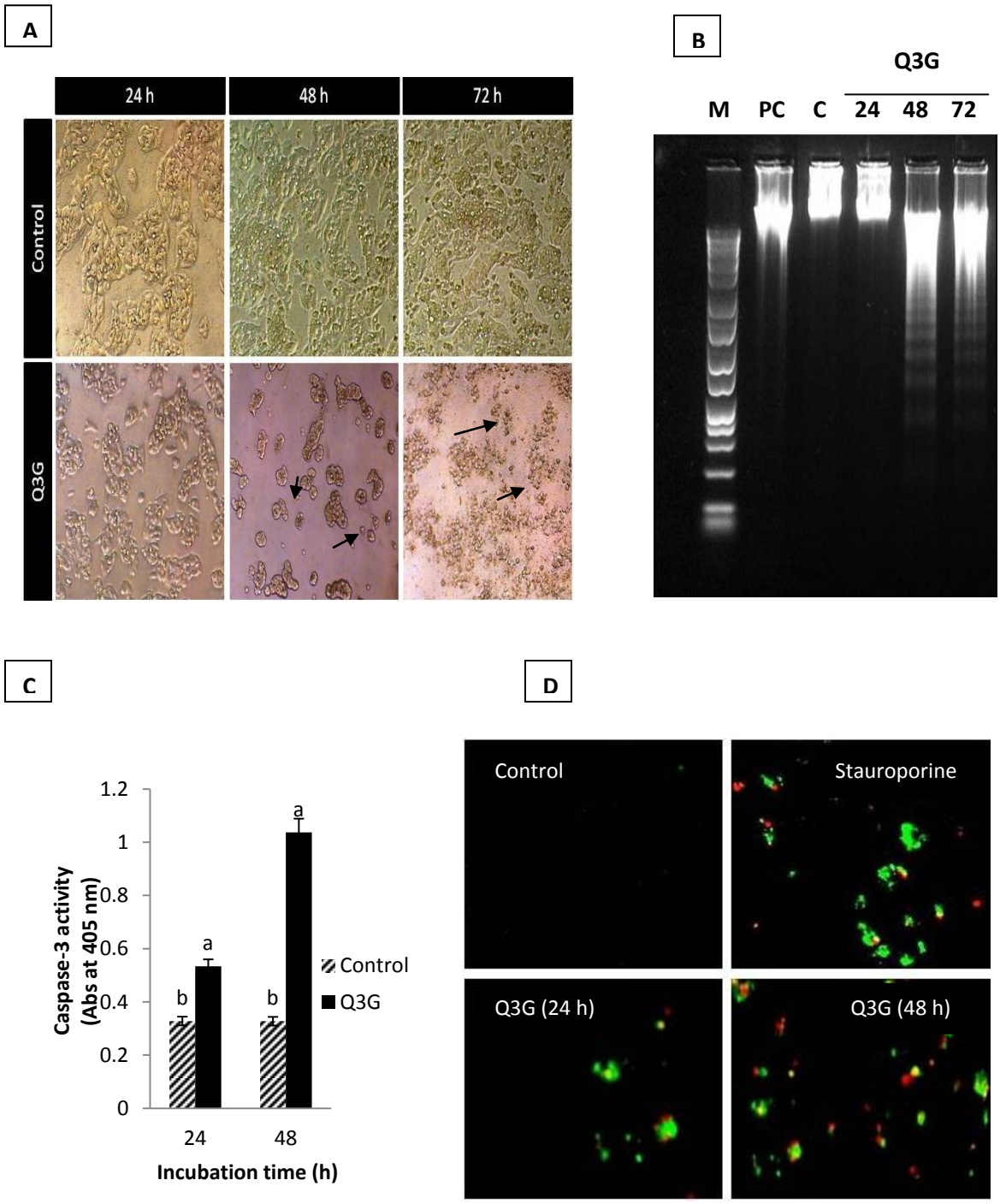


Figure 4.3. Detection of morphological changes and apoptotic induction by Q3G in HepG2 cells

(A) Cells were treated with 100 μM Q3G for 24, 48 and 72 h. After incubation, cells were observed and photographed by Nikon eclipse TS 100 phase contrast microscope equipped with Infinity 1 camera at 10x magnification. The arrows show possible formation of apoptotic bodies; (B) DNA was isolated and apoptosis was examined through DNA gel electrophoresis, M:DNA marker (10 kbp), PC: positive control (2 μM Staurosporine) and C:control without treatment; (C) cells were treated with 100 μM Q3G as indicated, then incubated at 37°C for 24 and 48 h. The treated cells were then suspended and stained with annexin V/PI and apoptotic cells were scored under fluorescent microscope and (D) caspase-3 activation was determined after 24 and 48 h of treatment with Q3G as described in materials and methods. Mean separation between groups was conducted using Tukey's test (n=3). Values with different letters differ significantly ($P<0.05$).

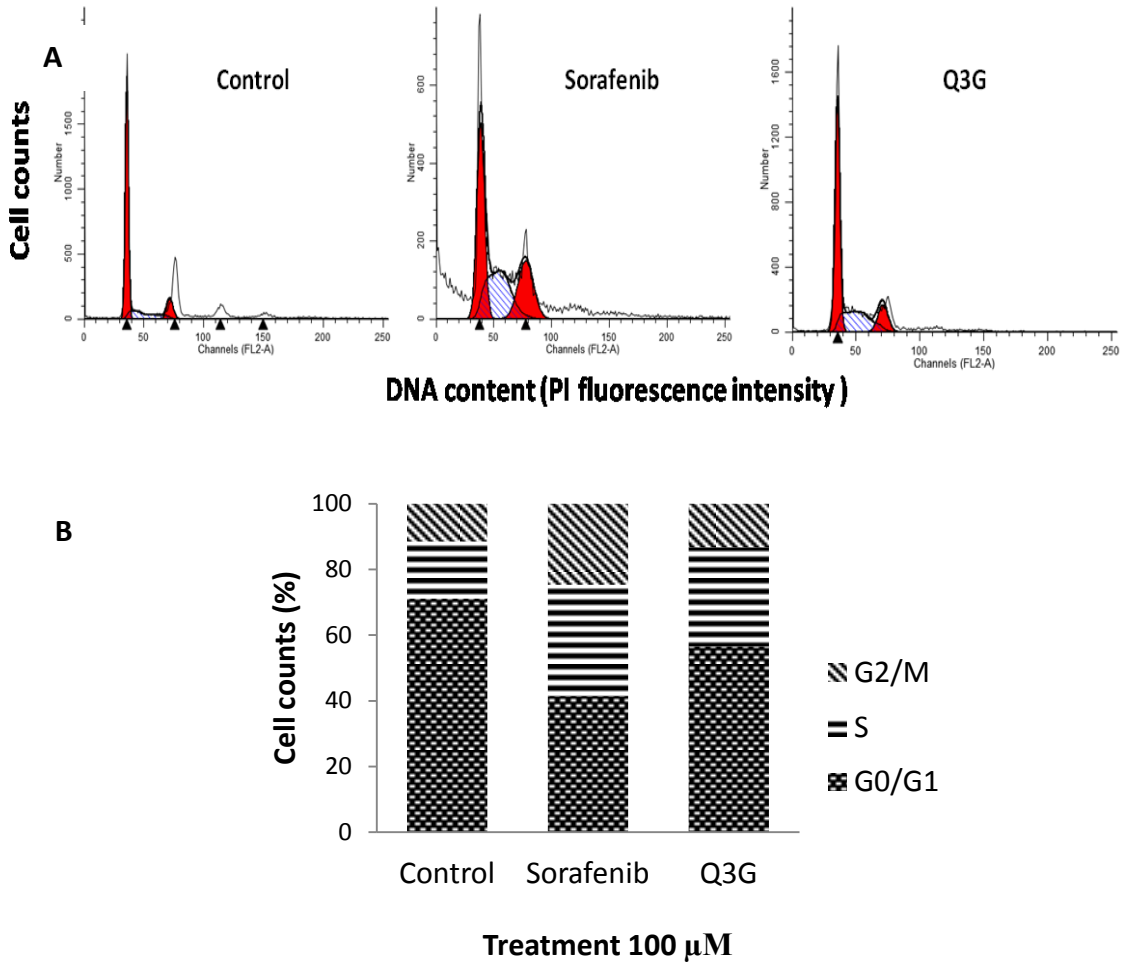


Figure 4.4. Cell cycle analysis of Q3G-treated HepG2 cells

Cells were treated with 100 µM Q3G as indicated and then incubated at 37°C for 24 h. Cells were harvested and fixed in 70% alcohol and then stained with propidium iodide. Stained cells were analyzed for cell cycle phase distributions using a flow cytometer as explained in materials and methods. (A) DNA histograms. (B) Plot of summarized values from DNA histogram.

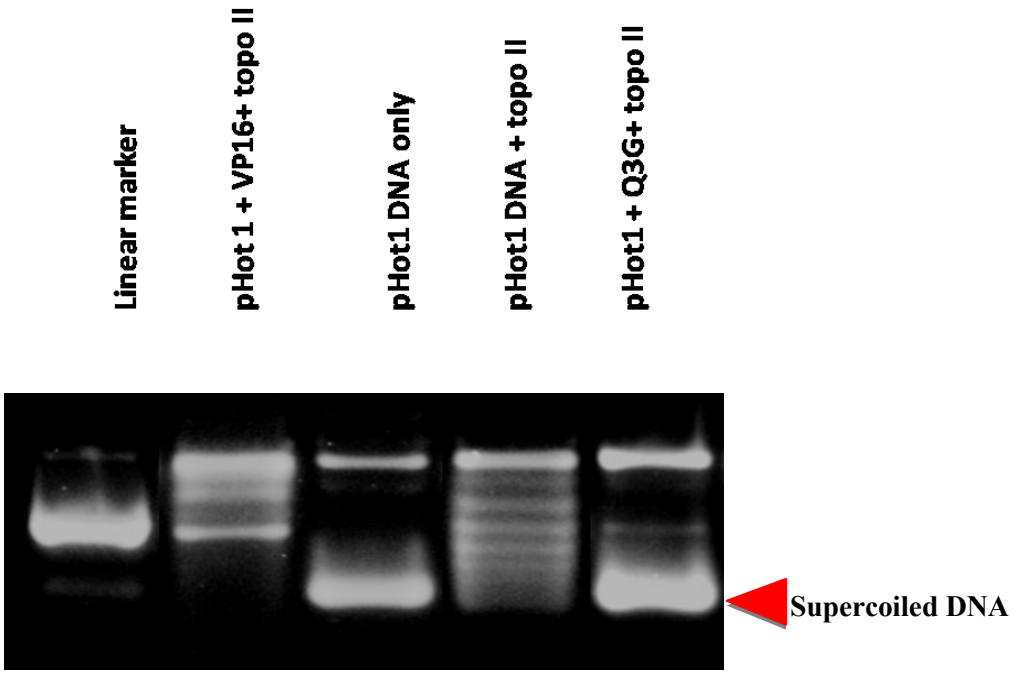


Figure 4.5. Q3G-induced DNA topoisomerase II inhibition

To assess the effect of Q3G on DNA topoisomerase II DNA relaxation activity, supercoiled circular pHot1 DNA (0.25 µg) was taken in test and control tubes and incubated with 4 units of the DNA topoisomerase II enzyme. The reactions were kept at 37º C for 30 min followed by 1% agarose gel electrophoresis. The gel was then stained with GelRed™ stain for two h and then destained with TBE buffer for 15 to 20 min. The gel was imaged in Bio Rad Gel doc system. Lane 1, linear DNA marker; lane 2, pHot1 DNA + positive control drug VP16 (etoposide) + topoisomerase II; lane 3, supercoiled pHot1 DNA; lane 4, pHot1 DNA + topoisomerase II; lane 5, pHot1 DNA+ 100 µM Q3G + topoisomerase II. DNA topoisomerase inhibition was detected by comparing the ability of DNA topoisomerase II to relax the supercoiled DNA in presence and absence of Q3G.

4.9 REFERENCES

Agarwal C, Sharma Y and Agarwal R: Anticarcinogenic effect of a polyphnolic fraction isolatd from grape seeds in human prostate carcinoma DU145 cells: cell-cycle regulatiors and induction of G1 arrest and apoptosis. *Mol. Carcinogenesis* **28**, 129–138, 2000.

Bischoff SC: Quercetin: potentials in the prevention and therapy of disease. *Curr Opin Clin Nutr Metab Care* **11**, 733-740, 2008.

Cantero G, Campanella C, Mateos S and Cortés F: Topoisomerase II inhibition and high yield of endoreduplication induced by the flavonoids luteolin and quercetin. *Mutagenesis* **21**, 321-5, 2006.

Chang J, Hsu Y, Kuo P, Kuo Y, Chiang L and Lin C: Increase of Bax/ Bcl-XL ratio and arrest of cell cycle by luteolin in immortalized human hepatoma cell line. *Life Sci* **76**, 1883–93, 2005.

Cheong E, Ivory K, Doleman J, Parker ML and Rhodes M: Synthetic and naturally occurring COX-2 inhibitors suppress proliferation in a human oesophageal adenocarcinoma cell line (OE33) by inducing apoptosis and cell cycle arrest. *Carcinogenesis* **25**, 1945–1952, 2004.

Chi YS, Jong HG, Son KH, Chang HW, Kang SS and Kim HP: Effects of naturally occurring prenylated flavonoids on enzymes metabolising arachidonic acid: cyclooxigenases and lypoxygenases. *Biochem Pharmacol* **62**, 1185–91, 2001.

Choi JA, Kim JY, Lee, JY, Kang CM and Kwon HJ: Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. *Int J Oncol* **19**, 837–844, 2001.

El-Awady RA, Ali MM, Saleh EM and Ghaleb FM: Apoptosis is the most efficient death-pathway in tumor cells after topoisomerase II inhibition. *Saudi Med J* **29**, 558-64, 2008.

Fernando J, Sancho P, Fernández-Rodriguez CM, Lledó JL, Caja L, et al.: Sorafenib sensitizes hepatocellular carcinoma cells to physiological apoptotic stimuli. *J Cell Physiol* **227**, 1319-25, 2012.

Granado-Serrano AB, Martín MA, Bravo L, Goya L, Ramos S: Quercetin Induces Apoptosis via Caspase Activation, Regulation of Bcl-2, and Inhibition of PI-3-Kinase/Akt and ERK Pathways in a Human Hepatoma Cell Line (HepG2). *J. Nutr* **136**, 2715–2721, 2006.

Hosokawa N, Hosokawa Y, Sakai T, Yoshida, M and Marui, N: Inhibitory effect of quercetin on the synthesis of a possibly cell-cycle-related 17-kDa protein, in human colon cancer cells. *Int J Cancer*. **45**, 1119–1124, 1990.

Hsu CP, Lin CC, Huang CC, Lin YH, Chou JC, Tsia YT, Su JR and Chung YC: Induction of apoptosis and cell cycle arrest in human colorectal carcinoma by Litchi seed extract. *J Biomed Biotechnol*. 341479, 2012.

Moon YJ, Wang X and Morris ME: Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. *Toxicol in Vitro* **20**, 187-210, 2006.

Mu C, Jia P, Yan Z, Liu X, Li X and Liu H: Quercetin induces cell cycle G1 arrest through elevating Cdk inhibitors p21 and p27 in human hepatoma cell line (HepG2). *Methods Find Exp Clin Pharmacol* **29**, 179-83, 2007.

Nguyen T, Tran E, Nguyen T, Do P, Huynh T and Huynh H: The role of activated MEK-ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells. Carcinogenesis **25**, 647–59, 2004.

Ong C, Tran E, Nguyen T, Ong C, Lee S, Lee J, Ng C, Leong C, Huynh H: Quercetin-induced growth inhibition and cell death in nasopharyngeal carcinoma cells are associated with increase in Bad and hypophosphorylated retinoblastoma expressions. *Oncol Rep* **11**, 727–33, 2004.

Patra N, De U, Kang JA, Kim JM, Ahn MY, Lee J: A novel epoxypropoxy flavonoid derivative and topoisomerase II inhibitor, MHY336, induces apoptosis in prostate cancer cells. *Eur J Pharmacol* **658**, 98-107, 2011.

Razavi SM, Zahri S, Zarrini G, Nazemiyeh H and Mohammadi S: Biological activity of quercetin-3-O-glucoside, a known plant flavonoid. *Bioorg Khim* **35**, 414-6, 2009.

Robert C, Soria JC, Spatz A, Le Cesne A, Malka D, Pautier P, Wechsler J, Lhomme C, Escudier B, Boige V, Armand JP and Le Chevalier T: Cutaneous side-effects of kinase inhibitors and blocking antibodies. *Lancet Oncol*. **6**, 491-500, 2005.

Roberts L and Gores G: Hepatocellular carcinoma: molecular pathways and new therapeutic targets. *Semin Liver Dis* **25**, 212–25, 2005.

Shen S, Chen Y, Hsu F and Lee W: Differential apoptosis-inducing effect of quercetin and its glycosides in human promyeloleukemic HL-60 cells by alternative activation of the caspase 3 cascade. *J Cell Biochem* **89**, 1044–55, 2003.

Singhal R, Yeh YA, Praja N, Olah E, Sledge GJ and Weber G: Quercetin down-regulates signal transduction in human breast carcinoma cells. *Biochem Biophys Res Commun* **208**, 425–31, 1995.

Surh Y-J: Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* **3**, 768–80, 2003.

Tselepi M, Papachristou E, Emmanouilidi A, Angelis A, Aligiannis N, Skaltsounis AL, Kouretas D and Liadaki K: Catalytic inhibition of eukaryotic topoisomerases I and II by flavonol glycosides extracted from Vicia faba and Lotus edulis. *J Nat Prod* **74**, 2362-70, 2011.

van Erk M, Roepman P, van der Lende T, Stierum R, Aarts J, van Bladeren P and van Ommen B: Integrated assessment by multiple gene expression analysis of quercetin bioactivity on anticancer-related mechanisms in colon cancer cells in vitro. *Eur J Nutr* **44**, 143–56, 2005.

Vijayababu M, Kanagaraj P, Arunkumar A, Ilangovan R, Aruldhas M and Arunakaran J: Quercetin-induced growth inhibition and cell death in prostatic carcinoma cells (PC-3) are associated with increase in p21 and hypophosphorylated retinoblastoma proteins expression. *J Cancer Res Clin Oncol* **131**, 765–71, 2005.

Wang I, Lin-Shiau S and Lin J: Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. *Eur J Cancer* **35**, 1517–25, 1999.

Watson W, Cai J and Jones D: Diet and apoptosis. *Annu Rev Nutr* **20**, 485–505, 2000.
Yang C, Landau J, Huang M and Newmark H: Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu Rev Nutr* **21**, 381–97, 2001.

Yang J and Liu RH: Synergistic effect of apple extracts and quercetin 3-beta-d-glucoside combination on antiproliferative activity in MCF-7 human breast cancer cells in vitro. *J Agric Food Chem* **57**, 8581-6, 2009.

Yoon H and Liu RH: Effect of selected phytochemicals and apple extracts on NF-kappaB activation in human breast cancer MCF-7 cells. *J Agric Food Chem* **155**, 3167-73, 2007.
Yoshida M, Sakai T, Hosokawa N, Marui N, Matsumoto K, Fujioka A, Nishino H and Aoike A: The effect of quercetin on cell cycle progression and growth of human gastric cancer cells. *FEBS Lett* **260**, 10–3, 1990.

You HJ, Ahn HJ and Ji GE: Transformation of Rutin to Antiproliferative Quercetin-3-glucoside by Aspergillus niger. *J Agric Food Chem* **58**, 10886–10892, 2008.

CHAPTER 5. ANTIPROLIFERATIVE PROPERTIES OF LONG CHAIN ACYLATED ESTERS OF QUERCETIN-3-*O*- GLUOCOSIDE IN HEPG2 CELLS

5.1 ABSTRACT

Despite their strong role in human health, poor bioavailability of flavonoids limits their biological effects in vivo. Enzymatically catalyzed acylation of fatty acids to flavonoids is one of the proposed ways of increasing cellular permeability and hence, biological activities. In this study six long chain fatty acid esters of quercetin-3-*O*-glucoside (Q3G) acylated enzymatically were used for determining their antiproliferative action in comparison to precursor compounds (quercetin, Q3G and six fatty acids namely, stearic acid, oliec acid, linolec acid, alpha-linolenic acid, eicosapentaenoic and docosahexanoic acids) using HepG2 cells *in vitro*. Long chain fatty acid esters of Q3G showed significant inhibition of cell proliferation (approximately 85% to 90%) in HepG2 cells compared to the precursor compounds and two prescribed drugs (Sorafenib and Cisplatin) for liver cancer chemotherapy (P-value < 0.05) after 6 h and 24 h of treatment. The cell death due to these novel compounds was associated with cell cycle arrest and apoptosis based on DNA fragmentation, cell cycle analysis by flow cytometer, fluorescent microscopy and elevated caspase-3 activity. Additionally, the long chain fatty acid esters of Q3G exhibited strong DNA topoisomerase II inhibition. Among the six fatty acid esters of Q3G, oleic acid ester displayed the greatest antiproliferation action and a high potential as a cancer therapeutic. Overall, the results of the study suggest strong antiproliferative action of these novel compounds in treatment of cancer.

5.2 INTRODUCTION

Plant flavonoids in our diet are widely found in fruits, vegetables, and plant derived beverages such as tea, coffee and wine. Research has revealed that some of these compounds are not only strong antioxidants but also strong therapeutics with anti-cancer activity. Previous epidemiology and cohort studies have suggested various phytochemicals, mainly flavonoids in the human diet might decrease the risk of various lifestyle and age related chronic diseases including cancer, cardiovascular disease, and diabetes (Hertog, 1993; Arts, 2001; Knekt, 2002; Le Marchand, 2006).

One of the most ubiquitous and very well studied flavonoid is quercetin which has been shown to exhibit antiproliferative effects and induce apoptosis in different types of cancer cell lines. Quercetin has been shown to induce apoptosis by activating caspase 3 cascade in human promyeloleukemic HL-60 cells (Shen et al., 2003). Quercetin induced cytotoxicity and reduction in cell viability of cancer cells has been shown as result of alteration in the signal transduction pathways by affecting various kinases which includes Akt/PI3K (Singhal et al., 1995 and Nguyen et al., 2004), epidermal growth factor receptor (EGF-R) (Agullu et al., 1997), Mitogen-activated protein kinases/Extracellular signal-regulated kinases (MAPK/ERK) (Granado-Serrano et al., 2006).Additionally, quercetin has been shown to inhibit DNA topoisomerase II activity strongly *in vitro* leading to cell division malfunction thereby arresting cell proliferation (Lanoue et al., 2010). The naturally occurring form, quercetin-3-*O*-glucoside has been shown to exhibit strong antioxidant activity (Razavi et al., 2009). In another study, quercetin-3-β-d-glucoside was shown to exhibit stronger antiproliferative activity than its aglycone, quercetin (You et al., 2010). However, its mechanism of action remains unclear.

Despite their strong health benefits and biological activity which includes the antiproliferative action, the major problem associated with the use of flavonoids and glucosides, is its low bioavailability (Passamonti et al., 2009) which limits their effect in vivo. To enhance these properties several researchers have performed modifications in flavonoid structures chemically or enzymatically. The number of hydroxyl groups, presence of a methoxy group in the B ring of flavonoids and lipophilicity have been shown to influence the bioavailability of flavonoids (Lin et al., 2003). Structural modification of the flavonoids by introducing hydrophobic groups could increase the solubility and absorption and therfore, bioavailability of glycosylated flavonoids. One of the effective approaches to enhance the membrane permeability is to increase the lipophilicity of these compounds by acylation (Salem et al., 2011). Essential fatty acids have unique biological activities. In previous studies, omega-3 and omega-6 polyunsaturated acids have been shown to exhibit antiproliferative nature (Mainou-Fowler et al., 2001 and Hardman 2002); however, their use is very limited as they are prone to autooxidation. However, combining the essential fatty acids with the certain falvonoids with strong antioxidant properties will not only preserve them from oxidation but also improve properties of the flavonoid. Salem (2011) in their recent study has revealed that Q3G esters showed increased antioxidant properties by enhancing lipophilicity. Compounds for this study were prepared using enzymatic acylation with the idea of increasing the lypophilicity. For this study, Q3G was acylated with six long chain fatty acid esters as previously described in (Ziaullah et al., 2012).

The aim of the current study was to determine the antiproliferative action of these long chain fatty acid esters of Q3G (namely, stearic acid ester, oleic acid ester, linoleic acid

ester, alpha-linolenic acid ester, eicosapentaenoic (EPA) ester, docosahexanoic acids (DHA) ester of Q3G) and to evaluate the mechanism of their action using human hepatocellular cell line HepG2. As stated above, flavonoid quercetin and its glucosides have been shown to suppress cell proliferation through apoptosis. Furthermore, activation of caspases induces various cellular changes which are hallmarks of apoptosis. Additionally caspase-3 family is quite distinguished in apoptosis. I hypothesised that the structural modification of Q3G into long chain fatty acids of Q3G will effectively enhance its lipophilicity and hence, *in vitro* antiproliferative activity which could be driven by cell cycle arrest leading to apoptosis. To test this, in the first phase, correct cytotoxic dosage of fatty acid esters of Q3G was determined through MTS and LDH antiproliferative assays. The second phase followed determination of the mechanism of action by evaluating hallmarks of apoptosis. This was done by checking difference in expression of caspase-3 family in treated and control cells, endonucleolytic cleavage of DNA via DNA fragmentation assay, production of apoptotic bodies through fluorescent microscopy. This was a comparative study which involved the evaluation of the antiproliferative nature of six long chain fatty acid esters of Q3G in comparison to the precursor compounds (quercetin, Q3G and six fatty acids) and currently prescribed drugs used in chemotherapy for liver cancer (Sorafenib and Cisplatin).

5.3 MATERIALS AND METHODS

5.3.1 Materials and chemicals

Quercetin, quercetin-3-*O*-glucoside, cisplatin, propidium iodide, fatty acids namely, oleic, stearic, linoleic, linolenic, eicosapentaenoic, docosahexanoic acids and two-well chambered cover slides were purchased from Sigma-Aldrich (Mississauga, ON, Canada).

Sorafenib/ Nexovar® was purchased from Cayman Chemical (Michigan, USA). Cell Titer 96TM Aqueous One solution cell proliferation (MTS) assay and CytoTox 96® non-radioactive cytotoxicity (LDH) Assay kits were purchased from Promega (Madison, WI). ApoTarget™ Quick apoptotic DNA ladder detection kit from Invitrogen (Burlington, ON). Caspase 3 colorimetric assay kit was purchased from BioVision, Inc. (California, USA). All cell culture vessels and plates were purchased from BD Biosciences (San Jose, CA). BCA protein purification kit was purchased from Thermo Scientific (Burlington, ON).

5.3.2 Synthesis of long chain fatty acid esters of Q3G

The compounds for the study were synthesized as previously described by Ziaullah et al., (2012). Briefly, in a round bottom flask, defined quantities of Q3G and individual fatty acids were dissolved in acetone. Enzymatic reactions were initiated by the addition of lipase (Novozyme 435®); with an activity of 10,000 propyl laurate units). The mixture was stirred and heated at 45 ºC for 12–24 h. and was monitored by thin layer chromatography (TLC), followed by staining with anisaldehyde spray reagent and then heating at 110 ºC. After completion of reaction, it was filtered, evaporated and column chromatography (acetone/toluene; 35:75 to 50:50) was performed to get the pure fatty acid esters of Q3G. The pure compounds were then analyzed by IR, ^{1}H NMR and ^{13}C NMR spectroscopy to confirm the structures.

5.3.3 HepG2 cell culture system

HepG2 cells were obtained from American Type Culture Collection (ATCC, 8065) and maintained according to ATCC's instructions. Briefly, the cells were cultured in Eagle's Minimum Essential Growth Medium (EMEM) with 2 mM L-glutamine and 10% Fetal

Bovine Serum (FBS) at 37°C and 5% CO_2. T-75 tissue culture flasks with 12-15 ml of media were used for regular culturing. Sub-culturing was performed in 1:4 or 1:5 ratio every 3 to 4 days when cells reached a confluency of 70-80%. Cells were counted under Nikon Eclipse TS 100 phase contrast microscope using haemocytometer and then transferred to fresh flasks.

5.3.4 Measurement of cell proliferation

The assay was performed as described previously by Talib et al., (2010) and Shan et al., (2009). Commercially available Cell Titer 96TM Aqueous One solution cell proliferation (MTS) assay was employed for the assay. Manufacturer's instructions were followed for performing assay. Briefly, HepG2 cells in the exponential growth phase were collected and seeded in 96-well microplate in density of 2 x 10^4 cells per well, final volume being 200 µl/well with the help of multi-channel pipette. The microplates were placed in culture incubator in standard conditions (37°C with 5% CO_2) and cultured for 24 hrs. After incubation, 100 µM of long chain fatty acid esters of Q3G and control samples in fresh media were added to each well in triplicates. The DMSO concentration for 100 µM of test compounds in all assays was less than 1%. The plates were then incubated for different time intervals (6 and 24 hrs) in culture incubator (37° C, 5% CO_2, 90% humidity). According to manual instructions, 20 µl of MTS was added to each well (5 g/L in PBS) and again incubated for 1-4 hrs. Absorbance was recorded directly at 490 nm using Fluostar Optima micro plate reader (BMG Labtech, Ortenberg, Germany).

5.3.5 Measurement of cell cytotoxicity

Commercially available CytoTox 96® Non-Radioactive Cytotoxicity (LDH) Assay kits from Promega was utilised for this assay. The manufacturer's instructions were followed

for the assay. Briefly, HepG2 (5000 cells/well) were plated in 96-well microplates, the final volume per well was kept at 100 µl. The controls included (i) Assay medium without cells, (ii) low control with cells and assay medium to see spontaneous or normal LDH activity and (iii) high control with cells treated with Triton X-100 to achieve maximum LDH release which served as positive control. The microplates were placed in culture incubator in standard conditions (37° C with 5% CO_2) and cultured for 24 hrs. After incubation, 100 µM of the long chain fatty acid esters of Q3G and control samples in fresh media were added to each well in triplicates. The plates were incubated for different time intervals (mainly, 6 and 24 hrs) in culture incubator (37° C, 5% CO_2, 90% humidity). After treatment, the 96-well microplate was centrifuged and supernatant was transferred to a fresh 96-well microplate and subjected to LDH assay. Absorbance was taken at 490-492 nm using Fluostar Optima micro plate reader (BMG Labtech, Ortenberg, Germany).

5.3.6 DNA fragmentation

This assay was performed by utilizing commercially available ApoTarget™ Quick Apoptotic DNA Ladder Detection Kit. The manufacturer's instructions were followed for the assay. Briefly, HepG2 cells (5 x 10^5 cells/well) were grown in 12 well culture plate (75-80%) confluency and then treated with 100 µM of test compounds for 24 and 48 hrs. Cells were collected and total DNA was isolated from each sample. Extracted DNA pellet was dissolved in 30 µl of DNA suspension buffer (provided with the kit) and resolved on 1.2% agarose gel containing GelRed™ stain (used 1X) in 1X TAE buffer (pH 8.5, 20 mM Tris-acetic acid, 2 mM EDTA) in BioRad mini-gel electrophoresis kit. The DNA gel

was then moved to UV sample tray for examining the bands visualised and photographed by BioRad's Gel Doc™ EZ system (Mississauga, ON).

5.3.7 Caspase Assay

The caspases activation was quantified by utilising Caspase-3/CPP32 Colorimetric Assay Kit. The assay was performed according to the manufacturer's instruction. Briefly, HepG2 cells (1 x 10^6 cells/well) were plated in six-well tissue culture plate. After treatment with the test compounds for 24 h, the cells were lysed with lysis buffer provided by the manufacturer and centrifuged at 13000 rpm. After collecting the supernatant, the protein was quantified using BCA protein quantification kit and 250 µg of protein per treatment sample was used for the assay. Reaction buffer (50 µl) was added to each treatment well of microplate reader followed by addition of 5 µl DEVD-pNA (Asp-Glu-Val-Asp p-nitroanilide) caspase substrate. The microplate was incubated at 37° C for 1-2 h. The absorbance of the samples was read at 405 nm in Fluostar Optima micro plate reader (BMG Labtech, Ortenberg,Germany). After subtracting background readings from all the samples (induced and uninduced), fold-increase in CPP32 activity was determined by comparing these results with the level of the uninduced control.

5.3.8 Topoisomerase Assay

Commercially available topoisomerase II drug screening kit (TopoGEN, Inc., Columbus, Ohio, U.S.A) was utilized and assay was performed as explained by Patra et al., (2011). Briefly, substrate supercoiled pHot1 DNA (0.25 µg) was incubated with four units (2 µl) of human DNA topoisomerase II, test compounds (2 µl) and assay buffer (4 µl) in 37° C for 30 min. The reaction was terminated by the addition of 10% sodium dodecyl sulphate (2 µl) followed by digestion with proteinase K (50 µg/µl) at 37° C for 15 min. After

incubation DNA was run on 1% agarose gel in BioRad gel electrophoresis system at 70V for 1-2 h. The gel was stained with GelRed™ stain for two h and destained for 15 minutes with TAE buffer followed by gel imaging via BioRad's Gel Doc™ EZ system. Supercoiled DNA and linear strand DNA were incorporated in the gel as markers for DNA topology and DNA topoisomerase II poison (inhibitor). Additionally, a positive control drug VP16 (provided with the kit) a known DNA toposiomerase II inhibitor (poison) was also incorporated in the gel for reference. The presence of a single linear band in the positive control reaction confirmed the inhibitory effect of the VP16 on DNA topoisomerase II activity. The inhibitory activity was calculated as relative activity of topoisomerase enzyme (in this case, intensity of the supercoiled band and presence/absence of single linear band) in the presence of test compounds in comparison to that in the negative control solution (DMSO control).

5.3.9 Fluorescence microscopy

GFP-Certified™ Apoptosis/Necrosis Detection Kit was purchased from Enzo Life Sciences Inc. (Farmingdale, NY) for the detection of adherent apoptotic cells. Briefly, 2 x 10^5 HepG2 cells were seeded on two-well chambered cover slides (Sigma-Aldrich) and grown to about 75% confluency; this was followed by treatment with 100 μM long chain fatty acid esters of Q3G for 24 h. Adherent cells were stained according to the manufacturer's instructions by dual detection reagent (containing annexin V coupled with PI). The dual-labeled cells were visualized by fluorescence microscopy with a Leica DMBL (20x/.040) fluorescent microscope (Houston, TX) incorporated with Nikon Cool Pix 4500 Digital camera (Mississauga, Ontario). Cells with bound annexin-V show green staining in the plasma membrane. Cells that have lost membrane integrity show red

staining (PI) throughout the cytoplasm with an impression of green staining on the plasma membrane. Cells with green staining were scored as apoptotic, cells with both green and red staining were scored as late apoptotic, whereas those with only red staining were considered necrotic.

5.3.10 Cell cycle analysis

HepG2 cells were plated in six well culture plate (1 x 10^6 cells/well). After 24 h of incubation at 37º C, 5% CO_2, the cells were treated with 100 µM long chain fatty acid esters of Q3G for another 24 h. Briefly, cells were trypsinized and centrifuged at 1200 rpm at 4 º C for 10 minutes followed by another PBS wash. The pellet was re-suspended in 0.3 ml of PBS. The cells were then fixed by adding 0.7 ml of ice cold ethanol for 2 h. After fixation, the cells were centrifuged again at 1200 rpm at 4 º C for 10 min and cell pellet was re-suspended in 0.25 ml of PBS with the addition of 5 µl of 10 mg/ml Rnase A (the final concentration being 0.2-0.5 mg/ml) and incubation at 37°C for 1 h. After incubation 10 µl of 1 mg/ml PI solution (the final concentration being 10 µg/ml) was added to the cell suspension and kept in the dark at 4°C until analysis. The cells were then analysed for cell cycle using flowcytometer FACS Caliber (BD Biosciences, San Jose, California) with an excitation wavelength at 488 nm and emission at 670 nm. DNA content was determined by MotFit LT™ software, version 4.0 (Topsham, ME), which provided histograms to evaluate cell cycle distribution.

5.3.11 Statistical Analysis

Data was analysed using Minitab 16 statistical software. The assays were replicated three times using a completely randomised design (CRD) model. Data was analysed using one way ANOVA. All treatments were checked for normality and constant variation check

before running ANOVA. Tukey's test was performed for achieving significant difference between different treatment compounds. Significance level in all assays was taken at $P < 0.05$. All data were expressed as mean ± SD with at least three independent experiments.

5.4 RESULTS

5.4.1 Antiproliferative and cytotoxic effects of long chain fatty acid esters of Q3G on HepG2 cells

The potential effects of long chain fatty acid esters of Q3G on cell proliferation were investigated by the MTS assay which measures the metabolically live cells based on their mitochondrial dehydrogenase activity (van de Loosdrecht et al., 1994). Incubation of HepG2 cells with 100 μM of long chain fatty acid esters of Q3G for 6 h and 24 h resulted in significant decrease (approximately 85% to 90%) in cell proliferation ($P < 0.05$) in comparison to the precursor compounds quercetin and Q3G, free fatty acids and prescribed drugs Sorafenib and Cisplatin (Figure 5.1). Within 6 h of incubation, all long chain fatty acid esters of Q3G except stearic acid ester of Q3G treated cells showed a drastic reduction in cell viability as compared to the respective controls (Figure 5.1.A). After 24 h of incubation, viability of HepG2 cells further decreased significantly in Q3G fatty acid esters treated cells as compared to the precursor compounds and control drugs treated cells (Figure 5.1.B). Also, at low concentrations (mainly, 10 and 50 μM) of long chain fatty acid esters of Q3G, longer incubations of 48, 72 and 96 h were necessary to obtain a significant reduction in cell viability (data not included).

Further, to evaluate the potential cytotoxic effects of long chain fatty acid esters of Q3G on HepG2 cells, LDH assay was performed as described in materials and methods section. This method estimates the cell viability by measuring the LDH enzyme release

from the cell membrane upon loss of membrane integrity. Incubation of HepG2 cells with 100 μM of long chain fatty acid esters of Q3G for 6 h and 24 h resulted in significant increase in LDH enzyme release ($P < 0.05$) in comparison to the precursor compounds quercetin and Q3G and control drugs, sorafenib and cisplatin (Figure 5.3). The LDH release data was consistent with the MTS data. Within 6 h of incubation, all long chain fatty acid esters of Q3G except steric acid ester of Q3G treated cells showed a strong increase in LDH release as compared to the respective controls (Figure 5.3.A). On the other hand after 24 h of incubation, LDH release was seen relatively lower than 6 h incubation data (Figure 5.3.B). This may be explained through the fact that LDH has a half-life of 8-9 hrs (Lisa et al., 2004 and Riss et al., 2004) and since the compounds cause a significant amount (over 85%) of cell death within 6 h, by 24 hrs LDH gets degraded in the medium and hence 24 h incubation readings were seen comparatively lesser than 6 h readings. Nevertheless, the LDH release which signifies the cytotoxic action of the long chain fatty acid esters of Q3G upon cell damage was significantly greater than the precursor compounds and control cancer drug, cisplatin ($P <0.05$) (Figure 5.3.B). Interestingly, oleic acid ester of Q3G emerged as the most effective compound showing the greatest antiproliferative action (over 95%), while all other long chain fatty acid esters of Q3G except steric acid ester showed relatively lesser antiproliferative action (~ over 85%).

To see the cytotoxicity effect of the long chain fatty acid esters of Q3G on normal hepatocytes, 100 μM long chain fatty acid esters of Q3G esters were incubated with normal rat hepatocytes cells and MTS assay was performed as described earlier. The results showed a significantly higher viability in long chain fatty acid esters of Q3G

treated normal rat hepatocytes compared to the long chain fatty acid esters of Q3G treated transformed HepG2 cells. Additionally, the long chain fatty acid esters of Q3G showed a significant lower cytotoxicity than control drug Sorafenib (Figure. 5.2).

5.4.2 Long chain fatty acid esters of Q3G cause a drastic change in cellular morphology of HepG2 cells with decreased cell number

HepG2 cells were cultured (as described in materials and methods) in squared chamber slides and incubated with 100 μM of long chain fatty acid esters of Q3G, precursor compounds (quercetin and Q3G) and control cancer drugs (Sorafenib and Cisplatin) for 6 and 24 h. After incubation, cells were observed and photographed under phase contrast microscope. Observation under the phase contrast microscope after 6 h showed a dramatic decrease in cell number of the cells treated with long chain fatty acid esters of Q3G as compared to the control cells with no treatment (Figure 5.4). Interestingly, it revealed that the long chain fatty acid esters of Q3G treated cells were undergoing an excessive morphology change in comparison to the precursor compounds, control drugs and control with no treatment. This was accompanied by a very low cell number. Cell membrane shrinkage, blebbing, clustering and lysis were clearly visible (Figure 5.4). Similarly, severe changes in cell number and morphology were observed after 24 h of treatment as well (Figure 5.4). However, consistent with above results, stearic acid ester of Q3G failed to show any major effect on cell number and morphology.

5.4.3 Long chain fatty acid esters of Q3G induce apoptosis in HepG2 cells via activation of Caspase-3 family

Intense morphological changes were observed under the phase contrast microscope. Further assessments were carried out to examine whether the cells were getting growth

arrested upon incubation with the long chain fatty acid esters of Q3G driving the cells to apoptosis. DNA fragmentation assay was performed as explained in materials and methods. After incubation of cells with 100 μM long chain fatty acid esters of Q3G, precursor compounds and control drugs for 24 h and 48 h, DNA was extracted and resolved on 1.2 % agarose gel. As expected, the long chain fatty acid esters of Q3G treated cells showed consistent DNA damage and fragmentation within 24 h as seen on the gel image. The intensity of the damage and fragmentation increased at 48 h incubation (Figure 5.5). This result revealed one of the basic hallmarks of apoptosis by the long chain fatty acid esters of Q3G.

DNA fragmentation data further prompted me to examine the mechanism of apoptosis induction caused by the long chain fatty acid esters of Q3G. As caspase-3 family of proteases is the principle effector caspases leading cells to apoptosis (Riedl et al., 2004), I examined their activity and level of expression upon long chain fatty acid esters of Q3G treatment as explained in materials and methods. After 24 h of treatment of HepG2 cells with 100 μM of long chain fatty acid esters of Q3G, a significant increase in the caspase-3 family was observed as compared to the control untreated cells (Figure 5.6). This relative change in caspase-3 activity data was consistent with the MTS and LDH release assays. Consistent with above results oleic ester of Q3G showed greatest caspase activity among the other esters of Q3G.

Further to analyse whether the long chain fatty acid esters of Q3G induced inhibitory effects on cell growth and the morphological changes observed were due to apoptosis or necrosis (or both), the cells were examined by annexin V FITC and PI staining after treatment for 24 h with 100 μM long chain fatty acid esters of Q3G via fluorescent

microscopy. The experiment was performed as described in materials and methods. Figure 5.7 shows representative images of long chain fatty acid esters of Q3G treated and untreated HepG2 cells. The untreated cells did not show any staining, suggesting that these cells did not undergo significant apoptosis or necrosis. Long chain fatty acid esters of Q3G treated cells displayed some staining for both annexin V and PI (yellow) which signifies late apoptotic cells and for PI only (red) which signifies necrotic cells and significant staining for annexin V only (green) which signifies apoptotic cells except for stearic acid ester of Q3G which did not show apparent staining. This data suggests that the inhibitory effects of the long chain fatty acid esters of Q3G may be due to the apoptosis leading to late apoptosis.

5.4.4 Long chain fatty acid esters of Q3G cause alterations in HepG2 cell cycle progression via induction of S-phase arrest

To assess whether long chain fatty acid esters of Q3G esters induced cell growth inhibition is mediated through changes in cell cycle progression, HepG2 cells were incubated with 100 μM of long chain fatty acid esters of Q3G and control drug Sorafenib for 24 h. The effect of long chain fatty acid esters of Q3G on cell cycle phase distribution was determined by flowcytometery analysis as explained in materials and methods. A representative histogram is shown in Figure 5.8.A, and the data are summarized in Figure 5.8.B. Consistent with their growth inhibitory effects, long chain fatty acid esters of Q3G increased the population in the S phase with a corresponding decrease of cells in the G1 phase after 24 h of treatment, implying that the DNA synthesis was retarded. In addition to increasing the population of cells in the S phase from 11.1% (control) to 35.4% (Oleic acid ester of Q3G), long chain fatty acid esters of Q3G also appeared to increase the cell

population in the G2-M phase, implying the cell mitosis stage was inhibited for the cells that managed to move from S phase to G2-M phase. These data support the potent inhibitory effect of long chain fatty acid esters of Q3G on DNA synthesis and possibly in parts, cell mitosis.

5.4.5 Long chain fatty acid esters of Q3G behave as potent Topoisomerase II inhibitor

DNA topoisomerase II is essential for cell division and proliferation as it marks the completion of mitosis. As mentioned earlier, quercetin has been shown to inhibit DNA topoisomerase II activity *in vitro*. To further examine whether the long chain fatty acid esters of Q3G were also able to catalytically inhibit DNA topoisomerase II activity which could inturn be a reason to activate caspases and drive cells to apoptosis. DNA topoisomerase catalytic activity was monitored via gel electrophoresis as explained in materials and methods. A representative gel image of the relaxation assay for determining the catalytic inhibition on human DNA topoisomerase II of 100 μM of long chain fatty acid esters of Q3G with controls is presented in Figure 5.9. The figure presents the pHot1 DNA linear marker (lane 1) which is diagnostic for DNA topoisomerase inhibitor (poison), relaxed pHot1 DNA (lane 2), positive control drug VP16 showing linear band (lane 3), negative control DMSO showing relaxed bands (lane 4), long chain fatty acid esters of Q3G with topo II (lane 5 to 10), quercetin and Q3G + topo II (lane 11 and 12 respectively), supercoiled substrate pHot1 DNA (lane 13) and Sorafenib + topo II (lane 14). The assay was performed to test whether long chain fatty acid esters of Q3G act as a poison and increase the DNA cleavage via topoisomerase II. As shown in Fig 9, the esters did not stabilize topoisomerase II cleavage complexes and failed to exhibit the

formation of single linear DNA, and increased the supercoiled DNA intensity, whereas, positive control drug VP 16 increased the formation of linear DNA. This result shows that long chain fatty acid esters of Q3G do not act as human topoisomerase II poison but as a catalytic inhibitor by inhibiting the DNA relaxation activity. The intensity of the supercoiled bands in comparison to the negative control (DMSO) is very high clearly suggesting that due to DNA topoisomerase II inhibition, the supercoiled DNA did not get relaxed (Figure 5.9).

5.5 DISCUSSION

Flavonoids are polyphenolic plant secondary metabolites which have been shown to have strong antioxidant, antiproliferative and other biological activities beneficial to human health by both epidemiological and *in vitro* studies (Gibelleni et al., 2011). Over the years these properties of plant secondary metabolites have gained a lot of interest in scientific research to use them as drug leads. Antiproliferative property of flavonoids has prompted researchers throughout the world to develop alternative natural medicines to replace synthetic chemotherapeutic agents which apparently are known to have many side effects. Quercetin and its glucosides have been studied extensively for their anticancerous and toxicological properties in various transformed cell lines making them promising candidates for cancer therapeutics (Gibelleni et al., 2011). However, poor bioavailability limits their biological effects in vivo (Passamonti et al., 2009), due to low membrane permeability and hence, limit their applications as therapeutic agents (Kitagawa et al., 2009). One of the effective approaches to enhance the membrane permeability is to increase the lipophilicity of these compounds by acylation (Salem et al., 2011). Q3G was acylated with long chain fatty acid esters as previously described in Ziaullah et al.,

(2012). In the current study, we investigated the antiproliferative and cytotoxic properties of these long chain fatty acid esters of Q3G to check if the acylation enhances its biological action and finally elucidated the mechanism of action of these compounds.

Over the years, researches on the dietary plant compounds have been focused essentially on their ability to influence cell cycle in cancer cells and driving cells to apoptosis. This makes cell cycle arrest and apoptosis induction a significant preventive approach. In this study, we show that the novel synthesized long chain fatty acid esters of Q3G can inhibit cell proliferation (HepG2 cells) through induction of apoptosis by the intensive activation of caspase-3 family of caspases followed by necrosis, through cell cycle changes, and possibly through inhibition of DNA topoisomerase II activity. Interestingly, as hypothesized, long chain fatty acid esters of Q3G exhibited much stronger anticancer property than precursor compounds (quercetin, Q3G and free fatty acids) and prescribed chemotherapy drugs (Sorafenib and Cisplatin).

Using HepG2 cell lines as a model, I showed that 100 μM of long chain fatty acid esters of Q3G significantly inhibited the cell proliferation in HepG2 cells within 6 h of incubation in comparison to the precursor compounds (quercetin and Q3G), fatty acids and chemotherapy drugs (Sorafenib and Cisplatin) of similar concentration. The cell proliferation was shown to further reduce by 24 h of incubation (Figure 5.1A and 5.1B). Also, for concentrations below 100 μM of long chain fatty acid esters of Q3G (mainly, 10 and 50 μM), 48 and 72 incubations were necessary to obtain a significant reduction in cell viability (Appendix A and B). Based on the effects on cell viability and morphology my data suggested that the test compounds caused cytotoxicty to the HepG2 cells resulting in the cell membrane shrinkage and eventually breakage (Figure 5.4). This

result was further confirmed by the membrane integrity test via LDH release assay which showed that there was clear membrane breakage when compared with untreated control cells (Figure 5.3A and 5.3B). Interestingly, the strong inhibition of cell proliferation by fatty acid esters of Q3G as compared to the precursor compounds alone and currently prescribed chemotherapy drugs (Sorafenib and Cisplatin) is noteworthy (Figure 5.1 and 5.3). Interestingly, oleic acid ester of Q3G emerged as a derivative with the strongest antiproliferative action whereas, stearic acid ester of Q3G showed the least growth inhibitory action among all the other esters of Q3G. The experimental results support the assumption that there is a structural-activity relationship due to the fact that stearic acid is the only saturated fatty acid among the six fatty acids tested. Once the stearic acid is attached to the Q3G skeleton, the change in the orientation may not be favourable for membrane interaction thereby, getting less absorbed by cells and inturn showing less activity. Overall, this data suggested the potential of long chain fatty acid esters of Q3G as strong antiproliferative agents. Interestingly, it is noteworthy to point out that the precursor compounds (quercetin and Q3G) that have been shown to display strong antiproliferative action by previous studies are infact concentration and time dependent. The peak growth inhibitory action displayed by the precursor compounds has usually been shown to range from 48-72 h (Shan et al., 2009 and You et al., 2010, chapter-4). In this study I showed that the long chain fatty acid esters of Q3G display the growth inhibitory effect on HepG2 cells within 6 h of treatment (Figure 5.1 and 5.3). This data clearly suggests, that acylation of Q3G with long chain fatty acid esters enhances its antiproliferative activity *in vitro*.

The long chain fatty acid esters of Q3G also showed significantly lower cytotoxic effect on normal hepatocytes as compared to the transformed HepG2 (Figure 5.2) suggesting their specific action on cancer cells. Additionally, cell membrane breakage suggested symptoms of late apoptosis and necrosis where the membrane is intact in apoptosis and with delayed apoptosis it leads to necrosis thereby rupturing the cell membrane. However, distinction between the apoptosis and necrosis is very challenging and could be difficult to confirm as other mechanisms of various death routes and symptoms of cell death may intersect (Broker et al., 2005). To distinguish between apoptosis and necrosis, cells were analyzed after staining with annexin V and PI through fluorescence microscopy. After 24 h of treatment of HepG2 cells with long chain fatty acid esters of Q3G showed that, some treated cells were positive for PI and some for both annexin V and PI (Fig. 5.7), suggesting that the cells at this time present necrotic and also apoptotic features presumably late apoptosis. This finding may be explained if treatment with the fatty acid esters of Q3G within 6 h triggers the activation of apoptotic proteins for phagocytosis and gradually with the overload of dying cells, early apoptotic cells progress to late apoptosis or secondary necrosis, where the membrane becomes more permeable weakening phagocytosis (Poon et al., 2010). Alternatively, this could also be explained as apoptosis, the programmed cell death taking over necrosis caused by the initial response of HepG2 cells to incubation of long chain fatty acid esters of Q3G.

To further analyse and confirm the apoptotic effect of the Q3G esters, I examined the caspase-3 activity which is a key enzyme in apoptotic signalling (Riedl et al., 2004). Consistent with the fluorescent data, an intensive activation of caspase-3 was observed in long chain fatty acid esters of Q3G treated cells in comparison to the uninduced control

and precursor compounds after 24 h of incubation (Figure 5.6). This data was also consistent with the MTS and LDH assays data. Additionally, this data was supported by the DNA fragmentation analysis. After 24 and 48 h of treatment of HepG2 cells with long chain fatty acid esters of Q3G, the fragmented DNA like pattern was observed (Figure 5.5) which is a basic hallmark of apoptosis (Saraste et al., 2000). These data suggested the apoptotic action of the novel long chain fatty acid esters of Q3G on HepG2 cells.

Cell cycle analysis showed that long chain fatty acid esters of Q3G blocked the HepG2 cells in the S phase (Fig. 5.8), this effect being consistent with the potent inhibition of DNA synthesis. Previous studies have shown that quercetin can induce cell cycle arrest in the S phase (Cheong et al., 2004) as well as in the G1/S phase (Hosokawa et al., 1990) or G2/M phase (Choi et al., 2001) depending on the cancer cell type. Apparently, a considerable increase in cell population in G2/M phase was also seen in the long chain fatty acid esters of Q3G treated HepG2 cells (Fig 5.8). This suggests that the cells that were infact able to cross S phase got arrested in G2/M phase. This data is thus in accordance with the previous studies that the cell cycle alterations are cell type and treatment dependent.

DNA topoisomerase II is essential for the basic cellular function and proliferation including mitosis and has been shown to be expressed in much higher levels in proliferating tumor cells (Coss et al., 2009) which makes it a promising target for chemotherapy drugs. Additionally, apoptosis has been shown to be the most efficient death-pathway in tumor cells after topoisomerase II inhibition (El-Awady et al., 2008). This prompted me to think that if long chain fatty acid esters of Q3G were causing topoisomerase II inhibition that was driving cells to apoptosis. As expected, data showed

that long chain fatty acid esters of Q3G were infact behaving as strong topoisomerase inhibitors as compared to the negative control (Figure 5.9). It was also interesting to note that stearic acid ester of Q3G which failed to show any significant response in any of the assays showed a significant reduction in topoisomerase activity. This shows that the compound is an active and potent topoisomerase II inhibitor and since the drug screening topoisomerase II assay was a cell free assay, stearic acid ester of Q3G showed its direct inhibitory action without the interaction with the cells or cellular membranes. Nonetheless, a reduction in DNA topoisomerase II activity observed with a reduced ability to relax the supercoiled DNA, indicates that the reduced cell proliferation was infact may be in parts a result of topoisomerase II inhibition which generated double strand breaks in DNA. DNA topoisomerase II-mediated DNA damage may have activated the cell cycle checkpoint causing growth arrest which inturn triggered and activated apoptotic signalling through caspases and hence caused cell death. With the current data generated this seems to be the most plausible antiproliferative mechanism of action of long chain fatty acid esters of Q3G which is consistent with one of the reviews published by Niida et al., (2006). It should be pointed out that these effects may or may not be specific for the HepG2 cells. However, additional studies need to be performed in order to understand the specific mechanism of action.

In conclusion, this study provides preliminary data for these novel compounds' high antiproliferative action on cancer cells (HepG2) and very low cytotoxic action on normal cells (rat hepatocytes) as compared to the precursor compounds and currently used chemotherapy drugs at same concentration. Based on this data, additional studies using other established transformed cell lines should be performed for determining

bioavailability, confirming anticancer and tumor suppression property *in vivo* using animal model systems.

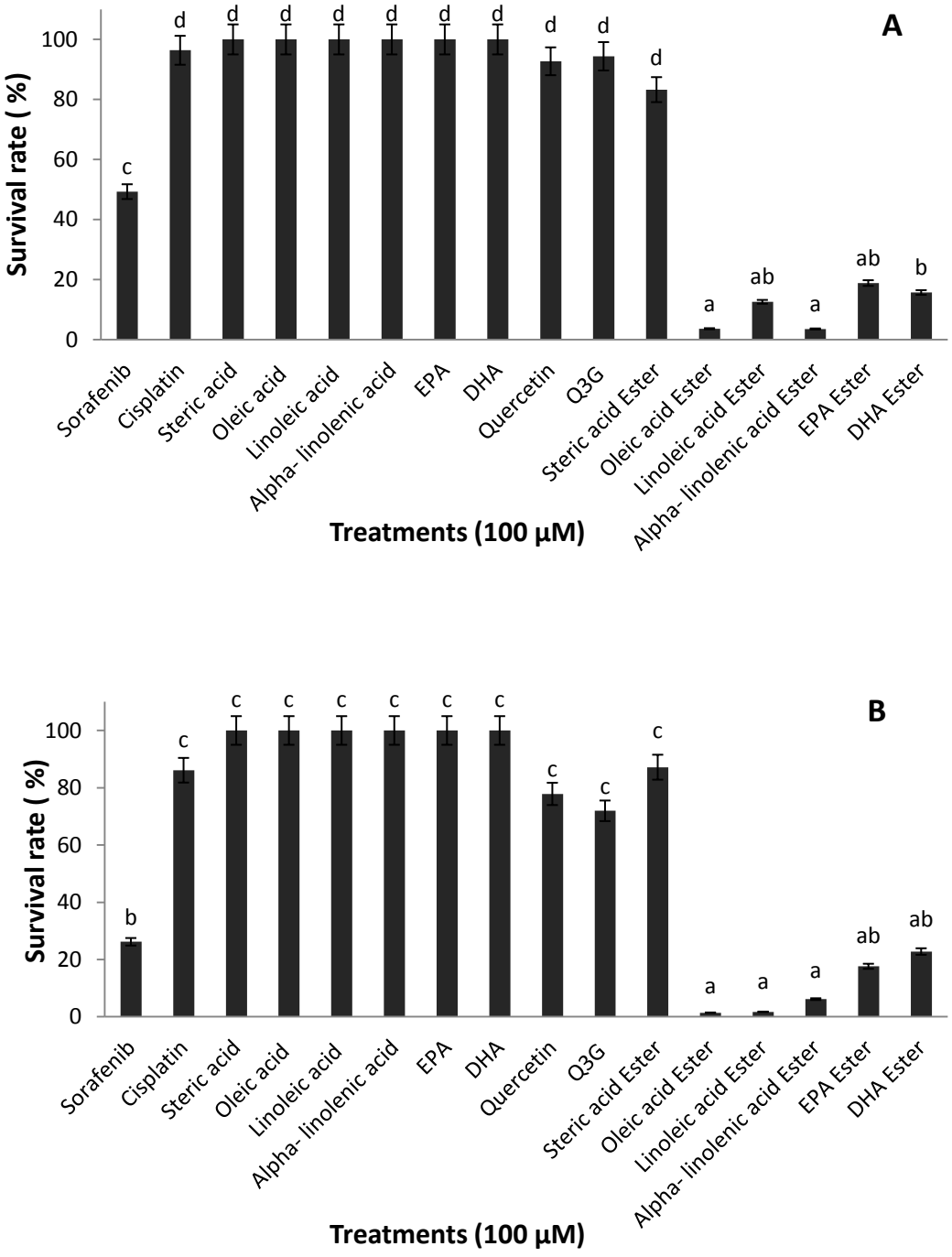


Figure 5.1. Antiproliferative effects of long chain fatty acid esters on HepG2 cells

The figure describes the percentage of viable HepG2 cells after treatment with long chain fatty acid esters of Q3G. Cells (2 x 104 cells per well; 96-well plate) were treated with 100 µM of long chain fatty acid esters of Q3G, precursor compounds (quercetin;

Q3G; free fatty acids) and chemotherapy drugs (Sorafenib and Cisplatin) for 6 (A) and 24 h (B). After treatment viable cell percentage was determined by MTS assay as described in Materials and Methods. All long chain fatty acid esters of Q3G except stearic acid ester show over 85% inhibition of proliferation as seen by the viability data of 6 h and 24 h. The antiproliferative activity of the esters (85% to 90%) is significantly greater than the precursor compounds, cancer drugs and fatty acids alone . Mean separation between groups was conducted using Tukey's test (n=6). Values with different letters differ significantly ($P<0.05$).

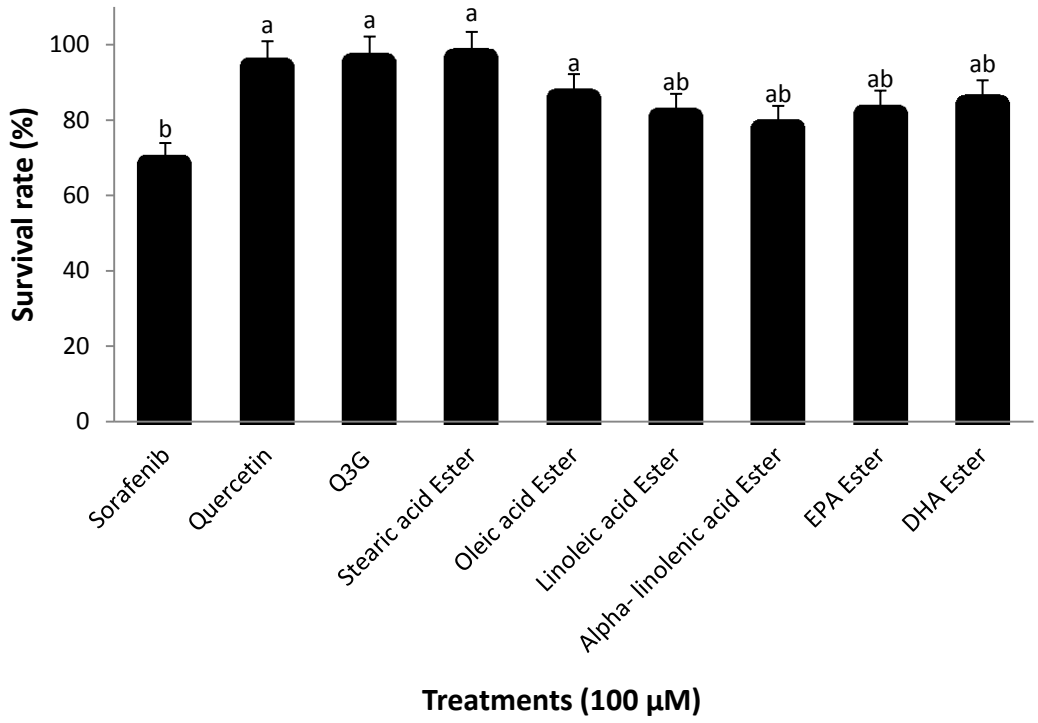


Figure 5.2. Effect of long chain fatty acid esters of Q3G on viability of normal rat hepatocytes.

The figure describes the percentage of viable rat hepatocytes cells after treatment with long chain fatty acid esters of Q3G. Cells (1 x 10^4 cells per well; 96-well plate) were treated with 100 µM of long chain fatty acid esters of Q3G, precursor compounds (quercetin and Q3G) and chemotherapy drug (Sorafenib) for 24 h. After treatment viable cell percentage was determined by MTS assay as described in materials and methods. Results are expressed relative to control (24-h incubation without test compounds). Mean separation between groups was conducted using Tukey's test (n=6). Values with different letters differ significantly ($P<0.05$).

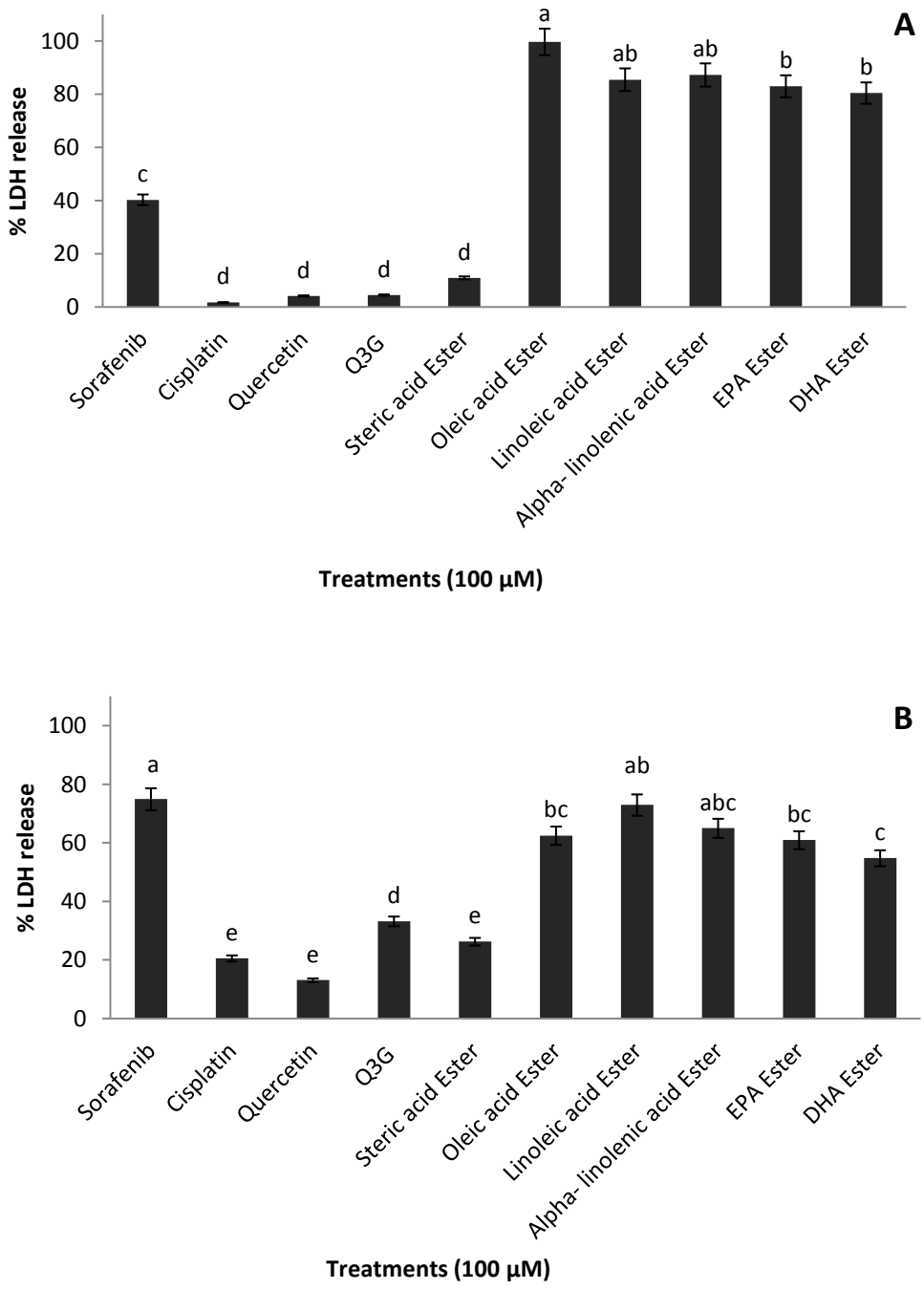


Figure 5.3. Effect of long chain fatty acid esters of Q3G on cytotoxicity in HepG2 cells.

The figure describes the percentage of LDH release from HepG2 cells after treatment with long chain fatty acid esters of Q3G. Cells (5 x 10^3 cells per well; 96-well plate) were treated with 100 μM of long chain fatty acid esters of Q3G, precursor compounds (quercetin and Q3G) and chemotherapy drugs (Sorafenib and Cisplatin) for 6 and 24 h. After treatment the cells were centrifuged and the percentage of LDH release from the cells was determined by LDH assay as described in Materials and methods. All long chain fatty acid esters of Q3G except stearic acid ester show over 80% cell death via LDH release within six h of treatment (Figure 3A). Considering LDH half life (8-9 h), the LDH release after 24 h is comparatively less (Figure 3B). The LDH release which signifies the cytotoxic action of the esters is significantly greater than the precursor compounds and control cancer drug, Cisplatin. Mean separation between groups was conducted using Tukey's test (n=6). Values with different letters differ significantly ($P<0.05$).

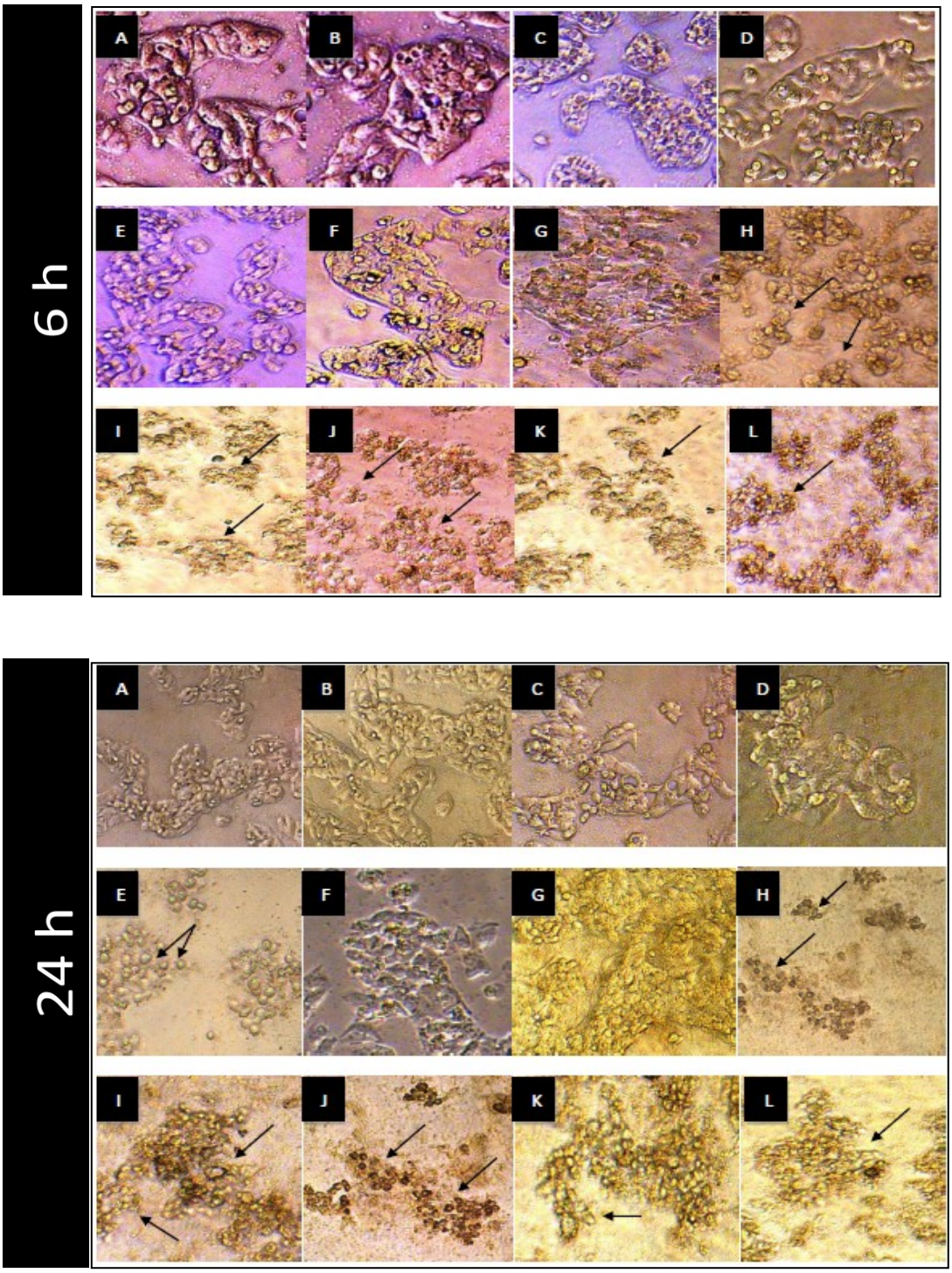


Figure 5.4. Morphological changes observed in HepG2 cells after 6 h and 24 h of treatment with long chain fatty acid esters of Q3G

Cells (1 x 10^4 cells per well; 6-well plate) were treated with 100 μM of long chain fatty acid esters of Q3G, precursor compounds (quercetin and Q3G) and chemotherapy drugs (Sorafenib and Cisplatin) for 6 and 24 h. After incubation, cells were observed and photographed by Nikon eclipse TS 100 phase contrast microscope equipped with Infinity 1 camera at 10x magnification. The arrows in the pictures show the change in the morphology of cells upon treatment with Q3G esters. As compared to control, the long chain fatty acid esters of Q3G treated HepG2 cells show a great decrease in cell number and complete loss of morphology. A) No treatment control B) DMSO control (0.1%), C) quercetin, D) Q3G, E) Sorafenib, F) Cisplatin, G) stearic acid ester of Q3G, H) oleic acid ester of Q3G, I) linoleic acid ester of Q3G, J) alpha-linolenic acid ester of Q3G, K) EPA ester of Q3G and L) DHA ester of Q3G.

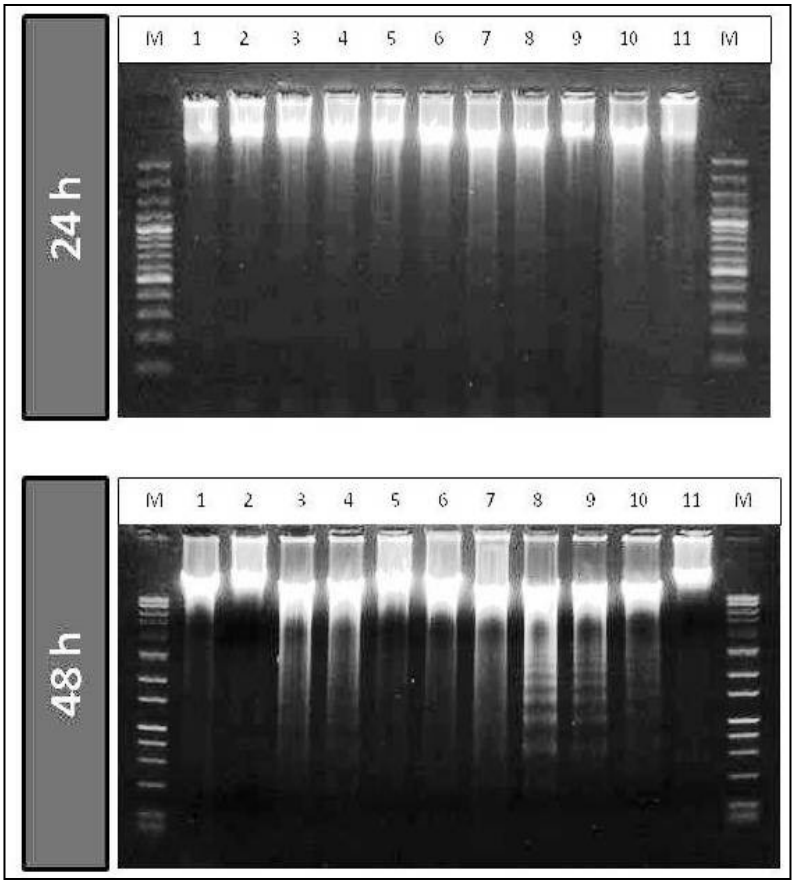


Figure 5.5 DNA fragmentation in HepG2 cells after 24 and 48 hrs of treatment with long chain fatty acid esters of Q3G

HepG2 Cells (5 x 10^5 cells; 12-well plate) were treated with 100 µM of the long chain fatty acid esters of Q3G and control cancer drugs for 24 and 48 h. Cells were collected, lysed and DNA was extracted and run on agarose gel containing GelRed™ DNA staining solution for fragmentation analysis as explained in materials and methods. Lane 1, Cisplatin; lane 2, stearic acid ester; lane 3, oleic acid ester; lane 4, linoleic acid ester; Lane 5, alpha-linolenic acid ester; lane 6, EPA ester; lane 7, DHA ester; lane 8, Q3G; lane 9, quercetin; lane 10, Sorafenib; lane 11, control (no treatment). After 24 and 48 h of treatment all long chain fatty acid esters of Q3G except stearic acid ester treatment showed substantial amount of apoptosis as seen by the fragmented DNA pattern.

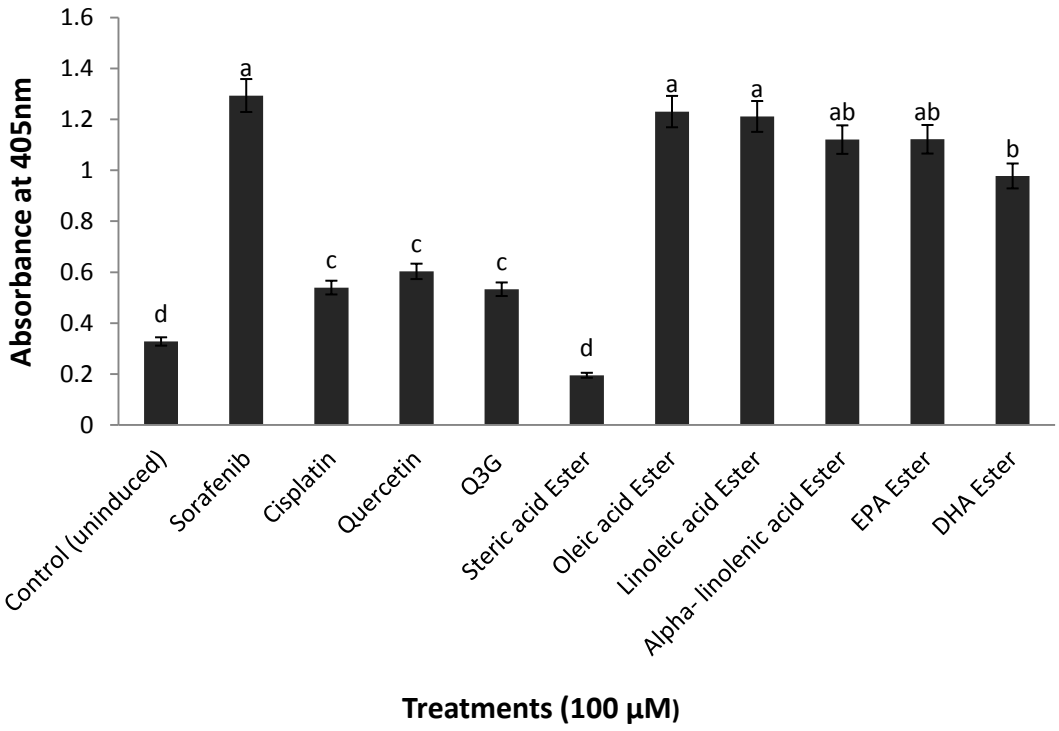


Figure 5.6. Effect of long chain fatty acid esters of Q3G on caspase-3 activation.

HepG2 cells (1 x 10^6 cells/ well) were incubated with long chain fatty acid esters of Q3G in a six well plate for 24 h. Cells were lysed and protein was quantified. After quantification, 250 µg of protein was used for detection of caspase-3 activity. Absorbance was taken at 405 nm in BMG microplate reader. Mean separation between groups was conducted using Tukey's test (n=3). Values with different letters differ significantly ($P<0.05$).

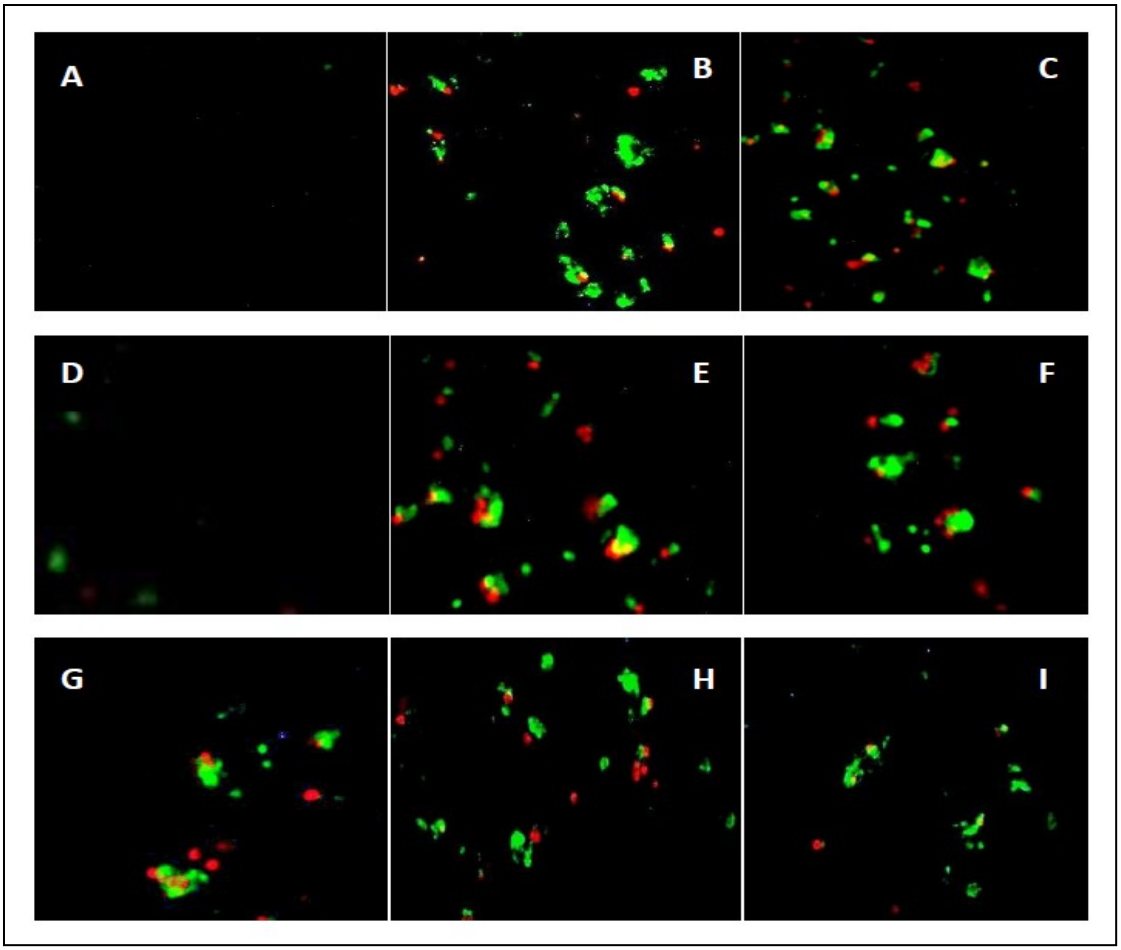


Figure 5.7. Apoptosis detection through fluorescence microscopy

HepG2 cells were treated for 24 hrs with 100 μM long chain fatty acid esters of Q3G (D-I) and 100 μM Sorafenib (C) in complete medium. After staining, necrotic and apoptotic cells were detected by fluorescence microscopy (20x). A) control (no treatment), B) positive control (Staurosporine 2μM) , C) Sorafenib, D) stearic acid ester, E) oleic acid ester, F) linoleic acid ester, G) alpha-linolenic acid ester, H) EPA ester, I) DHA ester. The figure shows annexin V and PI staining in HepG2 cells.

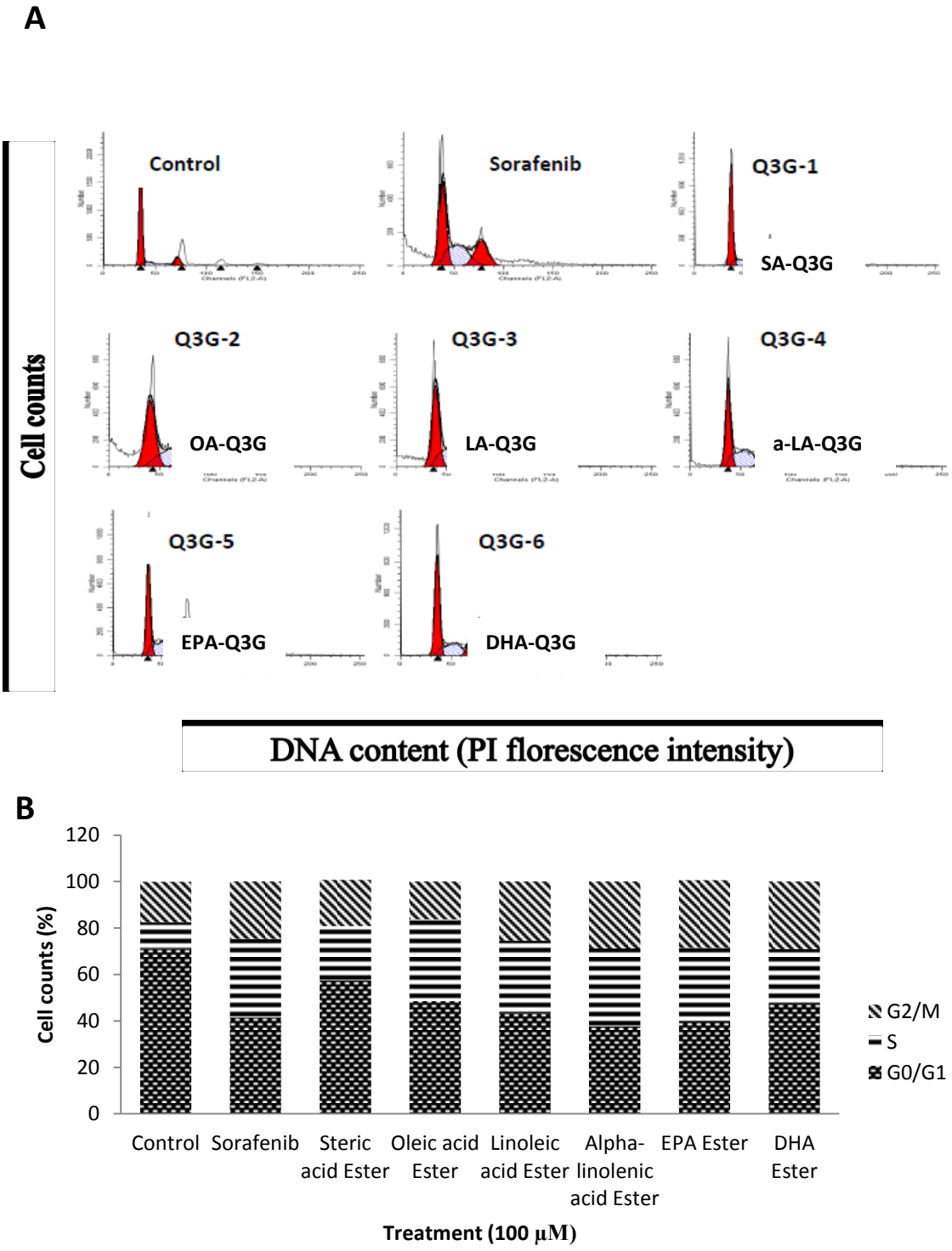


Figure 5.8. Effect of long chain fatty acid esters of Q3G on cell cycle distribution of HepG2 cells.

After treatment with 100 μM of long chain fatty acid esters of Q3G for 24 hrs in complete medium, cells were fixed and stained with propidium iodide, and the cell cycle distribution was analyzed by flowcytometry as explained in materials and methods. (A) DNA histograms. (B) Plot of summarized values from DNA histograms.

Abbreviations: Stearic acid ester of Q3G (SA-Q3G), Oleic acid ester of Q3G (OA-Q3G), Linoiec acid ester (LA-Q3G) of Q3G, alpha-linoleniec acid ester of Q3G (a-LA-Q3G), EPA ester of Q3G (EPA-Q3G) and DHA ester of Q3G (DHA-Q3G).

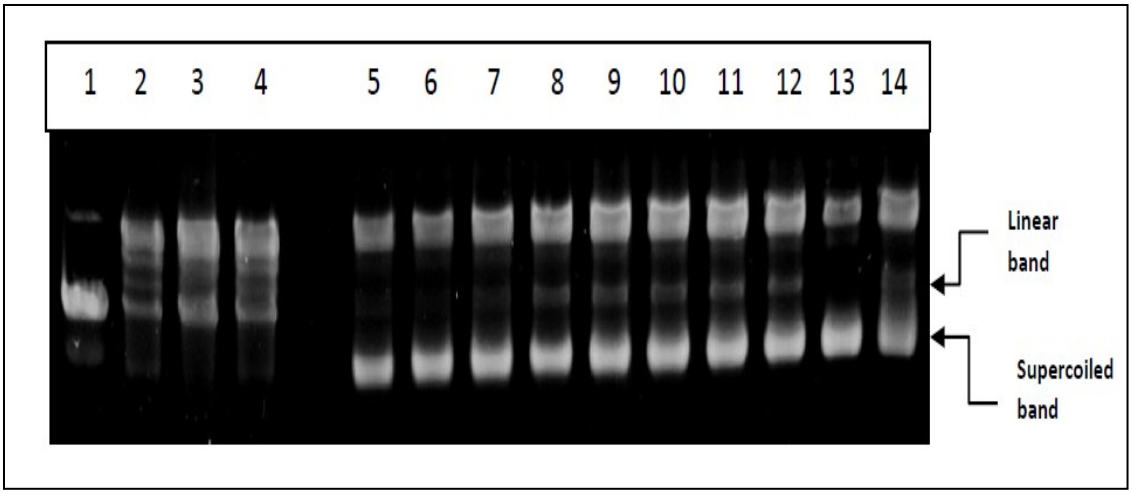


Figure 5.9. Effect of long chain fatty acid esters of Q3G on DNA topoisomerase II activity

Supercoiled circular pHot1 DNA (0.25 µg) was taken in test and control tubes and incubated with 4 units of the DNA topoisomerase II enzyme. The reactions were kept at 37º C for 30 min followed by 1% agarose gel run without DNA stain. The gel was stained with GelRed^{TM} DNA staining solution for 2 h and then destained with TBE buffer for 15 to 20 min. The gel was imaged in Bio Rad Gel doc system. Lane 1, Linear DNA marker; lane 2,DNA + topo II; lane 3, VP-16 + DNA+ topo II; lane 4, DMSO control; lane 5, stearic acid ester + DNA + topoII; lane 6, oleic acid ester; lane 7, linoleic acid ester + DNA + topo II; lane 8, alpha-linolenic acid ester + DNA + topoII; lane 9, EPA ester + DNA + topoII; lane 10, DHA ester + DNA + topoII; lane 11, Q3G + DNA + topo II; lane 12, quercetin + DNA + topo II; lane 13, Control (no topo); lane 14- Sorafenib + DNA + topo II. All long chain fatty acid esters of Q3G show DNA topoisomerase II inhibition as there is clear depiction of the linear band. Also, the intensities of the supercoiled bands in comparison to the negative control (DMSO) are shown very high clearly suggesting high DNA topoisomerase II inhibition.

5.8 REFERENCES

Agullo G, Gamet-Payrastre L, Manenti S, Viala C, Remesy C, Chap H and Payrastre B: Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. *Biochem. Pharmacol* **53**, 1649-1657, 1997.

Arts IC, Hollman PC, Bueno De Mesquita HB, Feskens EJ and Kromhout D: Dietary catechins and epithelial cancer incidence: the Zutphen elderly study. *Int J Cancer* **92**, 298-302, 2001.

Bröker LE, Kruyt FAE and Giaccone G: Cell death independent of caspases: a review. *Clin Cancer Res* **11**, 3155–3162, 2005.

Cheong E, Ivory K, Doleman J, Parker M L and Rhodes M: Synthetic and naturally occurring COX-2 inhibitors suppress proliferation in a human oesophageal adenocarcinoma cell line (OE33) by inducing apoptosis and cell cycle arrest. *Carcinogenesis* **25**, 1945–1952, 2004.

Choi J-A, Kim JY, Lee JY, Kang CM and Kwon HJ : Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. *Int J Oncol* **19**, 837–844, 2001.

Coss A, Tosetto M, Fox EJ, Sapetto-Rebow B, Gorman S, Kennedy BN, et al: Increased topoisomerase IIα expression in colorectal cancer is associated with advanced disease and chemotherapeutic resistance via inhibition of apoptosis . *Cancer Letters* **276**, 228-238, 2009.

El-Awady A, Ali MM, Saleh EM and Ghaleb FM: Apoptosis is the most efficient death-pathway in tumor cells after topoisomerase II inhibition. *Saudi Med J* **29**, 558-64, 2008.
Gibellini L, Pinti, M and Nasi M: Quercetin and Cancer ChemopreventionL: *Evid Based Complement Alternat Med* **2011,** 591356, 2011.

Granado-Serrano AB, Martín MA, Bravo L, Goya L, Ramos S: Quercetin Induces Apoptosis via Caspase Activation, Regulation of Bcl-2, and Inhibition of PI-3-Kinase/Akt and ERK Pathways in a Human Hepatoma Cell Line (HepG2). *J. Nutr* **136**, 2715–2721, 2006.

Hardman WE: Omega-3 fatty acids to augment cancer therapy. *J Nutr* **132**, 3508–12, 2002.
Hertog M G, Feskens EJ, Hollman PC, Katan M B and Kromhout D: Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. *Lancet* **342**, 1007-11, 1993.

Hosokawa N, Hosokawa Y, Sakai T, Yoshida M and Marui N: Inhibitory effect of quercetin on the synthesis of a possibly cell-cycle-related 17-kDa protein, in human colon cancer cells. *Int J Cancer* **45**, 1119–1124, 1990.

Karawajczyk A, Drgan, V, Medic N, Oboh G, Passamonti S and Novic M: Properties of flavonoids influencing the binding to bilitranslocase investigated by neural network modelling. *Biochem. Pharmacol* **73**, 308-20. 2007.

Kitagawa S, Tanaka Y, Tanaka M, Endo K and Yoshii A: Enhanced skin delivery of quercetin by microemulsion. *JPP* **61**, 855–860, 2009.

Knekt P, Kumpulainen J, Järvinen R, Rissanen H, Heliövaara M, Reunanen A, et al: Flavonoid intake and risk of chronic diseases. *Am J Clin Nutr* **76**, 560-8, 2002.

Lanoue L, Green K, Kwik-Uribe C and Keen, CL: Dietary factors and the risk for acute infant leukemia: evaluating the effects of cocoa-derived flavanols on DNA topoisomerase activity. *Experi Biol and Medi* **235**, 77-89, 2010.

Le Marchand L, Murphy SP, Hankin JH, Wilkens LR and Kolonel LN: Intake of flavonoids and lung cancer. *J Natl Cancer Inst* **92**, 154-60, 2000.

Lin YT, Hsiu SL, Hou YC, Chen HY and Lee CP: Degradation of Flavonoid Aglycones by Rabbit, Rat and Human Fecal Flora. *Biol. Pharm. Bull* **26**, 747–751, 2003.

Lisa KM: Handbook of Assay Development in Drug discovery. **Series 5**, Pg. 395, 2004.
Mainou-Fowler T, Proctor SJ and Dickinson AM: Gamma-linolenic acid induces apoptosis in B-chronic lymphocytic leukaemia cells in vitro. *Leuk Lymph* **40**, 393–403, 2001.

Nguyen T, Tran E, Nguyen T, Do P, Huynh T and Huynh H: The role of activated MEK-ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells. *Carcinogenesis* **25**, 647–59, 2004.

Niida H and Nakanishi M: DNA damage checkpoints in mammals. *Mutagenesis*. **21**, 3-9, 2006.

Passamonti S, Terdoslavich M, Franca R, Vanzo A, Tramer F, Braidot E, et al: Bioavailability of flavonoids: a review of their membrane transport and the function of bilitranslocase in animal and plant organisms. *Curr. Drug. Metabol* **10**,369–394, 2009.

Patra N, De U, Kang JA, Kim JM, Ahn MY, Lee J: A novel epoxypropoxy flavonoid derivative and topoisomerase II inhibitor, MHY336, induces apoptosis in prostate cancer cells. *Eur J Pharmacol* **658**, 98-107, 2011.

Perez RM: Antiviral Activity of Compounds Isolated From Plants. *Pharma Biol* **41**, 107–57, 2003.

Poon IKH, Hulett MD and Parish CR: Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell Death and Differen* **17**, 381–397, 2010.

Rauha J, Remes S, Heinonen M, Hopia A, Kähkönen M, Kujala T, Pihlaja K, Vuorela H: Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Inter Jour of Food Microbiol* **56**, 3–12, 2000.

Razavi SM, Zahri S, Zarrini G, Nazemiyeh H and Mohammadi S: Biological activity of quercetin-3-O-glucoside, a known plant flavonoid. *Bioorg Khim*. **35**, 414-6, 2009.

Riedl SJ and Shi Y: Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol* **5**, 897–907, 2004.

Riss T L and Moravec RA: Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay Drug Dev Technol* **2**, 51-62, 2004.

Salem J, Chevalot I, Schiavo CH, Paris C, Fick M and Humeau C: Biological activities of flavonoids from Nitraria retusa (Forssk.) Asch. and their acylated derivatives. *Food Chem* **124,** 486–494, 2011.

Shan B, Wang M, Li R: Quercetin Inhibit Human SW480 Colon Cancer Growth in Association with Inhibition of Cyclin D1 and Survivin Expression through Wnt/β-Catenin Signaling Pathway. *Cancer Investi* **27**, 604–612, 2009.

Shen SC, Chen YC, Hsu FL and Lee WR: Differential apoptosis-inducing effect of quercetin and its glycosides in human promyeloleukemic HL-60 cells by alternative activation of the caspase 3 cascade. *J Cell Biochem* **89,** 1044–55, 2003.

Singhal R, Yeh YA, Praja N, Olah E, Sledge GJ and Weber G: Quercetin down-regulates signal transduction in human breast carcinoma cells. *Biochem Biophys Res Commun* **208**, 425–31, 1995.

Talib W and Mahasneh A: Antiproliferative Activity of Plant Extracts Used Against Cancer in Traditional Medicine. *Sci Pharm* **78**, 33–45, 2010.

Van de Loosdrecht AA, Beelen RHJ, Ossenkoppele GJ, Broekhoven MG and Langenhuijsen, MM: A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. *J Immunol Methods* **174**, 311–320, 1994.

You HJ, Ahn HJ and Ji GE: Transformation of Rutin to Antiproliferative Quercetin-3-glucoside by Aspergillus niger. *J. Agric. Food Chem* **58**, 10886–10892, 2010.

Ziaullah, Khushwant SB, Sumudu NW and Rupasinghe HP: Biocatalytic synthesis, structural elucidation, antioxidant capacity and tyrosinase inhibition activity of long chain fatty acid acylated derivatives of phloridzin and isoquercitrin. *Bioorg & Medic Chem* **21**, 684-692, 2013.

CHAPTER. 6 MOLECULAR TARGETS OF OLEIC ACID ESTER OF QUERCETIN-3-O-GLUCOSIDE IN HUMAN HEPATOCELLULAR CARCINOMA CELLS

6.1 ABSTRACT

Previous chapters showed that acylation of querectin-3-O-glucoside (Q3G) with oleic acid (18:1 cis-9) exhibited significantly enhanced antiproliferative effect than parent compounds Q3G, oleic acid and chemotherapy drug Sorafenib via induction of cell cycle arrest, DNA topoisomerase II inhibition and apoptosis in HepG2 cells. In this study, possible molecular targets of oleic acid ester of Q3G (OA-Q3G) was investigated in HepG2 cells. Three different approaches, namely, RT Profiler PCR array, sandwich ELISA and ELISA array were utilised for this investigation. Results from the cancer drug target RT-PCR array suggested that antiproliferative effect of OA-Q3G *in vitro* could be related to enhanced mRNA levels of pro-apoptotic genes, down-regulation of cell cycle genes, growth factor-receptor, protein kinases and DNA topoisomerase II (2.7fold). In addition, OA-Q3G treatment considerably down-regulated mRNA expression of certain genes which are found over-expressed in cancer cells such as HIF1A (2.4-fold), NFKB1 (2.2-fold), HDAC6 (25.9-fold) and BIRC5 (11.2-fold). Furthermore, results from ELISA and ELISA array revealed a significant increase in the protein expression of pro-apoptotic genes phospho-p53, phospho-Bad, cleaved-caspase-3, cleaved-parp, ERK1/2, and decrease in protein expression of anti-apoptotic and cell survival genes AKT, PRAS40 and p70S6-kinase in HepG2 cells upon treatment with OA-Q3G. In conclusion, this study provides possible molecular targets of OA-Q3G in HepG2 cells through the attenuated regulation of genes involved in cell cycle, survival and apoptosis.

6.3 INTRODUCTION

Flavonoids present in the diet have been consistently shown to inhibit growth of cancer cells with the potential to act as chemopreventers. Among chemopreventers, flavonoid quercetin has received a great importance due to its ability to act as pro-apoptotic flavonoid and inhibit tumor related processes such as cell proliferation and metastasis (Chirumbolo et al., 2013). Various mechanisms of actions of quercetin have been elucidated with multiple cellular targets. Quercetin acts as anti- as well as pro-oxidant (Gibellini et al., 2010). It has been shown that anti-/pro-oxidant effect of quercetin is concentration dependent (Robaszkiewicz et al., 2007). Apart from acting as a strong antioxidant, quercetin has been shown to regulate several cell cycle proteins such as p21, cyclin-dependent kinases and topoisomerase II (Gibellini et al., 2010). In HepG2 cells, quercetin has been shown to upregulate the expression of cyclin-dependent kinases inhibitors, p21and p27 and induce apoptosis (Mu et al., 2007).

Another significant mechanism of action of quercetin is its direct pro-apoptotic effect. By disrupting the membrane permeability of mitochondria, quercetin directly promotes the activation of caspase-3 family in cancer cells (Naithani et al., 2008). Additionally, quercetin has been shown to regulate expression of various genes in cell survival pathways such as Wnt/beta-catenin and PI3K-Akt/PKB (Gulati et al., 2006; Shan et al., 2009). Further, quercetin has also been shown to trigger apoptosis by activation of AMPKα1 and p38 (Lee et al., 2010). Many studies have examined the effect of quercetin on tumor suppressor gene p-53. It has been shown that quercetin inhibits cell cycle and causes apoptosis in HepG2 cells by the induction and stabilisation of p-53 (Tanigawa et

al., 2008). Thus, previous studies suggest the strong potential of quercetin to regulate intracellular signalling in cancer cells.

The naturally occurring form of quercetin in plant foods, quercetin-3-*O*-glucoside (Q3G) has been shown to exhibit strong antioxidant activity (Razavi et al., 2009). In another study, quercetin-3-β-d-glucoside was shown to exhibit stronger antiproliferative activity than its aglycone, quercetin (You et al., 2010). However, its low bioavailability limits the biological effects in vivo (Passamonti et al., 2009). It has been proposed that structural modification of the flavonoids by introducing hydrophobic groups through acylation could increase the cellular uptake and absorption and therefore, bioefficacy of glycosylated flavonoids (Salem et al., 2011). I previously showed that acylation with long chain fatty acid esters significantly increases the antiproliferative properties of Q3G (Chapter-5). In the same study I proposed that the long chain fatty acid esters of Q3G cause regulation of cell cycle and induce cell cycle arrest, apoptosis and DNA topoisomerase II inhibition. In the present study, one of the most effective long chain fatty acid esters of Q3G, oleic acid ester-Q3G (OA-Q3G) was studied for its molecular mechanism of action. The focus of the present study was to elucidate the molecular mechanism involved in OA-Q3G treated cell death in HepG2 cells. Based on previous studies and our previous results, I utilized three approaches, namely, a mutli-target liver cancer specific drug screening RTPCR-array, multi-target apoptosis sandwich ELISA and a multi-target intracellular signalling ELISA array analysis to elucidate and highlight the prime molecular targets of OA-Q3G in HepG2 cells. Our results suggest that OA-Q3G influenced significant changes in levels (up or down-regulation) of key signaling molecules in pathways controlling cell growth, survival and apoptosis both at mRNA

level and protein-level such as cyclin dependent kinases (CDKs), DNA topoisomerase II, ERK1/2, PRAS40, Akt, Bad, PARP, caspase-3. Furthermore, OA-Q3G induced expression of p53 and p53 dependent pro-apoptotic genes in HepG2 cells.

6.2 MATERIALS AND METHODS

6.2.1 HepG2 cell culture system

HepG2 cells were obtained from American Type Culture Collection (ATCC, 8065) and maintained according to ATCC's instructions. Briefly, the cells were cultured in Eagle's Minimum Essential Growth Medium (EMEM) with 2 mM L-glutamine and 10% Fetal Bovine Serum (FBS) at 37°C and 5% CO_2. T-75 tissue culture flasks with 12-15 ml of the media were used for regular culturing. Cells were counted under Nikon Eclipse TS 100 phase contrast microscope (Mississauga, ON) using haemocytometer and then transferred to fresh flasks.

6.2.2 Cancer-related genes expression profiling

To identify possible molecular targets for antiproliferation action of OA-Q3G, Human Cancer Drug Target RT^2 Profiler™ PCR Array (SABioscience, Frederick, MD) was used. Assay was performed according to manufacturer's instructions. Briefly, HepG2 cells were treated with OA-Q3G for 24 h. Total RNA was isolated using Aurum™ Total RNA Mini Kit (Bio-Rad, Mississauga, ON) according to the manufacturer's protocol. RNA concentration was measured with NanoDrop 1000 Spectrophotometer (Thermo Scientific, Ottawa, ON), RNA quality was evaluated by electrophoresis. cDNA was synthesized from 500 μg total RNA using RT^2 First Strand cDNA Synthesis kit (Qiagen, Toronto, ON). Real-time PCR was performed using CFX96^{TM} Real-Time PCR detection System (Bio-Rad, Mississauga, ON) according to manufacturer's instructions. Obtained

data were analyzed with Excel-based PCR Array Data Analysis Software (SABioscience, Frederick, MD).

6.2.3 Multi-Target Sandwich ELISA analysis

To investigate the effect of OA-Q3G treatment on intracellular apoptotic markers, PathScan® Apoptosis Multi-target Sandwich ELISA kit (Cell Signaling Technology, Danvers, MA) was used. The assay is a solid phase is a solid phase sandwich enzymelinked immunosorbent assay (ELISA) that combines the reagents necessary to detect endogenous levels of p53 protein, phospho-p53 protein (Ser15), Bad, phospho-Bad (Ser112), Cleaved Caspase-3 (Asp175) and Cleaved PARP (Asp214), which are key signaling proteins of cell survival and apoptosis pathways. The assay was performed according to manufacturer's instructions. Briefly, HepG2 cells were treated with OA-Q3G for 0, 6 and 24 h in fresh media. Cells were harvested under non-denaturing conditions by adding 1X cell lysis buffer (provided with the kit) supplemented with 1X protease inhibitor (Sigma-Aldrich, Mississauga, ON) and kept on ice for 10 min. Lysates were then microcentrifuged for 10 min at 4 ºC and supernatents were transferred in fresh tubes and stored at -80 ºC in single-use aliquotes. The assay was performed using 500 µg/ml protein quantified by BCA Protein Assay (Thermo Scientific, Ottawa, ON) according to the manufacturer's instructions. To appropriate wells of ELISA microplate, 100 µl of each cell lysate was added and incubated the plate overnight at 4 ºC. After incubation, wells were washed four times with 1X wash buffer (provided with kit). After washing, 100 µl of detection antibody (provided with the kit) for each protein in respective wells and incubated the plate for 1 h at 37 ºC followed by washing with wash buffer. After washing, 100 µl of HRP-linked secondary antibody was added to

corresponding wells and incubated the plate for 45 min at 37 ºC followed by another wash procedure. After washing, 100 µl TMB substrate was added to each well and plate was incubated for 30 min at 37 ºC followed by addition of stop solution. Protein activity was measured by reading absorbance at 450 nm in Fluostar Optima micro plate reader (BMG Labtech, Ortenberg, Germany).

6.2.4 Multi-target ELISA Array analysis

To indentify intracellular protein level targets of OA-Q3G in HepG2 cells, PathScan® Intracellular Signaling Array kit (Cell Signaling Technology, Danvers, MA) was used. It is a slide-based antibody array founded upon the sandwich immunoassay principle. The array kit allows for the simultaneous detection of 18 important and well-characterized signaling molecules when phosphorylated or cleaved. The glass slides are nitrocellulose-coated spotted with target-specific capture antibodies. The assay was performed according to the manufacturer's instructions. Briefly, cell lysate were prepared and quantified similar to sandwich ELISA explained above. Protein (500 µg/ml) were incubated on the slide overnight at 4 ºC followed by addition of a biotinylated detection antibody cocktail (provided with kit). Streptavidin-conjugated HRP and LumiGLO® Reagent (provided with the kit) was then used to visualise the bound detection antibody by chemiluminesence. The bands were developed using Carestream® Kodak film (Sigma-Aldrich, Mississauga, ON) and were quantified using Image J 1.47 software according to NIH guidelines.

6.4 RESULTS

6.4.1. Cancer related gene expression profiling

To reveal possible molecular mechanisms of the antiproliferative effect of OA-Ester of Q3G in human liver cancer HepG2 cells in vitro, Human Cancer Drug Target RT Profiler PCR Array (SABiosciences) was used. This system profiles the expression of 84 actively sought targets for anticancer therapeutics and drug development which includes genes dysregulated during carcinogenesis, including those involved in key cellular growth pathways such as apoptosis, DNA damage repair, epigenetics, and growth factor and other signaling pathways. The relative modulation of mRNA levels of each gene in up- and down-regulation form is shown in Table 6.1. Results showed that 22 genes showed more than 2-fold change in mRNA levels (up- or down-regulated). Table 6.1 shows only the genes with significant and greater than 2-fold change in mRNA levels. Treatment of HepG2 cells with OA-Q3G influenced the expression of some key signalling genes involved in cancer cell survival and cell death pathways:

1. *Cell cycle and survival*: down-regulation of cell cycle genes, CDK1 (-2-fold), CDK2 (-2-fold), CDK (-12.2-fold), CDC25A (-2-fold), topoisomerase II TOP2A (-2.7-fold), growth factors and receptors, EGFR (-12.2-fold), GRB2 (-2-fold) receptor tyrosine kinase AKT2 (-2.1-fold), protein kinases, AURKB (-2-fold), PRKCA (-2-fold) and histone deacetylases HDAC2 (-2-fold), HDAC6 (-25.9-fold).

2. *Cell death and apoptosis*: up-regulation of pro-apoptotic genes BCL2 (+2.2-fold), cathepsins CTSB (+2.2-fold), IRF5 (+6.3-fold), RHOB (+3.3-fold) and down-regulation of anti-apoptotic genes BIRC5 (-11.2-fold), HIF1A (-2.4-fold), NFKB1 (-2.2-fold) and HRAS (-2-fold).

The obtained data suggested that the antiproliferative effect of OA-Q3G could be due to change in mRNA levels of cell cycle genes and apoptotic genes in HepG2 cells.

6.4.2. Intracellular signalling targets

Cell survival requires the active inhibition of apoptosis achieved via activation of pro apoptotic proteins by the upstream components of survival pathways (Zhang et al., 2011). To understand and map upstream intracellular targets of OA-Q3G, I utilized an intracellular signalling ELISA array. The array detects the indicated cellular proteins and signaling nodes only when phosphorylated or cleaved at the specific residues. HepG2 cells were treated with 100 μM of OA-Q3G for 6 and 24 h. Cell lysate was extracted and 500 μg/ml lysate was used for array analysis as explained in materials and methods. Results from the ELISA array are listed in Figure 6.1. Results showed an up-regulation in pro-apoptotic proteins ERK1/2, Bad, p-53, SAPK/JNK, PARP and caspase-3 active products expression and down-regulation of cell survival and growth proteins AKT, p70 S6 kinase, GSK 3β, Stat3 and PRAS40 within 6 h of treatment with OA-Q3G (Fig 6.1). On the other hand, results from 24 h treatment showed a decrease in the expression of ERK1/2, Stat1, HSP27 and p53 active products and an increase in the expression of p38, SAPK/JNK, PARP and caspase-3 active products (Fig 6.1). Results from this analysis suggest a strong role of OA-Q3G in active regulation of cell survival and cell death proteins.

6.4.3. OA-Q3G induces expression of p53 and p53 dependent pro-apoptotic genes in HepG2 cells

The tumor suppressor p53 plays an important role in cell cycle arrest and apoptosis (Vogelstein et al., 2000). HepG2 cells have been shown to possess the wild type p53 gene

(Muller et al., 1997). To examine whether OA-Q3G induced activation of p53, HepG2 cells were treated with 100 μM of OA-Q3G for 6 h and 24 h. Results from sandwich ELISA showed that total p53 and phosphorylated p53 protein levels significantly increased at 6 h of incubation and comparatively decreased after 24 h of incubation (Fig 6.2). Similar results were seen by densitometry analysis from ELISA array which showed a significant 2-fold increase in levels of phospho-p53 within 6 h of treatment with OA-Q3G (Fig 6.1).

To evaluate the effect of p53 and phospho-p53 expression on its downstream effectors, I examined the levels of CDK1, Bcl-2 and Bad. Results from the RT Profiler PCR array analysis showed a 2-fold downregulation in the mRNA levels of CDK1 (Table 6.1) and an up-regulation in the levels of pro-apoptotic Bcl-2 family as compared to untreated control cells within 24 h of treatment with OA-Q3G (Table 6.1). Additionally, results from sandwich ELISA showed a significant increase in the expression of pro-apoptotic protein Bad after 6 and 24 h of treatment with OA-Q3G (Fig 6.2).

Since activation of pro-apoptotic Bcl-2 family initiates the caspase cascade, I next examined caspase-3 activation in HepG2 cells upon treatment with OA-Q3G. Results from the sandwich ELISA showed a significant up-regulation of cleaved-caspase-3 expression after 6 and 24 h of treatment with OA-Q3G (Fig 6.2). Consistent results were observed by densitometry analysis from ELISA array (Fig 1). This was accompanied by an approximately 2-fold increase in cleaved PARP protein expression in HepG2 cells within 6 h and 1.6-fold increase after 24 h of treatment with OA-Q3G (Fig 6.1 and 6.2). These results suggest that OA-Q3G treatment in HepG2 cells induces p53 and phopho-p53 expression resulting in activation of downstream effectors for cell death response.

6.5 DISCUSSION

The primary aim of the study was to elucidate a possible molecular mechanism of action for the antiproliferative effect of OA-Q3G in HepG2 cells. In my previous work, I reported that OA-Q3G exhibits a strong antiproliferative effect in HepG2 cells which is significantly greater than a currently prescribed drug sorafenib and parent compound Q3G (Chapter-5). I also reported that OA-Q3G induces apoptosis via cell cycle arrest and DNA topoisomerase II inhibition. The current study aimed at revealing possible molecular targets for the antiproliferative activity. This study focused on changes in both gene level and protein level expressions of cancer related genes upon treatment with OA-Q3G. My results suggest that treatment with OA-Q3G induces significant changes in the regulation of mRNA level of the genes involved in drug metabolism, growth factors and receptors, G-protein signaling, receptor tyrosine kinase, cell cycle, transcription factors and apoptosis. This study further suggests that the treatment with OA-Q3G in HepG2 cells induces changes in protein level expression of various genes involved in cell survival and cell death signaling pathways including apoptosis. This is the first study to show the molecular drug targets of long chain fatty acid ester of Q3G (OA-Q3G).

Apoptosis is deregulated in many tumors and it has been shown that drugs that have the ability to interrupt cell division or induce normal apoptosis are very significant in treating cancers (Fesik et al., 2005). Several upstream signalling components are implicated in deregulation of apoptosis (Fesik et al., 2005). Therefore, to underline the cause of deregulation of apoptosis, various upstream signaling nodes in growth, cell survival and cell death are primarily focussed. In an attempt to reveal the molecular mechanisms of antiproliferative effect of OA-Q3G in liver cancer cells HepG2, PathFinder RT Profiler

PCR Array specifically designed for human cancer drug targets was applied. Analysis of the obtained results showed that molecular mechanisms of cell cycle control, signal transduction molecules, transcription factors, drug metabolism and apoptosis were affected in OA-Q3G treated cells. The growth factor receptor such as EGFR is up-regulated in broad range of epithelial tumors such as liver cancer (Klutz et al., 2011). Interestingly, results from RT Profiler PCR array showed significantly reduced mRNA level of EGFR (12-fold), AKT1 (2-fold), AURKB (2-fold) and PRKCA (2-fold) in OA-Q3G treated cells. Cell survival signaling molecule AKT has been shown to be a significant target for cancer drugs (Steelman et al., 2008). Additionally, protein kinases such as AURKB and Protein kinase C have also been seen up-regulated in cancers (Portella et al., 2011; Marengo et al., 2011). Furthermore, results showed that there was a 6.3-fold increase in IRF5 and 2.2-fold decrease in NFKB1 mRNA levels in OA-Q3G treated cells (Table 6.1). Up-regulation of transcription factor IRF5 have been shown to reduce cancer progression (Li et al., 2008) whereas, overexpression of NFKB1 gene has been seen in many human cancers (Rayat et al., 1999). Consistent with these previous findings, our data suggests that OA-Q3G treatment in HepG2 cells regulate key signalling molecules involved in cell survival pathways.

It is now clear that the checkpoints in cell-cycle can regulate the rate of cell division, therefore, drugs targeting cell cycle components are significant in chemoprevention (DiPaola et al, 2002). I previously showed that OA-Q3G induced cell cycle arrest and DNA topoisomerase II inhibition in HepG2 cells (Chapter-5). Data from RT Profiler PCR array showed a consistent result in the changes in mRNA levels of cell cycle genes as well (Table 6.1). Levels of mRNA of CDC25A, CDK1, CDK2 were down-regulated 2-

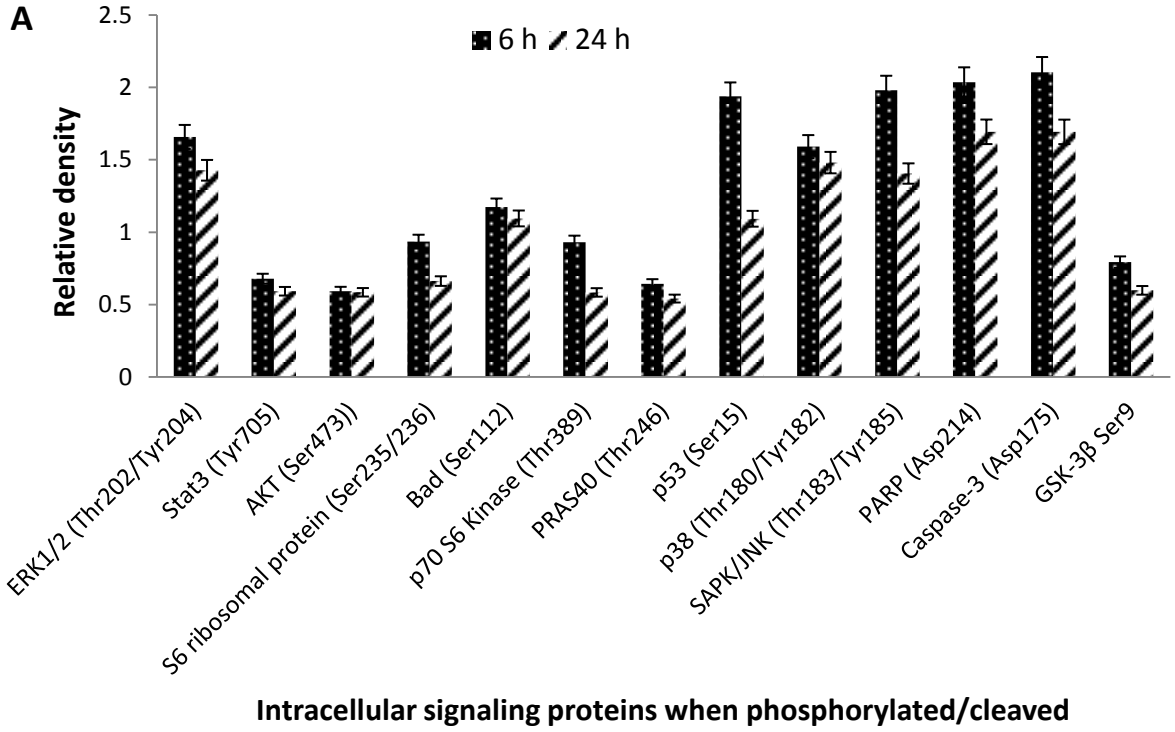
fold and CDK8 was down-regulated by 12.2-fold. In addition, mRNA level of DNA topoisomerase II was down-regulated 2.7-fold in HepG2 cells upon treatment with OA-Q3G. In the similar context, apoptosis has been shown to be the most efficient death-pathway in tumor cells after topoisomerase II inhibition (El-Awady et al., 2008). Results from RT Profiler PCR array showed a 2.2-fold increase in pro-apoptotic BCL2 and an 11.2-fold decrease in anti-apoptotic BIRC5 mRNA levels (Table 6.1). In addition, gene expression of PARP proteins which upon cleavage induce apoptosis up-regulated for PARP4 (2.1-fold) and down-regulated for PARP1 (2.5-fold). Furthermore, protein level expression of OA-Q3G treated cells by sandwich ELISA and ELISA array showed a 2-fold increase in cleaved PARP expression after 6 h of treatment and 1.6-fold increase after 24 h of treatment relative to the control (Fig 6.1 and 6.2). Protein expression of pro-apoptotic gene BAD was also seen up-regulated after 6 and 24 h of treatment (Fig 6.1 and 6.2). Furthermore, levels of phospho-p53 and p53 were also seen increased after 6 h and comparatively decreasing after 24 h of treatment with OA-Q3G (Figure 6.1 and 6.2). The mRNA level of whole p53 as obtained from RT Profiler PCR array also showed a decrease by 1.8-fold (data not shown) after 24 h of treatment. Quercetin has been previously shown to activate p53 and p53 dependent genes (Tanigawa et al., 2008). In the same study, quercetin had been shown to stabilise the p53 protein at both protein and mRNA level within 24 h of treatment. Results from our study suggest p53 induction as an early event upon treatment with and possibly through stabilisation by OA-Q3G in HepG2 cells, thereby activating its downstream effectors such as pro-apoptotic BCL2, Bad and CDK1 causing cell cycle arrest and apoptosis. Furthermore, results showed that OA-Q3G treatment increased and activated ERK1/2 and JNK protein. The increased expressions of

these proteins are known to induce apoptosis upon quercetin treatment in human lung cancer cells (Nguyen et al., 2004). OA-Q3G also increased the phosphorylation of p38 protein which is shown to be associated with induction of apoptosis upon quercetin treatment via caspase-3 activation (Nam et al., 2008). Akt phosphorylation of the proapoptotic protein Bad and kinase GSK 3β inhibits their activity and promotes cell survival (Song et al., 2005; Fang et al., 2000). It was seen that with the decrease in Akt phosphorylation caused by OA-Q3G, there was significant decrease in GSK 3β expression as well which may resulted in apoptosis via proapoptotic action of GSK 3β. Overall, the current data suggests that OA-Q3G treatment may involve multiple targets for its mode of action which include cell survival factors and cell death factors with the possible implication of Akt/GSK-3 pathway, p53 dependent apoptosis and ERK1/2 pathway. A schemetic of current data representing effect of OA-Q3G on intracellular signalling is shown in Fig 6.3. It should be pointed out that these effects may or may not be specific for the HepG2 cells. This study provides a preliminary analysis of the regulation of intracellular pathway components by OA-Q3G and further studies must be performed involving individual signaling pathways to reveal the specific targets and mode of action of OA-Q3G.

Table 1. Effect of OA-Q3G treatment on mRNA levels of the genes involved in human cancer drug targets in HepG2 cells

Signaling Group	*Gene*	*Description*	*Fold Change*
Apoptosis	*BCL2*	B-cell CLL/lymphoma 2	+2.2
	BIRC5	Baculoviral IAP repeat containing 5	-11.2
Growth factors and receptors	*EGFR*	Epidermal Growth Factor Receptor	-12.2
	PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	+2.0
Drug Metabolism	*TXN*	Thioredoxin	-2.0
G Protein Signaling	*RHOB*	Ras homolog gene family, member B	+3.3
Heat Shock Proteins	*HSP90AA1*	Heat shock protein 90kDa alpha (cytosolic), class A member 1	-2.1
Receptor Tyrosine Kinase	*AKT2*	V-akt murine thymoma viral oncogene homolog 2	-2.1
	GRB2	Growth factor receptor-bound protein 2	-2.0
Cathepsins	*CTSB*	Cathepsin B	+2.2
Cell Cycle	*CDC25A*	Cell division cycle 25 homolog A (S. pombe)	-2.0
	CDK1	Cyclin-dependent kinase 1	-2.0
	CDK2	Cyclin-dependent kinase 2	-2.0
	CDK8	Cyclin-dependent kinase 8	-12.2
Topoisomerase, Type II	*TOP2A*	Topoisomerase (DNA) II alpha 170kDa	-2.7
Transcription Factors	*HIF1A*	Hypoxia inducible factor 1, alpha subunit	-2.4
	IRF5	Interferon regulatory factor 5	+6.3
	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	-2.2
Protein Kinases	*AURKB*	Aurora kinase B	-2.0
	PRKCA	Protein kinase C, alpha	-2.0
RAS Signaling	*HRAS*	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	-2.0
Histone Deacetylases	*HDAC2*	Histone deacetylase 2	-2.0
	HDAC6	Histone deacetylase 6	-25.9
Poly ADP-Ribose Polymearses	*PARP1*	Poly (ADP-ribose) polymerase 1	-2.5
	PARP4	Poly (ADP-ribose) polymerase family, member 4	+2.1

A



B

Protein name	6 h	24 h
ERK1/2 (Thr202/Tyr204)	+1.7*	+1.5*
Stat3 (Tyr705)	-1.5*	-1.7*
AKT (Ser473))	-1.7*	-1.7*
S6 Ribosomal protein (Ser235/236)	-1.06	-1.5*
p70 S6 Kinase (Thr389)	-1.1	-1.7*
PRAS40 (Thr246)	-1.6*	-1.8*
p53 (Ser15)	+1.9*	+1.1
p38 (Thr180/Tyr182)	+1.6*	+1.5*
SAPK/JNK (Thr183/Tyr185)	+2.0*	+1.5*
PARP (Asp214)	+1.6*	+1.7*
Caspase-3 (Asp175)	+2.1*	+1.8*
GSK-3β Ser9	-1.2	-1.7*

Figure 6.1. Multi target intracellular signaling ELISA array analysis of HepG2 cells upon treatment with OA-Q3G

Briefly, HepG2 cells were treated with or without OA-Q3G for 6 and 24 h and lysates were prepared as described in Materials and Methods and analysed for ELISA array. The array was incubated with multi detection antibody cocktail for detection of 18 different key signalling molecules in pathways controlling cell growth, survival and cell death when phosphorylated or cleaved. The figure shows A) density of the bands corresponding to expression of each protein relative to untreated control and B) Fold change in the expression of each protein relative to untreated control. Results were analysed by using one way ANOVA in Minitab 16 statistical software. Results are expressed as mean ± SD with at least three independent experiments. * Proteins showing atleast 1.5-fold change (up- or down-regulation) relative to control and $P < 0.05$.

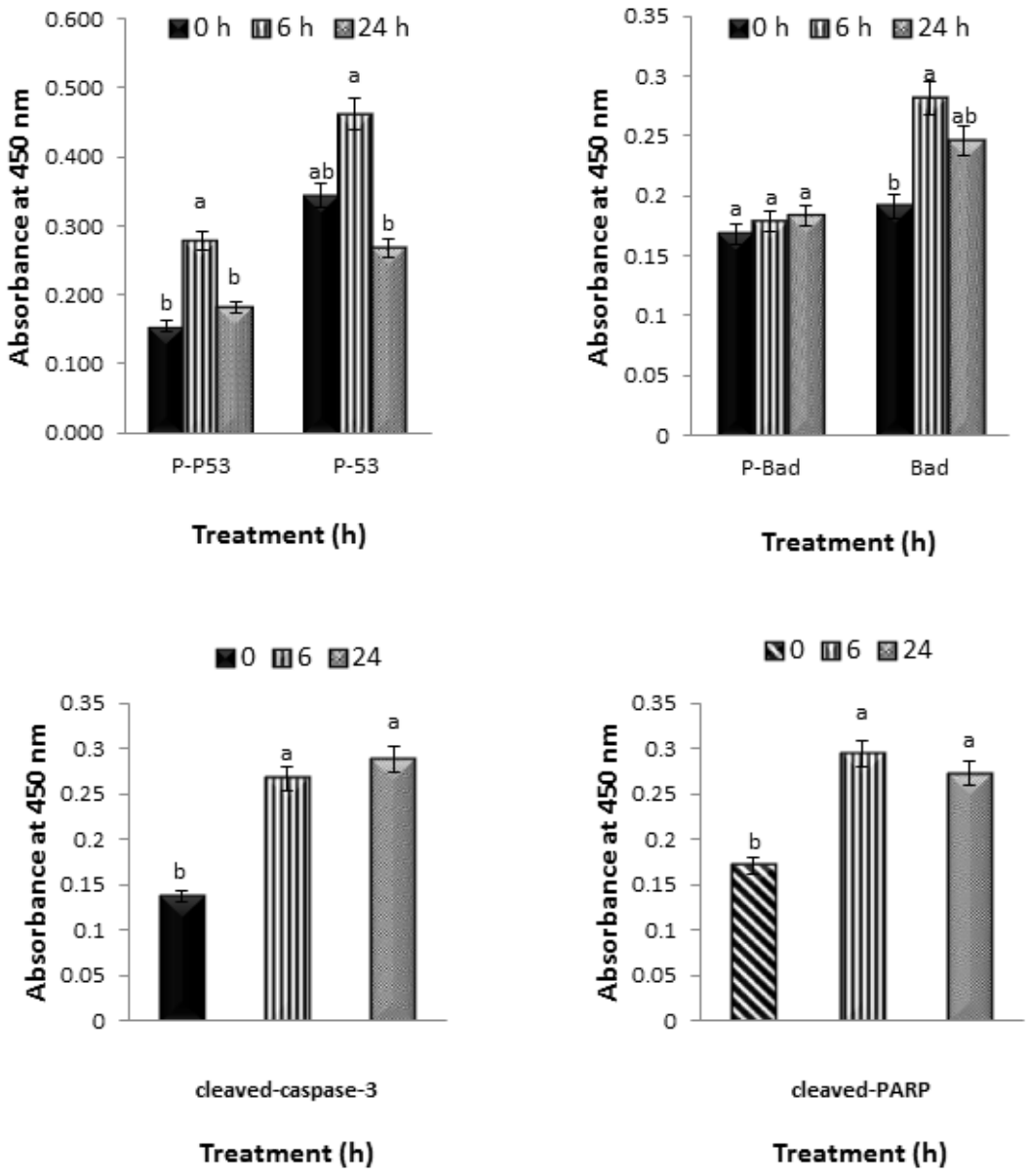


Figure 6.2. Multi-target sandwich ELISA analysis for apoptosis in HepG2 cells upon treatment with OA-Q3G.

Briefly**,** HepG2 cells were treated with OA-Q3G for 0, 6 and 24 h and lysates were prepared as described in Materials and Methods and analysed for sandwich ELISA detection of endogenous levels of key signaling molecules involved in cell survival and apoptosis [(p53 protein, phospho-p53 protein (Ser15), Bad, phospho-Bad (Ser112),

Cleaved Caspase-3 (Asp175) and Cleaved PARP (Asp214)]. Protein activity was measured by reading absorbance at 450 nm in microplate reader. The figure shows change in protein expression of indicated proteins in HepG2 cells upon treatment with OA-Q3G. Results were analysed by using one way ANOVA in Minitab 16 statistical software. Tukey's test was performed for achieving significant difference between different treatment times. Values with different letters differ significantly ($P<0.05$).

Results are expressed as mean ± SD with at least three independent experiments.

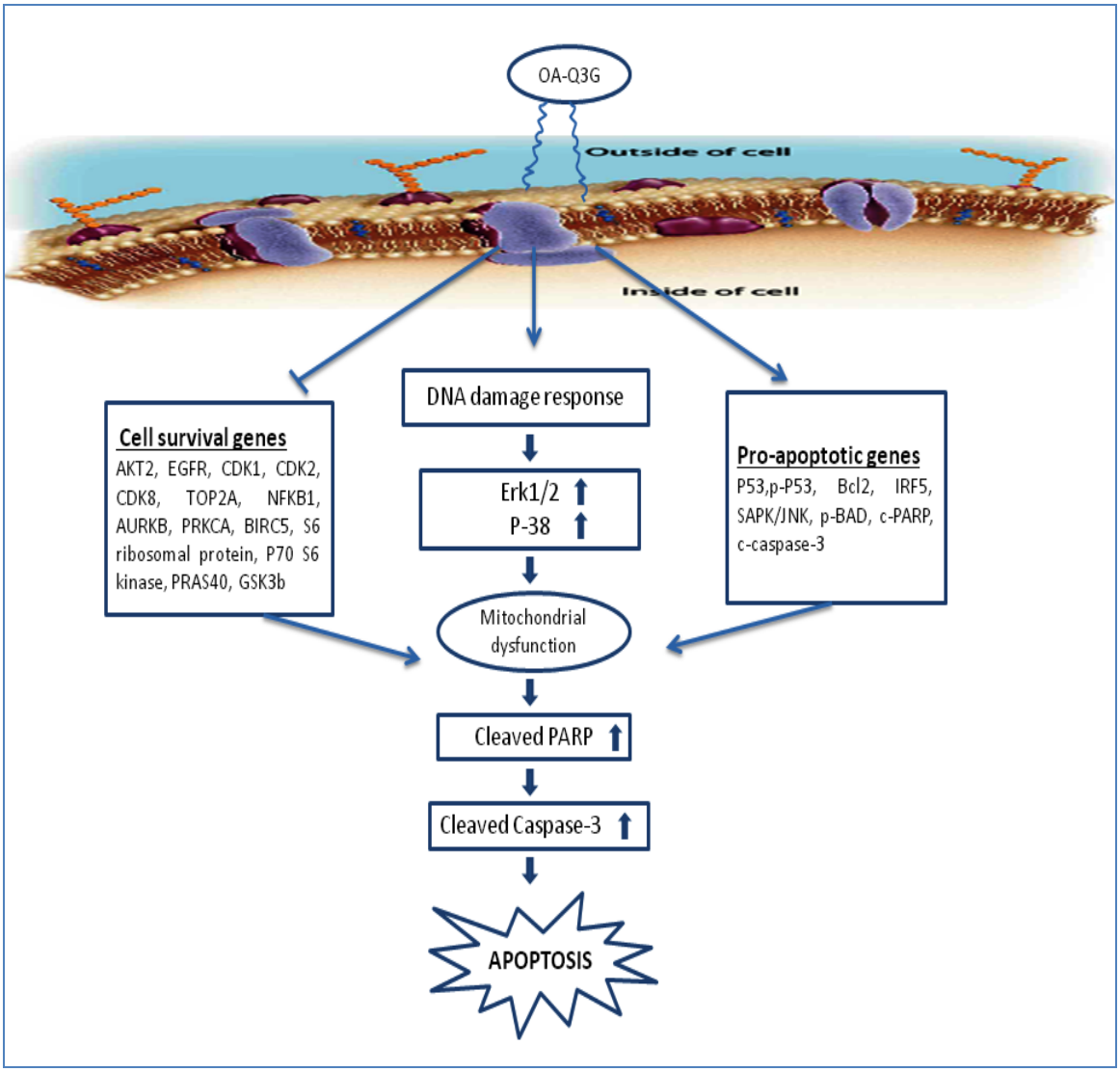


Figure 6.3 Schemetic representation of possible effect of OA-Q3G on intracellular signalling pathways responsible for cell survival and cell death in HepG2 cells.

6.7 REFERENCES

Chirumbolo S: Quercetin in cancer prevention and therapy. *Integr Cancer Ther* **12**,97-102, 2013.

DiPaola RS: To Arrest or Not To G2 M Cell-Cycle Arrest: Commentary re: A. K. Tyagi et al., Silibinin Strongly Synergizes Human Prostate Carcinoma DU145 Cells to Doxorubicin-induced Growth Inhibition, G2-M Arrest and Apoptosis. *Clin. Cancer Re* **8**, 3311–3314, 2002.

El-Awady RA, Ali MM, Saleh EM and Ghaleb FM: Apoptosis is the most efficient death-pathway in tumor cells after topoisomerase II inhibition. *Saudi Med J* **29**,558-64, 2008.

Fang X, Yu SX, Lu Y, Bast RC Jr, Woodgett JR, Mills GB: Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. *Proc Natl Acad Sci U S A*. **97**, 11960-5, 2000.

Fesik SW: Promoting apoptosis as a strategy for cancer drug discovery. *Nat Rev Cancer*. **5**,876-85, 2005.

Gibellini L, Pinti M, Nasi M, Montagna JP, De Biasi S, Roat E, et al: Quercetin and cancer chemoprevention. *Evid Based Complement Alternat Med* **2011**,591356, 2011.

Gulati N, Laudet B, Zohrabian VM, Murali R, Jhanwar-Uniyal M: The antiproliferative effect of Quercetin in cancer cells is mediated via inhibition of the PI3K-Akt/PKB pathway. *Anticancer Res* **26(2A)**,1177-81, 2006.

Klutz K, Schaffert D, Willhauck MJ, Grünwald GK, Haase R, Wunderlich N et al: Epidermal growth factor receptor-targeted (131)I-therapy of liver cancer following systemic delivery of the sodium iodide symporter gene. *Mol Ther*. **19**,676-85, 2011.

Lee YK, Hwang JT, Kwon DY, Surh YJ, and Park OJ: Induction of apoptosis by quercetin is mediated through AMPKalpha1/ASK1/p38 pathway. *Cancer Letters*, **292**,228–236, 2010.

Li Q, Tang L, Roberts PC, Kraniak JM, Fridman AL, Kulaeva OI, Tehrani OS, Tainsky MA. Interferon regulatory factors IRF5 and IRF7 inhibit growth and induce senescence in immortal Li-Fraumeni fibroblasts. *Mol Cancer Res* **6**,770-84, 2008.

Marengo B, Ciucis CD, Ricciarelli R, Pronzato MA, Marinari UM and Domenicotti C: Protein Kinase C: An Attractive Target for Cancer Therapy. *Cancers*, **3**,531-567, 2011.

Mu C, Jia P, Yan Z, Liu X, Li X, and Liu H: Quercetin induces cell cycle G1 arrest through elevating Cdk inhibitors p21 and p27 in human hepatoma cell line (HepG2). *Methods and Findings in Experim and Clini Pharma* **29**,179–183, 2007.

Muller M, Strand S, Hug H, Heinemann EM, Walczak H, Hofman WJ, et al: Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. *J. Clin. Invest* **99**,403–413, 1997.

Naithani R, Huma LC, Moriarty RM, McCormick DL, and Mehta RG: Comprehensive review of cancer chemopreventive agents evaluated in experimental carcinogenesis models and clinical trials. *Curr Medi Chem***15**,1044–1071, 2008.

Nam TW, Yoo CI, Kim HT, Kwon CH, Park JY, Kim YK: The flavonoid quercetin induces apoptosis and inhibits migration through a MAPK-dependent mechanism in osteoblasts. J *Bone Miner Metab*. **26**, 551-60, 2008.

Nguyen TT, Tran E, Nguyen TH, Do PT, Huynh TH, Huynh H: The role of activated MEK-ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells. *Carcinogenesis*. **25**, 647-59, 2004.

Portella G, Passaro C, Chieffi P: Aurora B: a new prognostic marker and therapeutic target in cancer. *Curr Med Chem* **18**,482-96, 2011.

Rayet B and Gélinas C: Abberant rel/nfkb genes and activity in human cancer. *Oncogene* **18**, 6938-47, 1999.

Robaszkiewicz A, Balcerczyk A, and Bartosz G: Antioxidative and prooxidative effects of quercetin on A549 cells. *Cell Biol Intern* **31**,1245–1250, 2007.

Shan BE, Wang MX, and Li RQ: Quercetin inhibit human SW480 colon cancer growth in association with inhibition of cyclin D1 and survivin expression through Wnt/beta-catenin signaling pathway. *Can Investi* **27**,604–612, 2009.

Song G, Ouyang G, Bao S: The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med*. **9**, 59-71, 2005.

Steelman LS, Stadelman KM, Chappell WH, Horn S, Bäsecke J, Cervello M, et al: Akt as a therapeutic target in cancer. *Expert Opin Ther Targets* **12**,1139-65, 2008.

Tanigawa S, Fujii M, and Hou DX: Stabilization of p53 is involved in quercetin-induced cell cycle arrest and apoptosis in HepG2 cells. *Bioscience, Biotech and Biochem* **72**,797–804, 2008.

Vogelstein B, Lane D, and Levine AJ: Surfing the p53 network. *Nature* **408**,307–310, 2000.

Zhang X, Tang N, Hadden TJ and Rishi AK: Akt, FoxO and regulation of apoptosis. *Biochim. Biophys. Acta* **1813**, 1978–86, 2011.

CHAPTER 7. CONCLUSIONS

Dietary flavonoids have been consistently shown to be associated with reduced risk of various types of cancers including liver cancer. In this thesis, I discussed the potential of the flavonoid quercetin as a natural anticancer agent. Its naturally occurring form, Q3G has been shown to possess anti-proliferative and strong anti-oxidant activities in vitro. Despite their strong biological effect in human health, poor bioavailability limits their biological effects in vivo. This suggests a need of developing strategies to improve the cellular uptake and bioavailability of flavonoids such as Q3G.

Our lab, in the previous work, modified the structure of Q3G by acylating it with six different long chain fatty acids as described in chapter 5 with the idea of increasing the lipophilicity of Q3G. It was hypothesised that acylation with long chain fatty acids will enhance the anti-proliferative effect of Q3G. Since, there has not been a time- and dose-dependent study for the anti-proliferative effect of Q3G in liver cancer cells, HEPG2 human hepatocellular carcinoma cells were used for the study. In the first phase of the study, I investigated the anti-proliferative effect of Q3G in HepG2 cells. It was seen that Q3G had little or no effect on cell viability after 6 h of treatment. Low doses of Q3G (1, 10 and 50 μM) did not have a significant anti-proliferative effect in 24 h of treatment. However, concentrations above 100 μM showed 30% - 60% of cell growth inhibition at 24 h which further increased to approximately 90% after 48 h of treatment. Investigation of the potential mechanism of cell death revealed that Q3G could induce cell cycle arrest, apoptosis and DNA topoisomerase II inhibition.

To test the hypothesis that acylation with long chain fatty acids enhances the antiproliferative effect of Q3G in HepG2 cells, I tested the cell viability of HepG2 cells in

a time- and dose-dependent treatment with long chain fatty acid esters of Q3G as described in chapter 5. As hypothesised, 100 μM of all long chain fatty acid esters of Q3G except stearic acid ester of Q3G showed a significant decrease in cell viability (approximately 90%) as compared to precursor compounds (quercetin, Q3G and free fatty acids alone) and chemotherapy drug Sorafenib (used for liver cancer therapy) within 6 h of treatment. The cell viability of HepG2 cells decreased from 96% (Q3G-treated) to 10-15% (Q3G esters-treated) within 6 h of incubation. Additionally, unlike Q3G, doses below 100 μM showed significant decrease in cell viability at and above 24 h after treatment. Interestingly, among tested esters, oleic acid ester of Q3G showed the greatest reduction in cell viabilty (approx. 5% in 6 h and 1% in 24 h); however, stearic acid ester of Q3G did not show any significant decrease. This also suggests that there may be a structural-activity relationship associated with the long chain fatty acid ester's interaction with the cell membrane or membrane bound cell signalling receptors. Stearic acid is the only saturated fatty acid among the six fatty acids tested. Once the stearic acid is attached to the Q3G skeleton, the change in the orientation may not be favourable for membrane interaction thereby, getting less absorbed by cells and in turn showing less activity. As a potential anticancer agent, it is necessary to investigate the effect of Q3G-esters in normal liver cells as well. Interestingly, data showed that treatment with long chain fatty acid esters of Q3G did not induce any significant cytotoxicity to normal liver cells (rat hepatocytes), whereas, sorafenib caused a significant amount of cytotoxicity. This suggests a specific action of the long chain fatty acid esters of Q3G towards cancer cells. Furthermore, to test the hypothesis whether the mechanism of action of long chain fatty acid esters of Q3G was similar to Q3G, basic hallmark apoptotic assays were

performed and it was revealed that the long chain fatty acid esters of Q3G induced cell cycle arrest, apoptosis with the activation of caspase-3 and DNA topoisomerase II inhibition similar to Q3G-treatment in HepG2 cells.

The results from the Chapter-5 of the study hence suggested that the long chain fatty acid esters of Q3G were causing cell death due to the induction of cell cycle arrest and apoptosis in HepG2 cells. This implied that the long chain fatty acid esters were not acting as poison and lysing the cells but were possibly causing changes in regulation of key molecules involved in cell cycle, cell growth, cell survival and cell death/apoptosis. To understand and investigate the molecular targets of long chain fatty acid esters of Q3G, the most efficaceous ester of Q3G, oleic acid ester (OA-Q3G) was used for further investigation. As explained in chapter 6, results obtained from RT-Profiler PCR array showed that treatment with OA-Q3G induced significant changes in the regulation of gene level expression of the genes involved in drug metabolism, growth factors and receptors, G-protein signaling, receptor tyrosine kinase, cell cycle, transcription factors and apoptosis. Additionally results obtained from sandwich ELISA and ELISA array showed that the treatment with OA-Q3G in HepG2 cells induced changes in protein level expression of various proteins involved in cell survival and cell death signaling pathways including apoptosis.

Apoptosis is deregulated in many tumors due to hyperactivation of various growth factors and molecules involved in cell growth and survival, and it has been shown that drugs that have the ability to interrupt cell division or induce normal apoptosis are very significant in treating cancers. OA-Q3G significantly decreased gene expression of cell survival genes EGFR (12-fold), AKT1 (2-fold), AURKB (2-fold) and PRKCA (2-fold) which are

seen highly up-regulated in cancers. Furthermore, OA-Q3G significantly decreased gene expression of cell cycle genes CDC25A, CDK1, CDK2 and CDK8 suggesting the involvement of these genes as possible targets of cell cycle arrest induced upon OA-Q3G treatment in HepG2 cells. Activation of tumor suppressor gene p-53 is known to cause cell cycle arrest and apoptosis via activation of caspase-3 and PARP which are principle mediators of apoptosis. Results showed that OA-Q3G increased the expression of p53 and phospho-p53 in HepG2 cells within 6 h of treatment. In addition, OA-Q3G significantly increased the protein expression of cleaved caspase-3 and PARP in HepG2 cells. This suggested that p53 may be one of the possible targets of OA-Q3G for its mode of action. Furthermore, results showed that OA-Q3G treatment increased activated ERK1/2 resulting in further activation of SAPK/JNK protein. The increased expressions of these proteins are known to induce apoptosis upon quercetin treatment. OA-Q3G also increased the phosphorylation of p38 protein which is known to induce apoptosis via caspase-3 activation. Akt phosphorylation of the proapoptotic protein Bad and kinase GSK-3 inhibits their activity and promotes cell survival. It was seen that with the decrease in Akt phosphorylation caused by OA-Q3G, there was significant decrease in GSK-3 expression as well which may resulted in apoptosis. Overall, the current data suggests that OA-Q3G treatment may involve multiple targets for its mode of action which include cell survival factors and cell death factors with the implication of Akt pathway, p53 dependent apoptosis and ERK1/2 pathway. This study provides a preliminary analysis of the regulation of intracellular pathway components by OA-Q3G and therefore, further studies must be performed involving individual signaling pathways to reveal the specific targets and mode of action of OA-Q3G. In addition, further studies

should be perfomed using animal models to confirm the antiprolifertaive effect and bioefficacy of these novel compounds in *vivo*.

7.1 Future recommendations

Antiproliferative activity

The antiproliferative effect of long chain fatty acid esters of Q3G must be confirmed using different cancer cell lines. This will provide a better understanding of the dosage required for treating different types of cancer.

Cytotoxic studies

The effect of long chain of fatty acid esters of Q3G on viability of normal cells must be further tested and analysed using different primary normal cell lines. This will provide a reliable understanding of dosage required for treating cancers and knowing whether the dosage required for inducing cell death is toxic to the normal cells. This study will rule out the problems associated with chemotherapy induced cytotoxicity.

Mechanism of action

RT PCR analysis along with the sensitive Western Blotting protein expression analysis must be further performed focusing on individual signaling pathways (cell survival and cell death) to reveal the specific targets of long chain fatty acid esters for their mode of action. The analysis must also be performed using different cancer cell lines to determine whether the mode of action is same or different for different cancer types.

Animal studies

Once the in vitro analysis have been performed successfully, it is highly recommended to test the potential of long chain fatty acid esters in *vivo*, using animal models. This study

will be used to confirm the antiproliferative effect of long chain fatty acid esters in vivo and to determine the mode of actions in vivo.

Bioavailability Assays

To understand and determine the cellular uptake of long chain fatty acid esters, different bioavailabilty assays both *in vitro* and in vivo should be performed.

In vivo testing in humans

The final determination of using Q3G and its derivatives as anticancer agents has to be done using human subjects. The test agents may have not caused toxicity in rat primary normal hepatocytes but may induce toxicity in human cells from different systems in the body.

REFERENCES

Ader P, Wessmann A and Wolffram S: Bioavailability and metabolism of the flavonol quercetin in the pig. *Free Radic Biol Med* **28**, 1056-1067, 2000.

Aggarwal BB, and Shishodia S: Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem. Pharmacol* **71**,1397–1421, 2006.

Agullo G, Gamet-Payrastre L, Manenti S, Viala C, Remesy C, Chap H and Payrastre B: Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. *Biochem. Pharmacol* **53**, 1649-1657, 1997.

Alía M, Ramos S, Mateos R, Granado-Serrano AB, Bravo L and Goya L: Quercetin protects human hepatoma HepG2 against oxidative stress induced by tert-butyl hydroperoxide. *Toxicology and Appl Pharma* **212**, 110 – 118, 2006.

AmericanCancersociety.http://www.cancer.org/acs/groups/cid/documents/webcontent/003114-pdf.pdf, 2010.

Ames BN, Shigenaga MK and Gold LS: DNA lesions, inducible DNA repair, and cell division: the three key factors in mutagenesis and carcinogenesis. *Environ. Health Perspect* **101** (suppl. 5), 35–44, 1993.

Andreadi CK, Howells LM, Atherfold, PA and Manson MM:Involvement of Nrf2, p38, B-Raf, and nuclear factor-kappaB, but not phosphatidylinositol 3-kinase, in induction of hemeoxygenase-1 by dietary polyphenols. *Mol. Pharmacol* **69**,1033–1040, 2006.

Appleton J. Enhancing Bioavailability of Quercetin. *Nat Med J* **2**, 1-6, 2010.

Arikawa E, Quellhorst G, Han Y, Pan H and Yang, J: RT2 Profiler™ PCR Arrays: Pathway-Focused Gene Expression Profiling with qRT-PCR. *SABioscience*. http://www.sabiosciences.com/manuals/pcrarraywhitepaper.pdf.

Arts IC and Hollman PC: Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr*. **81**(1 Suppl), 317S-325S, 2005.

Arts IC and Sesink AL, Faassen-Peters M and Hollman, PC: The type of sugar moiety is a major determinant of the small intestinal uptake and subsequent biliary excretion of dietary quercetin glycosides. *Br. J. Nutr* **91**, 841-847, 2004.

Bandele OJ, Clawson SJ, and Osheroff N: Dietary polyphenols as topoisomerase II poisons: B ring and C ring substituents determine the mechanism of enzymemediated DNA cleavage enhancement. *Chem Res in Toxicology* **21**,1253–1260, 2008.

Bartolomeo N, Trerotoli P and Serio G: Progression of liver cirrhosis to HCC: an application of hidden Markov model. *BMC Med Res Methodol* **4**, 11:38 2011.

Benz CC, and Yau C: Ageing oxidative stress and cancer: paradigms in parallax. *Nat Rev Cancer* **8**, 875-879, 2008.

Bidlack W R, Omaye S, Meskin M and Topham D: Phytochemicals as bioactive agents. *CRC Press* **1** edition, 2000.

Bravo L: Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr Reviews* **56**, 317–333, 1998.

Breuhahn K, Longerich T and Schirmacher P: Dysregulation of growth factor signaling in human hepatocellular carcinoma. *Oncogene* **25**, 3787-800, 2006.

Britton R: Metal-induced hepatotoxicity. *Semi. Liver dis* **16**, 3-12, 1996.

Bruix J, and Sherman M: Practice Guidelines Committee, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma. *Hepatology* **42**, 1208-1236, 2005.

Calle EE, Rodriguez C, Walker-Thurmond K and Thun MJ: Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* **348**, 1625–1638, 2003.

Cao G, Booth SL, Sadowsky JA and Prior RL: Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables. *Am J Clin Nutr* **68**, 1081–1087, 1998.

Cermak R, Landgraf S and Wolffram S: The bioavailability of quercetin in pigs depends on the glycoside moiety and on dietary factors. *J Nut* **133**, 2802-2807, 2003.

Chang Q, Zuo Z, Chow MS and Ho WK: Difference in absorption of the two structurally similar flavonoid glycosides, hyperoside and isoquercitrin, in rats. *Eur J Pharm Biopharm* **59**, 549-555, 2005.

Chen Y, Gelfond J, McManus L and Shireman PK: Reproducibility of quantitative RT-PCR array in miRNA expression profiling and comparison with microarray analysis. *BMC Genomics* **10**, 407, 2009.

Chirumbolo S: Quercetin in cancer prevention and therapy. *Integr Cancer Ther* **12**,97-102, 2013.

Chu YF, Sun J, Wu X and Liu RH: Antioxidant and antiproliferative activities of vegetables. *J Agric Food Chem* **50**, 6910–6916, 2002.

Chuang SC, Vecchia CL and Boffetta P: Liver cancer: Descriptive epidemiology and risk factors other than HBV and HCV infection. *Can Lett* **286:1**, 9–14, 2009.

Cos P, Ying L, Calomme M, Hu JP, Cimanga K, Van Poel B, et al.: Structure–activity relationship and classification of flavonoids as inhibitors of xantine oxidase and superoxide scavengers. *J Nat Prod* **61**,71–6, 1998.

Davila JA: Diabetes and Hepatocellular Carcinoma: What Role Does Diabetes Have in the Presence of Other Known Risk Factors? *Am J Gastroenterol* **105**, 632–634, 2010.

Deng R, Tang J, Xie BF, Feng GK, Huang YH, Liu ZC, et al.: SYUNZ-16, a newly synthesized alkannin derivative, induces tumor cells apoptosis and suppresses tumor growth through inhibition of PKB/AKT kinase activity and blockade of AKT/FOXO signal pathway. *Int J Cancer* **127(1),** 220-9, 2010.

Diebolt M, Bucher B and Andriantsitohaina R: Wine polyphenols decrease blood pressure, improve NO vasodilation, and induce gene expression. *Hypertension* **38**, 159–165, 2001.

dos Santos MD, Almeida MC, Lopes NP and de Souza GE: Evaluation of the Anti-inflammatory, Analgesic and Antipyretic Activities of the Natural Polyphenol Chlorogenic Acid. *Biol & Pharma Bulletin* **29**, 2236–40, 2006.

Duthie GG, Duthie SJ and Kyle JA: Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. *Nutr Res Rev* **13**, 79-106, 2000.

El-Serag HB, Tran T and Everhart JE:Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology. 2004, 126(2):460-8.

Erlund I, Freese R, Marniemi J, Hakala P and Alfthan G: Bioavailability of quercetin from berries and the diet. *Nutr Cancer* **54**, 13–17, 2006.

Erlund I, Kosonen T, Alfthan G, et al: Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. *Eur J Pharmacol* **56**, 545-554, 2000.

Ferrali M, Signorini C, Caciotti B, Sugherini L, Ciccoli L, Giachetti D, et al: Protection against oxidative damage of erythrocyte membranes by the flavonoid quercetin and its relation to iron chelating activity. *FEBS Lett* **416**, 123–9, 1997.

Ferry DR, Smith A, Malkhandi J, Fyfe DW, deTakats PG, Anderson D, et al: Phase I clinical trial of the flavonoid quercetin: pharmacokinetics and evidence for in vivo tyrosine kinase inhibition. *Clin Cancer Res* **2**, 659-68, 1996

Gao S and Hu M: Bioavailability challenges associated with development of anti-cancer phenolics. *Mini Rev Med Chem* **10**, 550-67, 2010.

García-Lafuente A, Guillamón E, Villares A, Rostagno MA and Martínez JA: Flavonoids as antiinflammatory agents: implications in cancer and cardiovascular disease. *Inflamm Res* **58**,537–552, 2009.

Gee JM, DuPont MS, Day AJ, Plumb GW, Williamson G and Johnson IT: Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. *J Nutr* **130**, 2765-2771, 2000.

Gerhouser, C: Cancer Chemopreventive Potential of Apples, Apple Juice, and Apple Components. *Planta Med.***74**, 1608-24, 2008.

Gibbs WW: Untangling the roots of cancer. *Scientific American* **289**, 57–65, 2003.

Gibellini L, Pinti M, Nasi M, Montagna JP, De Biasi S, Roat E, et al:. Quercetin and cancer chemoprevention. *Evid Based Complement Alternat Med* **2011**,591356, 2011.

Granado-Serrano AB, Martín MA, Bravo L, Goya L, Ramos S.. Quercetin Induces Apoptosis via Caspase Activation, Regulation of Bcl-2, and Inhibition of PI-3-Kinase/Akt and ERK Pathways in a Human Hepatoma Cell Line (HepG2). *J. Nutr* **136**, 2715–2721, 2006.

Hardman WE: Omega-3 fatty acids to augment cancer therapy. *J Nutr* **132**,3508–12, 2002.

Heck MM and Earnshaw WC: Topoisomerase II: A specific marker for cell proliferation. *J. Cell Biol* **103,** 2569-2581, 1986.

Hertog MGL, Hollman PCH, and Katan MB: Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. *J Agric Food Chem* **40**, 2379–2383,1992.

Hertog MGL, Hollman PCH, and van de Putte B: Content of potentially anticarcinogenic flavonoids of tea infusions, wines and fruit juices. *J Agric Food Chem* **41**, 1242–1246, 1993.

Hollman P, Buysman M P, van Gameren Y, Cnossen E, de Vries J and Katan M: The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Rad. Res*. **31**,569-573, 1999.

Hollman PC, van Trijp J, Buysman MN, van der Gaag M, Mengelers MJ, de Vries J, et al: Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett* **418**,152-156, 1997.

Huang S and He X: The role of microRNAs in liver cancer progression. *Bri J Cancer* **104**, 235–240, 2011.

Hussain P, Amstad P, Raja K, Ambs S, Nagashim M, Bennett WP, et al: Increased p53 load in noncancerous colon tissue from ulcerative colitis: a cancer prone chronic inflammation disease. *Cancer Res* **60**, 3333-3337, 2000.

Iakova P, Timchenko L and Timchenko AN: Intracellular signaling and hepatocellular carcinoma. *Semin Cancer Biol* **21**, 28–34, 2011.

Issa AY, Volate SR, Wargovich MJ, et al: The role of phytochemicals in inhibition of cancer and inflammation: New directions and perspectives. *J Food Comp Analysis* **19**,405–19, 2006.

Jayakumar R and Kanthimathi MS: Inhibitory effects of fruit extracts on nitric oxide-induced proliferation in MCF-7 cells. *Food Chem* **126**, 3, 956-960, 2011.

Jo JY, Elivera GM and Lila MA: Catalytic inhibition of human DNA topoisomerase II by interactions of grape cell culture polyphenols. *J Agric Food Chem* **54**, 2083-7, 2006.

Joseph JA, Shukitt-Hale B and Casadesus G: Reversing the deleterious effects of aging on neuronal communication and behavior: beneficial properties of fruit polyphenolic compounds. *Am J Clin Nutr* **81,**313–316, 2005.

Jover R, Roque Bort M, Joseè Goèmez-Lechoèn and Joseè V. Castell: Re-expression of C/EBPK induces CYP2B6, CYP2C9 and CYP2D6 genes in HepG2 cells. *FEBS Letters* **431,** 227-230, 1998.

Kew M: Hepatitis B virus x protein in the pathogenesis of hepatitis B virus-induced hepatocellular carcinoma. *Journal of Gastroenterology and Hepatology* **26** Suppl. 1, 144–152, 2011.

Khushwant S and Rupasinghe HPV: Polyphenols: Multipotent therapeutic agents in neurodegenerative diseases. *Oxid Medi and Cell Longevity* **2013**,891748 2013.

Kitagawa S, Tanaka Y, Tanaka M, Endo K and Yoshii A: Enhanced skin delivery of quercetin by microemulsion. *JPP* **61**, 855–860, 2009.

Knekt P, Kumpulainen J, Järvinen R, Rissanen H, Heliövaara M, Reunanen A, et al: Flavonoid intake and risk of chronic diseases. *Am J Clin Nutr* **76**,560-8, 2002.

Kuo PC, Liu HF and Chao JI: Survivin and p53 modulate quercetin-induced cell growth inhibition and apoptosis in human lung carcinoma cells. *J Biol Chem* **279**, 55875-85, 2004.

Kuper H, Ye W, Broome U, Romelsjo A, Mucci LA and Ekbom A: The risk of liver and bile duct cancer in patients with chronic viral hepatitis, alcoholism or cirrhosis. *Hepatology* **34**, 714–718, 2001.

Lagiou P, Kuper H, Stuver S, Tzonou A, Trichopoulos D and Adami H: Role of Diabetes Mellitus in Etiology of Hepatocellular carcinoma. *J Natl Cancer Inst* **92**,1096-1099, 2000.

Lanoue L, Green K, Kwik-Uribe C and Keen Carl: Dietary factors and the risk for acute infant leukemia: evaluating the effects of cocoa-derived flavanols on DNA topoisomerase activity. *Exp Biol and Medi* **235**,77-89, 2010.

Lau CS, Ho CY, Kim CF, Leung KN, Fung KP, et al: Cytotoxic activities of Coriolus versicolor (Yunzhi) extract on human leukemia and lymphoma cells by induction of apoptosis. *Life Sci* **75**,797–808, 2004.

Lin YT, Hsiu SL, Hou YC, Chen HY and Lee CP: Degradation of Flavonoid Aglycones by Rabbit, Rat and Human Fecal Flora, *Biol Pharm Bull* **26**,747–751, 2003.

Liu RH: Health benefits of fruits and vegetables are from additive and synergistic combination of phytochemicals. *Am J Clin Nutr* **78**, 517S–520S, 2003.

Liu Z, Hou J: Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) Dual Infection. *Int J Med Sci* **3**,57-62, 2006.

Lobner D: Journal of neuroscience methods, (2002), Vol 96, issue 2, Pages 147-152.

Lopez-Lazaro M, Willmore E and Austin CA: The dietary flavonoids myricetin and fisetin act as dual inhibitors of DNA topoisomerases I and II in cells. *Mutation Research* **696,** 41–47, 2010.

Lugli ER, Ferraresi E, Roat L, Troiano M, Pinti M, Nasi M et al: Quercetin inhibits lymphocyte activation and proliferation without inducing apoptosis in peripheral mononuclear cells. *Leukemia Res* **33**, 140–150, 2009.

Luqman S, Rizvi SI: Protection of lipid peroxidation and carbonyl formation in proteins by capsaicin in human erythrocytes subjected to oxidative stress. *Phytother Res* **20**,303–306, 2006.

Mainou-Fowler T, Proctor SJ and Dickinson AM: Gamma-linolenic acid induces apoptosis in B-chronic lymphocytic leukaemia cells in vitro. *Leuk Lymph* **40**,393–403, 2001.

Manach C, Williamson G, Morand C, Scalbert A and Rémésy C: Bioavailability and bioefficacy of polyphenol in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* **81**(Suppl 1), 230S–242S, 2005.

Manson MM: Cancer prevention – the potential for diet to modulate molecular signalling. *Trends Mol. Med* **9**, 11–18,2003.

Marrero JA, Kudo K and Bronowicki JP: The Challenge of Prognosis and Staging for Hepatocellular Carcinoma. *The Oncologist*, **15**, 23-33, 2010.

Marryann S, Curier S, Bailey E and Essingmann J: The chemistry and biology of aflatoxin B1, *Carcinogenesis* **22**,535-545, 2001.

Maurya PK and Rizvi SI: Protective role of tea catechins on erythrocytes subjected to oxidative stress during human aging. *Nat Prod Res* **23**, 1072-9, 2009.

Mellou F, Lazari D, Skaltsa H, Tselepis AD, Kolisis FN and Stamatis H: Biocatalytic preparation of acylated derivatives of flavonoid glycosides enhances their antioxidant and antimicrobial activity. *J Biotech*, **116**, 295–304,2005.

Miura T, Chiba M, Kasai K, Nozaka H, Nakamura T, Shoji T et al: Apple procyanidins induce tumor cell apoptosis through mitochondrial pathway activation of caspase-3. *Carcinogenesis* **29**, 585–593, 2008.

Moller H, Mellemgaard A, Lindvig K and Olsen JH: Obesity and cancer risk: a Danish record-linkage study. *Eur J Cancer* **30A**,344–350, 1994.

Morand C, Manach C, Crespy V and Remesy C: Quercetin 3-O-b-glucoside is better absorbed than other quercetin forms and is not present in rat plasma. *Free Rad Res* **33**,667-676, 2000.

National Cancer Institute (NCI), What You Need To Know About™ Liver Cancer booklet (NIH Publication No. 09-5009), (2009).

NCI, National Cancer Institute. Adult primary liver cancer treatment PDQ. July 8, (2010), www.cancer.gov.

Ng QS: Effect of nitric oxide synthase on tumour blood volume and vascular activity: A phase I study. *Lancet Onco* **8**, 111-118, 2007.

Nguyen T, Tran E, Nguyen T, Do P, Huynh T and Huynh H: The role of activated MEK-ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells. *Carcinogenesis* **25**, 647–59, 2004.

Nijveldt RJ, van Nood E, van Hoorn DEC, Boelens PG, van Norren K and van Leeuwen PAM: Flavonoids: a review of probable mechanisms of action and potential applications. *Am J Clin Nutr* **74**,418–25, 2001.

Noe´ V, Pen˜uelas S, Lamuela-Ravento´s RM, Permanyer J, Ciudad CJ and Izquierdo-Pulido M: Epicatechin and a Cocoa Polyphenolic Extract Modulate Gene Expression in Human Caco-2 Cells. *J Nutr* **134**, 2509–2516, 2004.

Olsen SK, Brown RS and Siegel AB: Hepatocellular carcinoma: review of current treatment with a focus on targeted molecular therapies. *Therap Adv Gastroenterol* **3**,55-66, 2010.

Olthof MR, Hollman PC, Vree TB and Katan MB: Bioavailabilities of quercetin-3-glucoside and quercetin-4'-glucoside do not differ in humans. *J Nutr* **130**,1200-3, 2000.

Padilla E, Ruiz E, Redondo S, Gordillo-Moscoso A, Slowing K and Tejerina T: Relationship between vasodilation capacity and phenolic content of Spanish wines. *Eur J pharma* **517**, 84–91, 2005.

Palumbo MO, Kavan P, Miller WH Jr, Panasci L and Assouline S:Systemic cancer therapy: achievements and challenges that lie ahead. *Front Pharmacol* **7**,4:57, 2013.

Pandey KB and Rizvi SI: Protective effect of resveratrol on markers of oxidative stress in human erythrocytes subjected to in vitro oxidative insult. *Phytother Res* **24** Suppl 1:S11-4, 2010.

Pandey KB, Mishra N and Rizvi SI: Protective role of myricetin on markers of oxidative stress in human erythrocytes subjected to oxidative stress. *Nat Prod Commun*. **4**, 221–226, 2009.

Parikh S and Hyman D: Hepatocellular Cancer: A Guide for the Internist. *The Am J Medi* **120,** 194-202, 2007.

Parkin DM, Bray F and Ferlay J: Estimating the world cancer burden: Globocan (2000), *Int J Cancer* **94**, 153-156, 2001.

Passamonti S, Terdoslavich M, Franca R, Vanzo A, Tramer F, Braidot E, et al: Bioavailability of flavonoids: a review of their membrane transport and the function of bilitranslocase in animal and plant organisms. *Curr. Drug. Metabol* **10**,369–394, 2009.

Paterlini P, Poussin K and Kew MC: Selective accumulation of the x transcript of hepatitis B virus in patients negative for hepatitis B surface antigen with hepatocellular carcinoma. *Hepatology* **21**, 313–21, 1995.

Perez R M: Antiviral Activity of Compounds Isolated From Plants". *Pharma Biol* **41**, 107–57, 2003.

Potenza L, Calcabrini C, De Bellis R, Mancini U, Cucchiarini L and Dachà M: Effect of quercetin on oxidative nuclear and mitochondrial DNA damage. *Biofactors* **33**,33-48, 2008.

Priyadarsini VR, Murugan SR, Maitreyi S, Ramalingam K, Karunagaran D and Nagini S: The flavonoid quercetin induces cell cycle arrest and mitochondria-mediated apoptosis in

human cervical cancer (HeLa) cells through p53 induction and NF-κB inhibition. *Eur J Pharmacol* **649**,84-91,2010.

Procházková D, Boušová I and Wilhelmová N; Antioxidant and prooxidant properties of flavonoids. *Fitoterapia* **82**,513-23, 2011.

Rauha J, Remes S, Heinonen M, Hopia A, Kähkönen M, Kujala T, et al: Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Intern J Food Microbiol* **56,**3–12, 2000.

Raynard B, Balian A, Fallik D, Capron F, Bedossa P, Chaput JC and Naveau S: Risk factors of fibrosis in alcohol-induced liver disease. *Hepatology* **35**,635-8, 2002.

Razavi SM, Zahri S, Zarrini G, Nazemiyeh H and Mohammadi S: Biological activity of quercetin-3-O-glucoside, a known plant flavonoid. *Bioorg Khim* **35**,414-6, 2009.

Rice Evans C, Miller NJ and Paganga G: Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med* **20**,933-956, 1996.

Rice-Evans C, Flavonoid antioxidants. *CurrMedi Chem* **8**,797–807, 2001.

Robert C, Soria JC, Spatz A, Le Cesne A, Malka D, Pautier P, et al: Cutaneous side-effects of kinase inhibitors and blocking antibodies. *Lancet Oncol* **6**,491-500, 2005.

Romagnolo DF and Selmin OI: Flavonoids and cancer prevention: a review of the evidence. *J Nutr Gerontol Geriatr* **31**,206-38, 2012.

Rudolph KL, Chang S, Millard M, Schreiber-Agus N and DePinho RA: Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery. *Science* **287**,1253–1258, 2000.

Rupasinghe HPV, Erkan N, and Yasmin A:. Antioxidant protection of eicosapentaenoic acid and fish oil oxidation by polyphenolic-enriched apple skin extract. *J. Agric. Food Chem* **58**,1233-1239, 2010.

Rupasinghe HPV, Thilakarathna S, and Nair S: 2012. Polyphenols of Apples and Their Potential Health Benefits. In: Polyphenols: Chemistry, Dietary Sources and Health Benefits (Eds.) J. Sun, K. N. Prasad, A. Ismail, B. Yang, X. You and L. Li, Nova Science Publishers, Inc. Hauppauge, NY, USA.pp. 333-368. ISBN: 978-1-62081-868-8.

Russo M, Spagnuolo C, Tedesco I, Bilotto S, Russo GL: The flavonoid quercetin in disease prevention and therapy: facts and fancies. *Biochem Pharmacol* **83**,6-15, 2012.

Russo M, Spagnuolo C, Tedesco I, Russo GL: Phytochemicals in cancer prevention and therapy: truth or dare? *Toxins (Basel)* **2**, 517-51, 2010.

Ryder S: Guidelines for the diagnosis and treatment of hepatocellular carcinoma (HCC) in adults. *Gut* **52**(Suppl 3): iii1–iii8, 2003.

Salem J, Chevalot I, Schiavo CH, Paris C, Fick M and Humeau C: Biological activities of flavonoids from *Nitraria retusa* (Forssk.) Asch. and their acylated derivatives. *Food Chem* **124**,486–494, 2011.

Salem J, Humeau , Chevalot I, Schiavo CH, Vanderesse R, Blanchard F and Fick M: Effect of acyl donor chain length on isoquercitrin acylation and biological activities of corresponding esters. *Process Biochem* **45**, 382–389, 2010.

Sawant SG, Gregoire V, Dhar S, Umbricht CB, Cvilic S, Sukumar S et al: Telomerase activity as a measure for monitoring radiocurability of tumor cells. *The FASEB J* **13**,1047-1054,1999.

Scalbert A, Johnson IT and Saltmarsh M: Polyphenols: antioxidants and beyond. *Am J Clin Nutr* **81**(1 Suppl), 215S-217S, 2005.

Serag BH and Rudolph KL: Hepatocellular Carcinoma: Epidemiology and Molecular Carcinogenesis. *Gastroenterology* **132**,2557–2576, 2007.

Shan B, Wang M, Li R: Quercetin Inhibit Human SW480 Colon Cancer Growth in Association with Inhibition of Cyclin D1 and Survivin Expression through Wnt/β-Catenin Signaling Pathway. *Can Investi* **27**,604–612, 2009.

Shen S, Chen Y, Hsu F and Lee W. Differential apoptosis-inducing effect of quercetin and its glycosides in human promyeloleukemic HL-60 cells by alternative activation of the caspase 3 cascade. *J Cell Biochem* **89**,1044-55, 2003.

Singhal R, Yeh YA, Praja N, Olah E, Sledge GJ and Weber G: Quercetin down-regulates signal transduction in human breast carcinoma cells. *Biochem Biophys Res Commun* **208**,425–31, 1995.

Soundararajan R, Rupasinghe HP, Wishart AD, Arcellana-Panlilio M, Nelson CM, Mayne M and Robertson GS: Quercetin 3-glucoside protects neuroblastoma (SH-SY5Y) cells in vitro against oxidative damage by inducing sterol regulatory element-binding protein-2-mediated cholesterol biosynthesis. *J Biol Chem* **283**,2231-45, 2008.

Stagos D, Amoutzias GD, Matakos A, Spyrou A, Tsatsakis AM and Kouretas D: Chemoprevention of liver cancer by plant polyphenols. *Food Chem Toxicol* **50**,2155-70, 2012.

Stern M C, Umbach D M and Yu MC: Hepatitis B, Aflatoxin B_1, and p53 Codon 249 Mutation in Hepatocellular Carcinomas from Guangxi, People's Republic of China, and a Meta-analysis of Existing Studies. *Cancer Epidemiol Biomarkers Prev* **10**,617-625, 2001.

Suh S and Kim KW: Diabetes and cancer: is diabetes causally related to cancer? *Diabetes Metab J* **35**,193-8, 2011.

Sun J, Chu YF, Wu X and Liu RH; Antioxidant and antiproliferative activities of fruits. *J. Agric. Food Chem* **50**,7449–7454, 2002.

Talib W and Mahasneh A: Antiproliferative Activity of Plant Extracts Used Against Cancer in Traditional Medicine. *Sci Pharm* **78**,33–45, 2010.

Tanigawa S, Fujii M and Hou DX: Stabilization of p53 is involved in quercetin-induced cell cycle arrest and apoptosis in HepG2 cells. *Biosci Biotechnol Biochem* **72**,797-804, 2008.

Temple NJ: Antioxidants and disease: more questions than answers. *Nutr. Res* **20**,449–459, 2000.

Theise N, Park Y and Kojiro M: Dysplastic nodules and hepatocarcinogenesis. *Clin Liv Dis* **6**,497–512, 2002.

Thorgeirsson S, Grisham J: Molecular Pathogenesis Of human hepatocellular carcinoma. *Nat genetics* **31**, 339-46, 2002.

Tommasi S, Pinto R, Pilato B and Paradiso A: Molecular pathways and related target therapies in liver carcinoma. *Curr Pharm Des* **13**,3279-87, 2007.

Vargas AJ and Burd R: Hormesis and synergy: pathways and mechanisms of quercetin in cancer prevention and management. *Nutr Rev* **68**,418-28, 2010.

Vitor RF, Mota-Filipe H, Teixeira G et al: Flavonoids of an extract of Pterospartum tridentatum showing endothelial protection against oxidative injury. *J Ethnopharmacol* **93**,363-370, 2004.

Wang X, Hussain S, Huo T, Wu C, Forgues, Hofseth L, Brechot C and Harris C: Molecular pathogenesis of human hepatocellular carcinoma. *Toxicology* **181–182**,43-47, 2002.

Wang X, Gibson M., Vermeulen W, Yeh H, Forrester K, Stürzbecher H, et al: Abrogation of p53 induced apoptosis by the hepatitis B virus X gene. *Cancer Res* **55**,6012-6, 1995.

Wilkening S, Stahl F and Bader A: Comparison of primary human hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. *Drug Metab.Dispos* **31**,1035-1042, 2003.

Willett WC: Balancing life-style and genomics research for disease prevention. *Science* **296,**695–698, 2002.

Williams L and Wilkins: Professional Guide to Diseases, Ninth edition, Chapter, **5**, *Hepatobiliary disorders* 299-301, 2009.

Williamson G and Manach C: Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am J Clin Nutr* **81**,243S-255S, 2005.

Wolk A, Gridley G, Svensson M, et al: A prospective study ofobesity and cancer risk (Sweden). *Cancer Causes Control* **12**,13–21, 2001.

World Health Organisation, February (2010),
www.who.int/mediacentre/factsheets/fs297/en/

www,atcc.org.

Yang KC, Tsai CY, Wang YJ, Wei PL, Lee CH, Chen JH et al: Apple polyphenol phloretin potentiates the anticancer actions of paclitaxel through induction of apoptosis in human hep G2 cells. *Mol Carcinog* **48**,420-31, 2009.

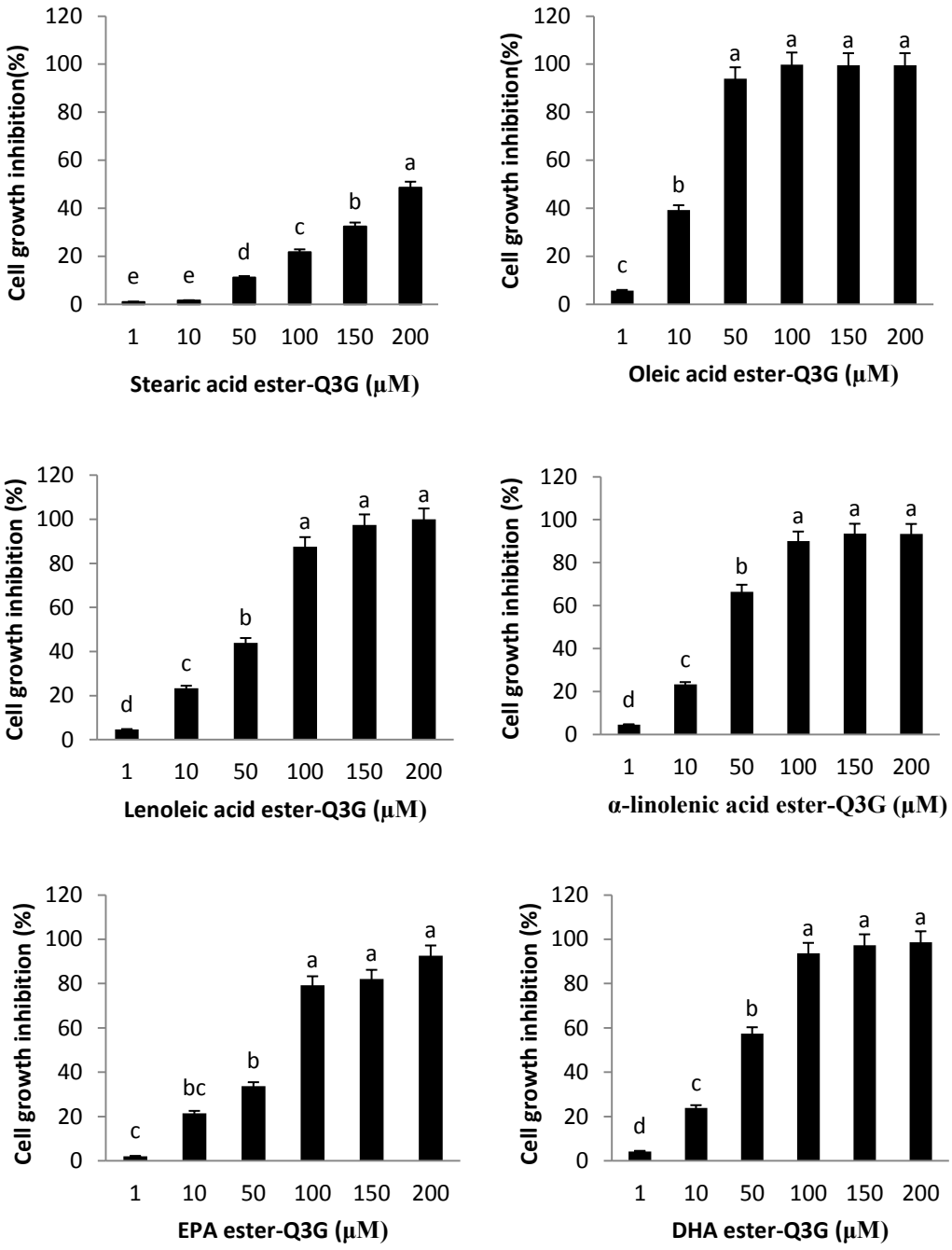
Yen GC, Duh PD, Tsai HL and Huang SL: Pro-oxidative properties offlavonoids in human lymphocytes. *Biosci Biotechnol Biochem* **67**,1215–22, 2003.

You HJ, Ahn HJ and Ji GE: Transformation of Rutin to Antiproliferative Quercetin-3-glucoside by Aspergillus niger. *J. Agric. Food Chem* **58**, 10886–10892, 2010.

Zhang KY, Tung BY and Kowdley KV: Liver transplantation for metabolic liver diseases. *Clin Liver Dis* **11**,265–281, 2007.

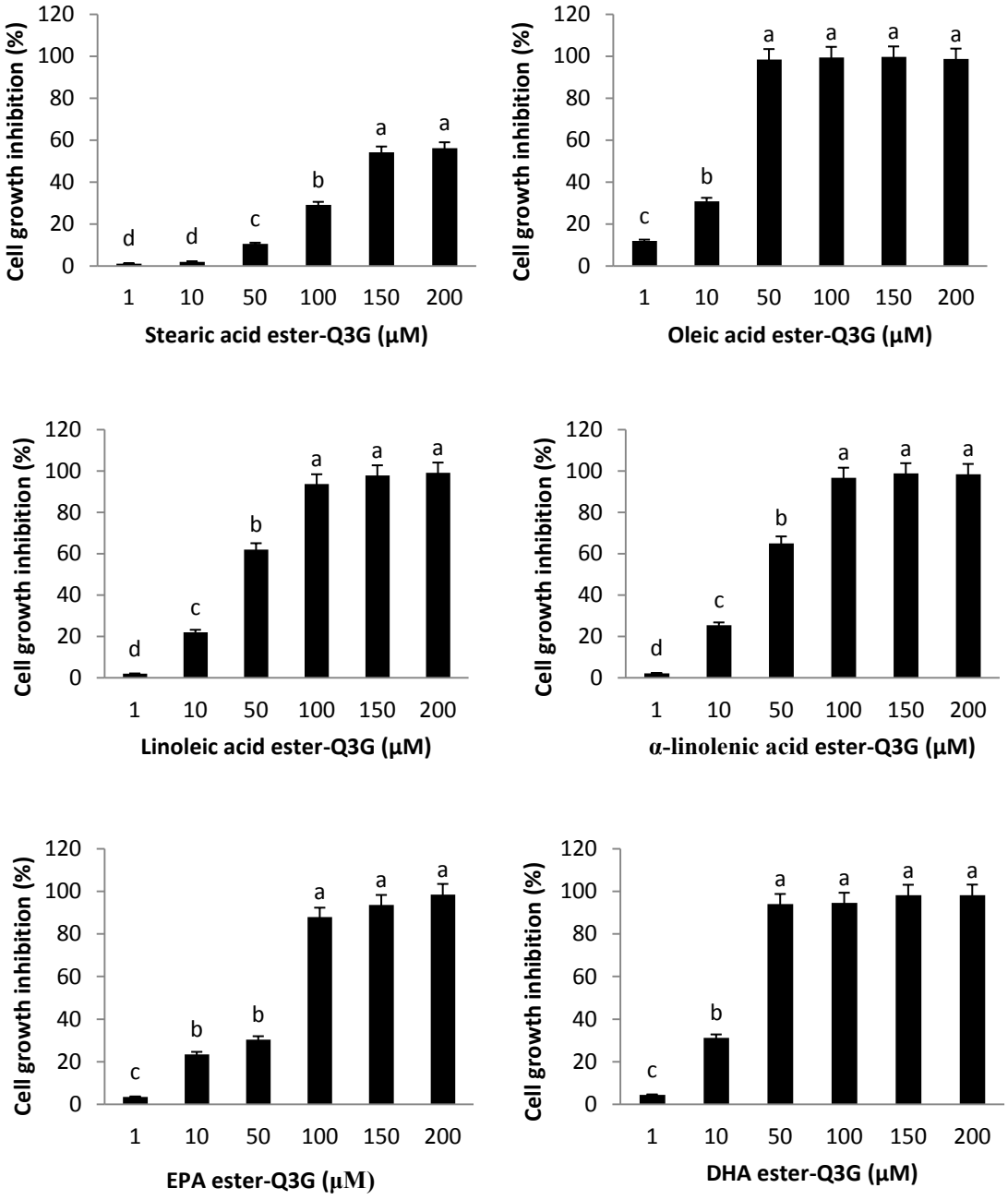
Ziaullah, Khushwant SB, Sumudu NW and Rupasinghe HPV: Biocatalytic synthesis, structural elucidation, antioxidant capacity and tyrosinase inhibition activity of long chain fatty acid acylated derivatives of phloridzin and isoquercitrin. *Bioorg & Medi Chem* **21**,684-692, 2013.

APPENDIX A Effect of long chain fatty acid esters of Q3G on HepG2 cells after 48 h of treatment.



Analysis of cell growth inhibition upon treatment with long chain fatty acid esters of Q3G in Hep G2 cells after 48 h. Values with different letters differ significantly ($p<0.05$)

APPENDIX B Effect of long chain fatty acid esters of Q3G on HepG2 cells after 72 h of treatment



Analysis of cell growth inhibition upon treatment with long chain fatty acid esters of Q3G in Hep G2 cells after 72 h. Values with different letters differ significantly ($p<0.05$)