**University of Alberta**

# Mitochondrial Dysfunction and Induction of Apoptosis in Hepatocellular Carcinoma and Cholangiocarcinoma Cell Lines by Thymoquinone

by

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**Abstract**

Thymoquinone, the plant-based bioactive constituent derived from the volatile oil of *Nigella sativa*, has been shown to possess considerable anti-neoplastic activity. The present study aimed to investigate the anti-tumour property of TQ against hepatocellular carcinoma (HepG2) and cholangiocarcinoma (HuCCT1) cells, the two most common primary hepatic tumours. All cell lines were treated with increasing concentrations of TQ for varying durations. The anti-proliferative effect of TQ was measured using the MTS assay and resulted in dose- and time-dependent growth inhibition in both cell lines. The analysis of cell cycle distribution, determination of apoptosis, assessment of morphological alterations and measurement of mitochondrial membrane potential changes were also investigated. TQ caused cell cycle arrest at different phases in the two cell types and induced apoptosis in both cell lines through the mitochondrial pathway. Overall, these findings suggest that TQ possesses promising therapeutic potential as an anti-tumour agent for treating hepatocellular carcinoma and cholangiocarcinoma.

## Dedication

I would like to dedicate this thesis to my beloved parents, Jafar Abdulmajid and Hayat Adham, for their constant support, encouragement and inspiration. To my wonderful siblings, Dr. Turki Abdualmjid and Roba Abdualmjid, for their unconditional love and support, which motivates me to work harder.

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## Abbreviations

| | |
|---|---|
| **ACF** | Aberrant crypt foci |
| **AF** | Aflatoxin |
| **AFB1** | Aflatoxin Bl |
| **AFP** | Alfa-fetoprotein |
| **AIF** | Apoptosis inducing factor |
| **AKT/PKB** | Protein Kinase B |
| **Apaf-1** | Apoptotic protease activating factor 1 |
| **AR** | Androgen receptor |
| **ATP** | Adenosine triphosphate |
| **Bcl-2** | B-cell lymphoma 2 |
| **BEGM** | Bronchial Epithelial Cell Growth Medium |
| **Bp** | Base pair |
| **BP** | Benzo(a)pyrene |
| **CA 125** | Carbohydrate antigen-125 |
| **CA 19-9** | Carbohydrate antigen 19-9 |
| **CCA** | Cholangiocarcinoma |
| **CDKs** | Cyclin-dependent kinases |
| **CEA** | Carcinoembrionary antigen |
| **CEA** | Carcinoembryonic antigen |
| **COX-2** | Cyclooxygenase-2 |
| **CT** | Computed tomography |
| **dCCA** | Extrahepatic or distal cholangiocarcinoma |
| **DD** | Death domain |
| **DIABLO** | Direct IAP Binding protein with Low pI |
| **DISC** | Death-Inducing Signaling Complex |
| **DMEM** | Dulbecco's Modified Eagle’s Medium |
| **DMSO** | Dimethyl sulfoxide |
| **DR** | Death receptor |
| **EGFR** | Epidermal growth factor receptor |
| **ER** | Endoplasmic reticulum |

| | |
|---|---|
| **ERKs** | Extracellular-signal-regulated kinases |
| **FADD** | Fas-associated death domain |
| **FBS** | Fetal Bovine Serum |
| **HBV** | Hepatitis B virus |
| **HCC** | Hepatocellular carcinoma |
| **HCV** | Hepatitis C virus |
| **HEK293T** | Human embryonic kidney |
| **HepG2** | Hepatocellular carcinoma cells |
| **HtrA2** | Omi/high temperature requirement protein A |
| **HuCCT1** | Cholangiocarcinoma cells |
| **IAPs** | Inhibitor of apoptosis proteins |
| **ICAD** | Inhibitor of caspase-activated deoxyribonuclease |
| **iCCA** | Intrahepatic cholangiocarcinoma |
| **IL-6** | Interleukin 6 |
| **iNOS** | Inducible nitric oxide synthase |
| **JNK** | c-Jun N-terminal kinase |
| **LT** | Liver transplantation |
| **MAPK** | Mitogen Activated protein kinase |
| **Mcl-1** | Induced myeloid leukemia cell differentiation protein-1 |
| **MDR** | Multi-drug resistant |
| **MMP/Δψm** | Mitochondrial membrane potential |
| **MPT** | Mitochondrial permeability transition |
| **MRI** | Magnetic resonance imaging |
| **NAFLD** | Nonalcoholic fatty liver disease |
| **NASH** | Nonalcoholic steatohepatitis |
| **NDEA** | N-nitrosodiethylamine |
| **NF-kB** | Nuclear factor kappa B |
| **NO** | Nitric oxide |
| **OLT** | Orthotropic liver transplantation |
| **PBS** | Phosphate buffer saline |
| **pCCA** | Perihilar or Klatskin cholangiocarcinoma |

| | |
|---|---|
| **PI** | Propidium iodide |
| **PIVKA II** | Serum protein induced vitamin K absence |
| **PLK1** | Polo-like kinase 1 |
| **PPARs** | Peroxisome proliferator-activated receptors |
| **PS** | Phosphatidylserine |
| **PSC** | Primary sclerosis cholangitis |
| **PTEN** | Phosphatase and tensin homolog |
| **ROS** | Reactive oxygen species |
| **RPMI-1680** | Roswell Park Memorial Institute |
| **Smac** | Second mitochondria-derived activator of caspase |
| **STAT3** | Signal transducer and activator of transcription 3 |
| **TACE** | Transarterial chemoembolization |
| **THLE-3** | Immortalized human liver cells |
| **TNFR1** | Tumor necrosis factor receptor 1 |
| **TQ** | Thymoquinone |
| **TRADD** | TNF receptor-associated death domain |
| **TRAF2** | TNF-receptor associated factor 2 |
| **USA** | United States of America |
| **VEGF** | Vascular endothelial growth factor |

# Chapter 1: Introduction

## 1.1. Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is a highly aggressive and lethal tumor that arises from the transformation of hepatocytes, the parenchymal cells of the liver. HCC is the most common form of primary hepatic malignancy, accounting for 85% to 90% of all hepatic tumours [1, 2]. Additionally, HCC is the fifth most common tumour diagnosed worldwide, with 1 million cases diagnosed yearly. Globally, HCC is the third frequent cause of cancer-related mortality [3-5]. Nevertheless, HCC is associated with poor prognosis, as most patients present with advanced stage of HCC at the time of diagnosis and have an estimated 5-year survival of less than 5% [6].

### 1.1.1. Epidemiology

The incidence of HCC is increasing around the world and its prevalence varies according to geographical location, based on existing risk factors [7]. It has been estimated that about 78% of HCC cases are attributable to infection with hepatitis B (HBV) or hepatitis C (HCV) viruses. The highest HCC frequency is found in Sub-Saharan Africa and Southeast Asia (> 80%), where 40-90% of HCC cases are attributed to HBV infection [1]. About 50% of global cases are in China, making it the third most infected country in the world [8, 9]. In other countries like Japan, Singapore, Australia/New Zealand, UK and France, the HCC incidence is high due to HCV infection [10-12]. HCV infection is also the main leading cause of HCC in the United States of America (USA) and Europe where HCC ranked ninth most common cause of cancer death [13].

Additionally, liver cancer is the fifth most common cancer in men and the sixth in women worldwide [2]. The prevalence of HCC is higher among men than women due to sex-specific difference in exposure to environmental risk factors such as alcohol consumption and cigarette smoking [14]. Moreover, it has been reported that the high levels of testosterone correlate with HCC occurrence [15]. In addition, the mortality rate is higher in men (48%) than in women (39%) [16].

Risk of developing HCC increases with age; however, age distribution varies depending on the incidence rate, risk factors, region and sex [4]. The majority of patients are diagnosed after

age 40, except in areas where HBV infection is common [13]. The mean age of patients at diagnosis is between 50 and 70 years with women averaging 5 years older than men [4].

### 1.1.2. Risk Factors

The etiological factors for HCC are well understood. The incidence of these risk factors is associated with the frequency of HCC in each geographical area. Any agents that cause chronic injury to the liver leading to cirrhosis are considered oncogenic [7].

The main leading cause of HCC is cirrhosis, which is the end result of chronic diffuse hepatic disease. It is present in 80-90% of all HCC cases leading to hepatocarcinogenesis [17]. The main feature of cirrhosis is the amendment of normal liver into abnormal nodules with the involvement of fibrosis [18]. Other factors that increase risk of developing cirrhosis include alcohol consumption, HBV and HCV infection, and nonalcoholic steatohepatitis (NASH) [19, 20]. The typical progression is from acute hepatitis to chronic hepatitis, leading to cirrhosis and eventually the development of HCC [21].

Viral infection with HBV and HCV is a major risk factor for HCC; HBV accounts for 50-80% of HCC cases and HCV is responsible for 10-25% of cases [22, 23]. The viruses can be transmitted through transfusion of blood products, vertical transmission from mother, infected tools during invasion process, sexual contact, and intravenous injections. In Asia, Africa and western pacific countries, HBV is predominant with most people infected at birth [2, 7]. Infection with HBV produces HCC via chronic inflammation and regeneration, increasing the risk of developing HCC by 10-25%; the majority of HBV-induced HCC is due to cirrhosis (up to 90%) [4, 24]. HCV is prevalent in developed countries; infection with HCV causes chronic inflammation and cellular proliferation, leading to cirrhosis and HCC. Fibrosis was found to be an important indicator for determining the risk of developing HCC induced by HCV [25].

Chronic alcohol consumption is considered an indirect risk factor for HCC since it results in liver injury that progresses to extensive fibrosis and cirrhosis [26]. Several studies have reported the association between alcohol use and the development of HCC and reported that alcohol consumption accounted for 32-45% of cases [27]. It is a major risk factor in the US, as around 18 million people abuse alcohol [13].

In Asia and Africa, exposure to aflatoxin (AF) is a risk factor for HCC development. Aflatoxin is a mycotoxin produced by some *Aspergillus* species including *A. flavus* and *A. parasiticus*. It grows on food stored in humid conditions like rice and corn. The most common

naturally occurring aflatoxin that causes liver disease is Aflatoxin Bl (AFB1) [28]. Intake of high levels of AFB1 leads to genetic mutations of the p53 gene [29, 30]. Down-regulation of p53 is responsible for 30-60% of HCC cases in Asia, Africa and some parts of Latin America [31]. In HBV infected individuals, exposure to AF further increases the risk of developing liver cancer [32].

Some patients with HCC and cryptogenic cirrhosis are diagnosed with a severe form of nonalcoholic fatty liver disease (NAFLD) called nonalcoholic steatohepatitis (NASH) [33]. Twenty percent of patients with NASH develop cirrhosis leading to HCC, along with other complication [34]. Other factors like obesity and diabetes have been found to increase the incidence of NAFLD, in the absence of cirrhosis or consumption of alcohol [35].

### 1.1.3. Pathogenesis of HCC

Hepatocarcinogenesis is a complex, multistep process that involves a sequential number of genetic and epigenetic alterations, ultimately leading to hepatocyte malignant transformation. Carcinogenesis of HCC is influenced by various external and environmental risk factors. Several studies have been conducted to identify the molecular pathogenesis of HCC development; however, these mechanisms are not well identified and vary depending on the underlying etiology [36, 37].

The majority of HCC cases result from chronic liver disease of various etiologies, which leads to activation of hepatic inflammation, necrosis/regeneration, accumulation of intrahepatic lipid (steatosis), oxidative stress and fibrosis, cirrhosis, and eventual development of HCC with accumulation of genetic mutations [38]. Cirrhosis is the most common condition that promotes the process of hepatocarcinogenesis through activation of growth signals, resistance to growth inhibitory signals, unlimited replication, escape from apoptosis, invasiveness/metastasis and persistent angiogenesis [39, 40].

HCC has been associated with several alterations in cellular signaling pathways and genetic and genomic mutations including point mutation, chromosomal rearrangement and genomic instability [1, 36]. Several pathways have been found to be dysregulated during the process of malignant transformation in hepatocytes, including activation of the MAPK-ERK pathway, NF-κB pathway and the Wnt/β-catenin signaling pathway. Additionally, many tumour suppressor genes and oncogenes are known to be mutated in HCC, p53, retinoblastoma protein pRb and c-Myc [30, 41-44].

Recently, new techniques have been used to identify numerous genes associated with hepatocarcinogenesis. Several studies have employed DNA-microarrays analysis to determine the different molecular subtypes of HCC, with the aim to improve the identification of molecular mechanisms and guide treatment [45, 46].

### 1.1.4. Clinical presentations

Generally, HCC is asymptomatic in the early stages of disease, becoming symptomatic in its advanced stages when metastases occur [3]. Clinical symptoms of HCC patients are similar to those of patients with chronic liver disease including right superior abdominal pain, upper abdominal lump, weight loss, fatigue, anemia, nausea, vomiting, fever, and jaundice characterized by yellow eyes and skin, dark urine and pale stool [47].

### 1.1.5. Laboratory tests and diagnostic tools:

HCC is a complex disease with wide heterogeneity related to underlying etiological factors. The surveillance of a population at high risk of developing HCC, such as patients with cirrhosis or chronic viral hepatitis B or C, alcoholism or NASH, is important for the early detection of HCC.

Surveillance involves regular screening of at-risk patients using hepatic ultrasonography and α-fetoprotein (AFP) serology every 6 months [48]. Ultrasonography can aid in the assessment of blood supply injury as well as the presence of tumour vascular invasion [49]. α-fetoprotein (AFP), the most important protein of fetal serum, usually decreases after birth to undetectable levels. Its elevation (>400 ng/mL) is associated with certain pathological disorders including HCC, bile duct cancer, and gastric cancer. Therefore, AFP can aid in diagnosis but it is not specific for HCC [50, 51]. In addition, serum AFP concentration lacks accuracy as 40% of HCC patients do not produce AFP; African populations usually have normal AFP levels at 500 ng/mL [52]. Other important serum markers found to be increased in HCC patients include carcinoembrionary antigen (CEA) and serum protein induced vitamin K absence (PIVKA II) [53, 54].

Surveillance has been shown to improve outcomes and reduce mortality rates [55]. Once surveillance screening demonstrates abnormal outcomes, further diagnostic modalities are warranted. Computed tomography (CT) scans and magnetic resonance imaging (MRI) are required for early diagnosis and for determination of the size of the tumour. Liver biopsy is

conducted if lesions are more than 2 cm and cannot be identified by CT or MRI. However, hemorrhage as well as needle track tumour seeding are major drawbacks of this percutaneous procedure [56].

### 1.1.6. Treatment of HCC

There are two treatment options for HCC: curative and palliative treatment, selection is dependent upon tumor characteristics, liver function, and presence of metastasis and invasion. Curative treatments include surgical resection, liver transplantation and percutaneous ablation procedures, which may be conducted in the early stages of HCC. However, most HCC patients are diagnosed in the late stages of disease and have poor liver function to which curative treatments are not amenable. Therefore, palliative therapies, including chemotherapy, immunotherapy and hormonal therapy are available options for these patients.

#### *1.1.6.1. Surgical resection of tumour*

Surgical resection of liver is the most successful modality of treatment for non-cirrhotic and cirrhotic patients with early HCC and normal liver function, providing a 60-70% 5-year survival rate [57]. However, surgical resection is associated with high incidence of recurrence following operation [58]. It has been reported that risk of recurrence in HCC patients at 5-years is 70% [59].

#### *1.1.6.2. Liver transplantation*

Orthotropic liver transplantation (OLT) is an optimal curative option as it is effective for the treatment of both HCC and underlying liver disease like cirrhosis [60, 61]. Patients must meet certain criteria in order to be eligible for OLT; tumour diameter must be 5 cm or less, a maximum of three nodules and the absence of tumour invasion and metastasis. The survival rate of patients undergone OLT has been found to exceed 70% [62]. Nevertheless, recurrence of disease after OLT has also been reported but is less than 15% [63].

#### *1.1.6.3. Tumour Ablation*

Percutaneous ablation is another treatment option in early stage HCC. The ablation of a tumour can be performed using chemicals such as ethanol, or through temperature modifying modalities including laser, radiofrequency, or microwaves. The most commonly used ablation method is ethanol injection and radiofrequency, which has been reported to induce 100% tumour

necrosis in HCC patients [64]. The 5-year survival rate in HCC individuals after ablation has been reported to be 50-75% [65].

#### *1.1.6.3. Palliative therapies*

Patients at advanced stage of disease with unresectable and asymptomatic HCC with multiple nodules are given palliative therapies in order to relieve symptoms and improve survival [66]. Such therapies include systematic chemotherapy and hormonal therapy like doxorubicin, cisplatin, sorafenib, interferon and 5-fluorouracil [67-69] and transarterial chemoembolization (TACE) which blocks oxygen and nutrient supply to the tumour, resulting in tumour necrosis [70, 71]. However, all of these therapeutic options are ineffective in improving overall survival [7].

## 1.2. Cholangiocarcinoma

Cholangiocarcinoma (CCA) is a rare adenocarcinoma that arises from the epithelial cells of the bile duct with cholangiocyte differentiation. CCA is the second most common primary neoplasm of liver following HCC and represents 3% of all gastrointestinal malignancies [72-74]. Although hepatobiliary tumors account for 13% of cancer-related deaths, 10-20% is due to CCA [74, 75]. Moreover, CCA is an aggressive and lethal cancer characterized by poor prognosis as it is typically diagnosed at advanced stages due to its high potential to infiltrate and metastasize within the liver [72, 73].

### 1.2.1. Classifications

CCA can be classified according to its anatomical location within the biliary tree into three subtypes: intrahepatic cholangiocarcinoma (iCCA), Perihilar or Klatskin cholangiocarcinoma (pCCA) and extrahepatic or distal cholangiocarcinoma (dCCA) **Figure 1** [76]. CCA is further classified according to morphological appearance into different subtypes based on growth pattern. Intrahepatic CCA is classified into periductal-infiltrating, mass-forming, intraductal-growing and superficial-spreading [77]. Extrahepatic CCA morphological classifications includes papillary, nodular or sclerosing tumors [78]. Histologically, most CCAs are considered to be well, moderately or poorly differentiated adenocarcinoma [79].

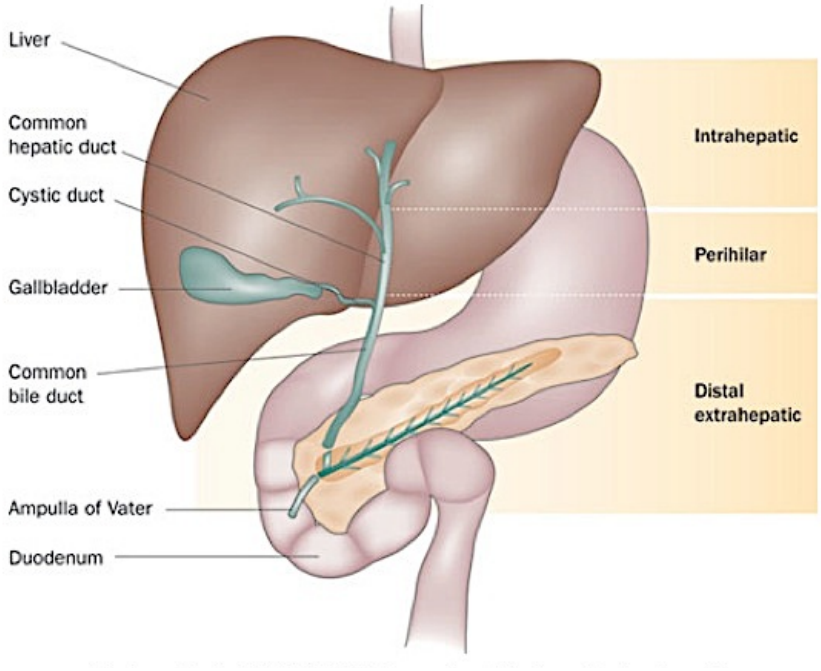



**Figure 1. Classifications of cholangiocarcinoma [80].**

### 1.2.2. Epidemiology

CCA is the second commonest primary hepatic tumor (10-15%) with 80-90% and 5-10% being attributed to extrahepatic and intrahepatic CCA, respectively [74, 75]. The incidence of CCA varies across the world with the highest reported prevalence in Thailand, China, Japan, Korea and Eastern Asia [81, 82]. In the US, around 5000 new cases of CCA per year are diagnosed, while in Europe, about 50,000 new cases of primary hepatic malignancies are diagnosed annually and 20% of these cases attributed to CCA [83]. Asian (3.3 per 100,000) and Hispanic (2.8 per 100,000) populations have higher incidences of CCA and African Americans have the lowest rate (2.1 per 100,000) [84]. These geographical variations in incidence of CCA proposed to be related to adverse environmental risk factors [75]. Additionally, it has been reported that the incidence and mortality of intrahepatic CCA is increasing globally and have a low 5-year survival rate following diagnosis (less than 5%) [83, 85, 86]. The incidence and mortality of extrahepatic CCA is decreasing, particularly in western countries, excluding Japan and Italy [87]. A slight improvement in the percentage of 5-year survival has been reported; from 11.7% in 1973-1977 to 15.1% in 1983-1987 [88].

CCA is male predominant, with 1.2-1.5 cases per 100,000 versus 1 case per 100,000 in females, with exception of Hispanic females (1.5 per 100,000 versus 0.9 per 100,000 in Hispanic

males). This is due to the prevalence of primary sclerosing cholangitis (PSC) in men. CCA is uncommon in children and the mean age of patients at time of diagnosis is 50 years [84].

### 1.2.3. Risk Factors

The majority of CCA cases are sporadic and *de novo* with unknown etiology; however, certain risk factors have been established. The most common predisposing causal factors of CCA are chronic inflammation of the biliary tree and cholestasis leading to cholangiocarcinogenesis.

PSC, an autoimmune disorder that causes cholestasis and inflammation of biliary tree, is the most definite risk factor for CCA development [89]. It is common in Western Countries with annual risk of 1.5 % and prevalence rate of 5-15% [90, 91]. Within 2 years of PSC diagnosis, patients have 30% chance of developing bile duct cancer [92].

CCA can be caused by infection with hepatic flukes including *Opisthorchis viverrini* and *Clonorchis sinensis* due to consumption of raw and undercooked fish, which is common in Southeast Asia and Japan [93, 94]. These parasites accumulate mainly in the bile duct and gallbladder and can remain for years in their hosts causing chronic inflammation [95]. Liver flukes have been associated with 8-10% of CCA cases and commonly seen in men due to high consumption of raw fish with alcohol [83].

Additionally, chronic hepatolithiasis (stones in the intrahepatic bile duct), has been linked with a 10-15% increase in risk of developing CCA [96]. It is endemic to East Asia but rare in west [97]. Hepatolithiasis is strongly associated with fluke infection [98].

Patients with biliary tree abnormalities are at higher risk of developing CCA (15-20%) including fibrocystic malformation of biliary system linked with choledochal cysts and Caroli's disease (a rare congenital disease of the bile and pancreatic ducts) [99]. Patients are usually diagnosed with cysts (intrahepatic or extrahepatic) at 32 years of age [100].

In addition, chronic HBV and HCV viral infections and cirrhosis have been implicated as risk factors for CCA [101-103].

Exposure to radionuclides and chemical agents can also lead to the development of CCA. It has been reported that thorotrast (thorium dioxide), which is used as a radiocontrast agent increases the risk of CCA up to 300 fold [104]. Other toxic agents include dioxin, vinyl chloride, radon, asbestos and nitrosamine [105].

Some other factors have a weak association with CCA risk such as alcohol, obesity, smoking, diabetes, fatty liver disease, inflammatory bowel disease, cholelithiasis and

choledocholithiasis [84, 106, 107]. Alterations in genes responsible for DNA repair may also contribute to CCA development [108].

### 1.2.4. Pathogenesis of CCA

The molecular mechanisms involved in CCA pathogenesis are not fully understood. However, some of proposed molecular pathways include autonomous or self-sufficiency proliferation, escape from senescence, resistance to apoptosis, unlimited replication as well as invasion and metastasis of tumor [74, 76].

The malignant transformation of cholangiocytes arises as a result of chronic biliary inflammation (cholangitis), associated with bile flow obstruction (cholestasis); these are unifying chronic features shared between all risk factors and thought to promote pathogenesis [109, 110]. This inflammatory environment causes defects in DNA mismatch repair genes/proteins and alterations in the expression of tumor suppressor genes and proto-oncogenes.

Additionally, chronic inflammation results in an increased release of cytokines, chemokines, tumor suppressor genes, growth factors and bile acids, which stimulates epithelial cells to contribute to the inflammation and maintain the cholangiocarcinogenic process [76, 110, 111]. A well-known pivotal cytokine is interleukin (IL-6), which plays an essential role in CCA pathogenesis. IL-6 was found to be associated with up-regulation of myeloid cell leukemia sequence 1 (MCL1), an anti-apoptotic protein of the BCL-2 family, which promotes CCA cell survival [74, 112]. Moreover, cytokines activate inducible nitric oxide synthase (iNOS), leading to overexpression of nitric oxide (NO) in CCA [113-115].

Furthermore, bile acids act as tumor promoters that induce activation of mitogen-activated protein kinase (MAPK), cyclooxygenase-2 (COX-2) and epidermal growth factor receptor (EGFR) [116, 117]. Additionally, the activation of PI3K/AKT pathway and NF-κB pathway reported to have a profound role in proliferation, metastasis, angiogenesis and inhibition of apoptosis of CCA cells [118, 119].

### 1.2.5. Clinical presentation

The clinical features of CCA depend on the intrahepatic or extrahepatic location of the tumor. Intrahepatic CCA patients are mostly asymptomatic and present with non-specific symptoms like anorexia, fatigue, abdominal pain, night sweets and palpable abdominal mass detected during physical examination or CT and/or MRI [80, 84, 120].

The signs, symptoms and biochemical markers of extrahepatic CCA resemble that of cholestasis where patients present with dark urine, pale stool, malaise, jaundice and pruritus; all signs of biliary obstruction [86].

### 1.2.6. Laboratory tests and diagnostic tools

For the diagnosis of CCA, laboratory investigation of serum and bile biomarkers is required. A widely used tumor marker is carbohydrate antigen 19-9 (CA 19-9), a glycoprotein that is elevated in 85% of CCA cases [121]. In CCA patients with PSC, a cutoff value of CA 19-9 of greater than 100 U/mL has 89% sensitivity and 86% specificity for CCA diagnosis. Additionally, carbohydrate antigen-125 (CA125) and carcinoembryonic antigen (CEA) markers are elevated in 40-50% and 30% of CCA cases, respectively. However, these biomarkers are not specific for CCA and diagnosis should not be made based on these values as they are elevated in patients with other diseases including gastrointestinal and pancreatobiliary neoplasms, hepatolithiasis and bacterial cholangitis [122]. In addition, several markers of cholestasis including bilirubin, gamma-glutamyl transferase and alkaline phosphatase are increased in CCA patients [80].

### 1.2.7. Treatment of CCA

#### *1.2.7.1. Surgical resection of tumour*

The only potential cure for CCA is surgical resection for early stage tumor [123]. Prior to surgery, evaluation of criteria including the size and location of the tumor, invasiveness and metastases, and involvement of main portal vein is required [124, 125]. Additionally, a preoperative assessment of patients' general condition, including nutritional status and diagnostic data from CT, MRI and laboratory results is essential. These evaluations are helpful in deciding whether surgery is required for patients. Also, a thorough pre-operative assessment may aid in predicting possible complications such as liver failure, and provide an indication of survival rate following surgery [126]. However, the prognosis remains poor even after bile duct resection and 5-year survival is less that 20% [123]. In some cases of CCA, patients require hepatectomy with or without pancreaticoduodenectomy. Furthermore, some cases have required more aggressive surgical resection such as semi-hepatectomy and trisegmentectomy. These resulted in a significant improvement in survival rate: 59-60% [123].

#### *1.2.7.2. Liver transplantation*

Liver transplantation (LT) is another potential therapeutic approach to CCA patients with unresectable tumour. Due to poor prognosis and possible recurrence of cancer, LT remains controversial. The initial studies for results of LT were unsatisfactory due to high recurrence rate. For example, a study done Meyer, Penn [127] involved 207 patients with CCA and they all had LT. The 2- and 5-year survival reported to be 48% and 23%, respectively. However, more than 50% of patients experienced recurrence within 2 years.

New strategies have been developed to improve the outcome of LT patients. A study carried out by Rea, Heimbach [128] reported the benefits of liver transplantation combined with neoadjuvant chemoradiation. Patients with localized hilar CCA exhibited significantly higher survival and later recurrence (average of 40 months) compared to those who had surgical resection (recurrence average of 21 months). Another study by Hong et al. (2011) reported a significant increase in 5-year survival rate (33%) following liver transplantation, without reported recurrence of CCA [129]. Moreover, the combination of liver transplant with other neoadjuvant and adjuvant therapies resulted in a survival rate of 45% and liver transplant with adjuvant therapy had a 33% survival rate. Treatment with liver transplantation alone resulted in a 20% survival rate.

#### *1.2.7.3. Palliative therapies*

Most CCA patients are diagnosed at advanced stages of disease and are not candidates for surgical treatment due to possible liver dysfunction and recurrent cholangitis associated with biliary obstruction. For such patients, other treatment options are available to relieve symptoms like jaundice, pruritus and cholangitis, prolong survival, and improve quality of life. Palliative biliary drainage is one option for treating patients with advanced stage of CCA. Other therapeutic approaches include chemotherapy such as gemcitabine [130], radiotherapy such as external beam radiation [131, 132], chemo-radiation therapy [133] and photodynamic therapy that involves the use of lasers in order to activate photosensitizing molecules that in turn generate oxygen free radicals [134].

### 1.3. Apoptosis

The mechanism by which cell death occurs involves either apoptosis or necrosis. Necrosis is passive (un-programmed) process of cell death due to a pathological response. In necrosis, the

disruption of the cell membrane occurs, causing the release of intracellular components into extracellular space, affecting neighboring cells. This results in inflammation in response to the presence of immune cells, which may stimulate the growth of tumors [135]. In contrast, apoptosis is a non-inflammatory, programmed cell death, where the cell membrane remains intact during the process [136, 137]. Distinctive features between apoptosis and necrosis are listed in **Table 1.**

Apoptosis is an important pathophysiological process of cell death that takes place in all living organisms to aid in the elimination of unwanted cells during intrauterine development and removal of damaged, infected and senescent cells [138-141]. Additionally, apoptosis plays a key role in maintaining a balance between newly growing cells and damaged cells, to preserve homoeostasis [142].

Several stimuli can trigger apoptotic cell death, such as exposure to chemotherapeutic agents, ultraviolet light or gamma radiation, death receptor signaling or withdrawal of growth factors [143].

Defects in apoptosis, on the other hand, mediate induction of several diseases such as cancer, autoimmune disorders including rheumatoid arthritis and multiple sclerosis, and neurodegenerative conditions including Parkinson's and Alzheimer's diseases [144]. Failure of normal apoptosis process plays an essential role in carcinogenesis. This deregulation could be due to imbalance between pro- and anti-apoptotic proteins, impaired caspase activity, mutations of p53 or damage to death receptor signaling [137].

### 1.3.1. Morphological hallmarks of apoptosis

The apoptotic process is regulated by several biochemical incidents, resulting in morphological alterations of cells. The morphological changes associated with apoptosis were first described in 1972 using electron microscopy [145]. Characteristic features of early apoptosis of cells include shrinkage of cells and reduction of cellular volume (pyknosis), retraction of pseudopods, condensation of chromatin and fragmentation of the nucleus [146]. Additional characteristic features of late apoptotic stage include blebbing of the plasma membrane, shrinkage of cytoplasm, loss of membrane integrity and formation of apoptotic bodies [146]. Ultimately, apoptotic cells are either phagocytosed and engulfed by macrophages or undergo another degradation process called "secondary necrosis" which resembles necrosis [147].

### 1.3.2. Biochemical hallmarks of apoptosis

Biochemical changes during apoptosis include plasma membrane alterations, DNA and protein cleavage, and caspase cascade activation [137, 141]. At the onset of the apoptotic process, the inner phosphatidylserine (PS) protein translocate to the outer surface of the plasma membrane, which allows the recognition of apoptotic cells by macrophages and permits the initiation of phagocytosis without the involvement of a secondary necrotic process or inflammation of tissues [148, 149]. Following this, DNA degradation and breakdown occurs, resulting in formation of 50-300 base pair (bp) fragments. Then, endonuclease enzymes further cleave DNA into oligonucleosomes of 180-200 bp [150]. However, internucleosomal DNA cleavage is a non-specific characteristic as it occurs in both apoptosis and necrosis [151].

Activation of the caspase cascade is another features of apoptosis. Caspases are a group of cysteine-aspartic protease enzymes which have proteolytic activity and the ability to cleave proteins specifically after an aspartate residue [152]. These caspases mainly exist in their inactive form in growing cells and their activation is necessary for the initiation and execution of apoptosis, which occurs as a result of activation of upstream (initiator) caspases (caspase-3, -8, -9 & -10) that lead to activation of downstream (effector or executioner) caspases (caspase-3, -6, & -7) [153].

**Table 1**. **Morphological and biochemical hallmark of apoptosis and necrosis [154]**

| Apoptosis | Necrosis |
|---|---|
| Regulated process | Accidental process |
| Induced by Physiological and pathological stimuli | Only pathological stimuli |
| Involves single cell | Involves cultures of cells |
| Intact plasma membrane, membrane blebbing | Impaired plasma membrane |
| Shrinkage of cell | Swelling of cell |
| Condensation of mitochondria | Swelling of mitochondria |
| Fragmentation of DNA | Swelling of nucleus |
| Formation of apoptotic bodies | No apoptotic bodies |
| Phagocytosed by phagocytes | No phagocytosis |
| No leakages of cellular components | Leakages of cellular components |
| No inflammatory response | Involvement of Inflammatory response |
| Preserved ATP | Depleted ATP |
| Involvement of oxidative stress | - |
| Release of cytochrome c | - |
| Degradation by caspases | - |

### 1.3.3. Apoptotic pathways

There are two common pathways by which apoptosis is initiated; the intrinsic (mitochondrial) pathway and the extrinsic (cytoplasmic or death receptor) pathway [155]. Both pathways are linked together and converge to a final caspase pathway, leading to activation of several caspase cascades resulting in apoptosis [137, 156]. A third pathway has been proposed called the intrinsic endoplasmic reticulum pathway; little is known about this pathway.

#### *1.3.3.1. The intrinsic or mitochondrial-mediated pathway*

The intrinsic signaling pathway is initiated within the cell by several intracellular events including severe oxidative stress, hypoxia, hormones, DNA damage, cytokines, viral infection, toxins, radiation and elevated levels of cytosolic calcium ions. These stimuli result in changes in mitochondrial membrane potential and the formation of pores. Additionally, these stimuli

mediate the release of important apoptogenic molecules from the mitochondrial membrane space into the cytoplasm such as cytochrome c, which is responsible for outer membrane permeabilization [157].

Other released apoptogens include apoptosis inducing factor (AIF), Omi/high temperature requirement protein A (HtrA2), direct IAP Binding protein with Low pI (DIABLO) and second mitochondria-derived activator of caspase (Smac) [158].

The intrinsic pathway is regulated by two subgroups of proteins belonging to the Bcl-2 family [159]. The first subgroup includes pro-apoptotic proteins such as Bid, Bax, Bad, Bak, Bim, Bcl-Xs and Hrk, which regulate apoptotic pathways by promoting the release of cytochrome c from the mitochondria. The second subgroup consists of anti-apoptotic proteins, including Bcl-2, Bcl-W, Bcl-XL, Mcl-1 and Bfl-1, which regulate apoptosis via blocking of cytochrome c release. Accordingly, in order to initiate apoptosis, a balance between these pro- and anti-apoptotic proteins must be determined [160].

Once cytochrome c is released into the cytoplasm, it binds to apoptotic protease activating factor 1 (Apaf-1) and caspase-9 leading to creation of the apoptosome. Subsequently, this complex activates downstream caspases (caspase-6, -7 & -3). Omio/HtrA2, Smac/ DIABLO induces apoptosis by inhibiting the activity of inhibitor of apoptosis proteins (IAPs) [158, 161].

#### *1.3.3.2. The extrinsic or death receptor-mediated pathway*

The extrinsic signaling pathway is initiated once a death ligand binds to its death receptor (DR) in response to extracellular stimuli such as cytotoxic drugs, chemicals, stressors or poisons. These DR are membrane-bound proteins present on cell surface that are responsible for apoptotic signaling transmission once ligated with appropriate ligands. Commonly known death receptor complexes include tumor necrosis factor receptor 1 (TNFR1) and its ligand TNF, Fas receptor and its ligand FasL [149], and Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (elmore, 2007). Each receptor has an intracellular, cytoplasmic death domain (DD), which recruits and binds adaptor proteins. These DD are TNF receptor-associated death domain (TRADD), Fas-associated death domain (FADD) and caspase 8 [162-164].

Once ligation occurs, the adaptor proteins with corresponding death domains are recruited, leading to the formation of a complex called Death-Inducing Signaling Complex (DISC) that triggers the assembly and activates caspase 8 [156]. The activation of caspase 8

further activates downstream (executioner) caspases without the involvement of mitochondria, resulting in cell death. This apoptotic process is known as the caspase-dependent pathway.

#### *1.3.3.3. Intrinsic endoplasmic reticulum (ER) pathway*

This ER pathway is the third apoptotic pathway, which is a less common pathway that is mitochondrial-independent and caspase-12-dependent. This pathway is initiated once the ER is injured by cellular stresses such as hypoxia, glucose starvation or exposure to free radicals, leading to ER unfolding and reduced cellular protein synthesis. Additionally, TNF-receptor associated factor 2 (TRAF2) is an adaptor protein present in its inactive form bound to procaspase-12 in normal cells. Following ER injury, dissociation of this bond occurs, activating caspase-12 and leading to caspase-9 and caspase-3 cleavage and eventually apoptosis [137, 165-167].

#### *1.3.3.4. Common or execution pathway*

Caspases play a central role in both the extrinsic and intrinsic pathway, as they are involved as both initiators (caspase-2,-8,-9,-10) and effectors or executioners (caspase-3,-6,-7). Eventually, the endpoint of both pathways is the final common pathway, where the execution phase starts. The initiator caspases for both the extrinsic and intrinsic pathway are caspase 8 and caspase 9, respectively. Once activated, they activate the executioner caspases, with caspase-3 being the most important. This leads to activation of endonucleases and proteases that cleave DNA repair proteins (e.g., PARP), inhibitory subunits of endonucleases, cytoskeletal proteins, and protein kinases [168]. Moreover, activated caspase-3 degrades the inhibitor of caspase-activated deoxyribonuclease (ICAD) through endonuclease activation, resulting in CAD release and DNA degradation [169]. These downstream caspases affect signaling pathways, the cell cycle and cytoskeleton. Consequently, these outcomes contribute to both the morphological and biochemical alterations involved in apoptosis [168, 170].

### 1.4. Cell cycle progression

Cell cycle is a well-controlled and highly coordinated series of phases in which a cell replicates, grows, and divides the genetic materials into daughter cells. The cell cycles through a set of 4 interphases: G1, S, G2 and M phases. G1 (gap 1) is the entry point of a cell into the cell cycle, where it prepares for the process of DNA replication. DNA synthesis occurs during S phase. G2, the second gap in the cell cycle involves cellular preparation for division. Mitosis, or

M phase, occurs when condensation of DNA takes place and chromosomes segregate into two daughter cells, each with identical copies of genetic material (**Figure 2**) [171, 172]. Additionally, there is a quiescent phase or resting state (G0), which cells in G1 enter, in the absence of cell cycling stimuli. Most non-growing human cells remain in G0 phase [173, 174].

The completion of each phase of the cell cycle prior to transition to the next phase is essential to ensure accurate DNA replication and chromosome separation. This process regulates cellular proliferation and prevents cells with damaged DNA from proceeding to the next phase. Mammalian cells have developed a regulatory system of checkpoints that control cell cycle. These checkpoints regulate the rate and quality of the division of cells and arrest cells with damaged DNA before proceeding to M phase [175]. There are three checkpoints for DNA damage; the G1 checkpoint, which occurs before entry into S phase, the G2/M checkpoint follows DNA replication and the M checkpoint takes place during mitosis [176]. Among these, the G1 and G2 checkpoints have the most significant role in the cell cycle as the G1 checkpoint prevents DNA replication of DNA damaged cells while the G2 checkpoint prevents damaged cells from progressing to M phase.

A family of protein kinases called cyclin-dependent kinases (CDKs) mediates cell cycle progression. Throughout cell cycle, various CDKs are activated, depending on the phase of cell cycle. For instance, CDK4, CDK6 and CDK2 are activated in the G1 phase, CDK2 in S phase and CDK1 in G2 and M phase [171, 175-177].

CDKs require association with regulatory proteins known as cyclins that trigger the activation of CDKs and move the cells through the cell cycle phases. Similar to CDKs, different cyclins are engaged, according to the phase of cell cycle. For example, cyclin-D binds to CDK4 and CDK6, which facilitates entry into the G1 phase, while cyclin-E binds to CDK2 to induce progression to S phase. The association of cyclin-A with CDK2 is essential during S phase. Cyclin-A and –B interaction with CDK1 allows the transition of cells from S phase to M phase [171, 178-180]. There are also negative regulators such as CDKs inhibitors (CDKIs) including p16, p21, p27 and p57, which stop transitions within phases of cell cycle [181].

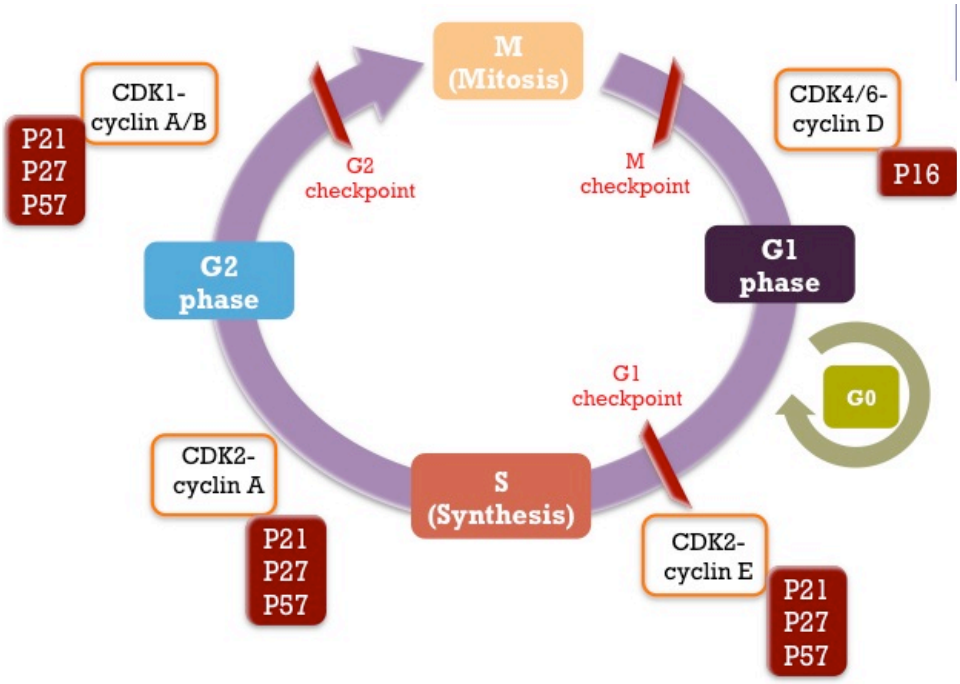



**Figure 2. Cell cycle progression and checkpoints**

### 1.4.1. Impact of cell cycle in cancer therapy

Damage to cell cycle genes or loss of checkpoint integrity will result in uncontrolled cell cycle events and hence uncontrolled proliferation. This leads to several abnormalities, alterations and mutations that can further cause human malignancies [174, 178, 180]. For instance, the deregulated expression and gene amplification of cyclin-D1 has been reported to mediate carcinogenesis in animal models [182]. Another example involves the deregulation of cyclin-E, which was found to contribute to several neoplasms such as colon and breast cancers [183, 184].

Disruption of the cell cycle is a major pathway to tumor development. Predominantly, genes of two signal transduction pathways are mutated in cancer; oncogenes (cell cycle accelerators) and tumor suppressor genes (cell cycle decelerators). Mutations in oncogenes such as Bcl-2 promote tumor proliferation. There are other abnormally activated oncogenes involved in cancer such as the intracellular signaling RAS and growth factor c-SIS. Mutation in tumor suppressor genes such as p53 causes damage to proteins responsible for cell cycle inhibition [176, 178]. Additionally, alterations in pro-apoptotic and anti-apoptotic signals like reduction of pro-apoptotic p53 protein or increased anti-apoptotic Bcl-2 protein will lead to tumorigenesis [185].

Understanding the mechanisms of cell cycle progression will aid in the development of new therapeutic agents that target genes governing cell cycle and its checkpoints, as these genes

play a critical role in the promotion and progression of tumors. For instance, treatments could be designed to cause arrest of cells in G1 and thereby slow proliferation, leading to cell death, or cause arrest of cells in S or M phase, preventing the propagation of DNA and allowing more time for DNA repair and enhancement of apoptosis.

**1.5. Limitation of current therapies:**

Cancer is the leading cause of mortality worldwide with an annually increasing incidence. Globally, hepatic cancer accounts for 5% of all cancers and is the 5$^{th}$ most common cancer in men and the 8$^{th}$ in women. Among hepatobiliary cancer, hepatocellular carcinoma and cholangiocarcinoma are the most common primary tumours of the liver [186]. Despite advances in diagnostic and therapeutic strategies, the incidence of these hepatobiliary cancers is on the rise and they are becoming a lethal pandemic worldwide. Currently available therapies, including surgery, chemotherapy, radiotherapy and liver transplantation, are still relatively ineffective due to associated side effects and toxicities, often resulting in organ failure and fatal outcomes.

The therapies most frequently employed in the early stage of cancer are surgical removal of the tumour and liver transplantation. However, the applicability of these treatments is limited since most patients tend to be diagnosed at advanced stages of the disease and are therefore not candidates for these therapies. Additionally, the recurrence rate of cancer after surgery is still high; more than 50% at 3 years post-surgery [7]. Moreover, liver transplantation is limited due to a scarcity of donors, this is a major issue associated with liver transplantation. Following transplant, graft rejections, viral reinfection and high recurrence rate are other drawbacks [24, 83].

Palliative therapies are proposed for patients with advanced stages of disease (unresectable or metastatic tumours) to maintain quality of life. For instance, chemotherapeutic agents which kill rapidly dividing cancerous cells are commonly used in cancer therapy; however, highly proliferative normal cells are also affected. Patients receiving chemotherapy suffer from other uncomfortable symptoms such as nausea, dysphoria and vomiting [187]. Additionally, these patients are vulnerable to viral infections due to immunosuppression. Another limitation of chemotherapy is the development of drug resistance due to enhancement of drug efflux pump, activation of drug detoxification mechanisms and stimulation of DNA damage repair, all of which lead to blocking of the apoptotic pathway and alteration of cell cycle progression and its checkpoints [188, 189]. In addition, radiotherapy is associated with dose-

related toxicity to normal tissues. Moreover, side effects such as swelling and erythema of treated skin, and fatigue could occur [190].

Due to the limitations and side effects of conventional therapies, an urgent need for the development of new, well-tolerated and safe therapeutic strategies is warranted. Recently, there has been greater interest in investigating the anti-cancer potential of phytochemical compounds extracted from natural sources. Naturally occurring compounds have been identified and used for treating cancers since 1967 [191, 192]. Thymoquinone, the bioactive ingredient purified from volatile oil of *Nigella Sativa*, has been utilized for more than 2000 years as a curative remedy for treating several illnesses including cancer. Its anti-neoplastic potential has been widely investigated *in vivo* and *in vitro* models [193, 194].

### 1.6. *Nigella Sativa*

*Nigella sativa* (*N. sativa*) is an annual herbaceous flowering plant that belongs to Ranunculaceae family. It is native to countries bordering the Mediterranean Sea and southwestern Asian countries like India, Pakistan, and Saudi Arabia. In English-speaking countries, *N. sativa* is also known as black seeds, black cumin, black caraway, "the blessed seed", nutmeg flower and Roman coriander. It is also known as Al-Habbah Al-Sawada, Kamon Aswad or Habbatul Baraka in Arab countries, and In India, Pakistan and Sri Lanka, it is known as Kalaunji, Kalajira or Kulaunji [195, 196].

Morphologically, *N. sativa* is a small shrub with divided tapering green leaves and delicate rosaceous flowers that are usually white, yellow or purple. It has large capsulated fruit that contains numerous tiny black seeds (**Figure 3**).

This miracle plant has an extensive history; it has been known for centuries since ancient Egyptians and Greeks. It has also been referenced in several religious and ancient texts: Prophet Mohammed (Peace be upon him) described the healing power of *N. sativa* as he said, "Hold on using the black seed, as it has a remedy for every illness except death" [197]. *N. sativa* was referred to as "black cumin" in the Holy Bible. In addition, Hippocrates and Dioscorides described *N. sativa*, calling it Melanthion and Pliny defined it as Gith [197].

*N. sativa* seeds have been used traditionally for thousands of years in cooking as a food preservative and as a spice in cheese, breads and soups [198, 199]. Additionally, it has been employed externally for cosmetic and beauty purposes. Seeds and oils of *N. sativa* have been well known for their therapeutic potential to promote and maintain health. For more than 2000

years, *N. sativa* was used as a natural remedy for treating several diseases and conditions such as cough, cold, headache, diarrhea, asthma, bronchitis, rheumatism, diabetes, hypertension, infection, eczema, dysentery, gastrointestinal problems and obesity [199-201].

Growing interest has been directed toward scientific research of this medicinal plant, in order to determine and isolate its active constituents. Black seeds have been shown to have a variety of chemical constituents, including fixed oil, volatile oil, protein, carbohydrate, alkaloid, saponin, crude fiber, and vitamin and mineral components, including iron, calcium, potassium and sodium (Khan, 1999). Fixed oil accounts for 37% of seeds constituents and contains unsaturated fatty acids, whereas volatile oil accounts for 0.4 to 2.5% and contains many other constituents of which thymoquinone is the most abundant [201] (**Table 2**). Other pharmacologically active components that have been identified beside thymoquinone include dithymoquinone, thymol, thymohydroquinone, carvacrol, alpha-hedrin, nigellidine and nigellicine [202-207].

**Table 2**. **Constituents of the volatile oil of black seeds**

| Component | Percent of volatile oil |
|---|---|
| Thymoquinone | 28 – 57% |
| ρ-cymene | 7.1 - 15.5% |
| Carvacrol | 5.8 -11.6% |
| 4-terpineol | 2.0 - 6.6% |
| Longifilone | 1.0 - 8.0% |
| t-anethole | 0.25 - 2.3% |

**Figure 3. Nigella sativa flowering plant (CC Nigella sativa by Yamada K. http://commons.wikimedia.org/wiki/File:Nigella_sativa.jpg).**

### 1.6.1. Thymoquinone

Thymoquinone (TQ) is the major bioactive constituent derived from the volatile oil of *N. sativa* and is found as a yellowish crystalline powder [201]. It was first extracted and identified in 1963 by El-Dakhakhny [208]. The molecular structure of TQ consists of benzene ring conjugated with para-substituted dione. A methyl side chain is present in position 2 and an isopropyl side chain in position 5 (2-methyl-5-isopropyl-1,4- benzoquinone) (**Figure 4**).

Most medicinal and therapeutic properties of black seeds have been attributed to TQ. Accordingly, TQ has been highly and extensively investigated in order to elucidate its therapeutic potential. Moreover, TQ has been demonstrated to exert numerous biological activities both *in vivo* and *in vitro* including anti-inflammatory, anti-neoplastic, anti-oxidant, anti-bacterial, anti-viral, anti-diabetic and immunomodulatory activities [193, 194, 209-212].

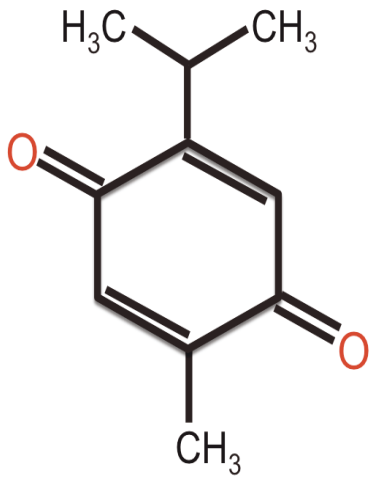



**Figure 4. Chemical structure of thymoquinone.**

***1.6.1.1. Potential anti-tumor activity of TQ***

The anti-tumor properties of TQ have been extremely investigated to determine the mode of action responsible for this activity. TQ has been shown to exert its anti-cancer effect through suppression of growth of cancerous cells; the main strategy of any anti-cancer drug. Additionally, the anti-proliferative effect of TQ has been reported in a wide variety of cancer cells including osteosarcoma [213], breast and ovarian adenocarcinoma [214], colorectal carcinoma [215], hepatocellular carcinoma [216], pancreatic adenocarcinoma [217], neoplastic keratinocytes [217, 218], fibrosarcoma and lung carcinoma [219], glioma/glioblastoma [220], prostate cancer [219, 221] and myeloblastic leukemia [222] (**Table 3**). The cytotoxic effect of TQ has also been reported in multi-drug resistant (MDR) cell lines; for example, TQ suppressed growth and induce apoptotic cell death in cisplatin-resistant canine osteosarcoma cell lines (COS31/rCDDP) [214]. In addition, TQ exerted a significant anti-tumour effect against multi-drug resistant uterine sarcoma, leukemic cell lines and pancreatic adenocarcinoma [217]. Furthermore, it has been reported that TQ causes little to no cytotoxicity in non-cancerous cell lines [219, 220, 223].

The mechanisms of the anti-proliferative effects of TQ have been further investigated and were proposed to be due to the induction of apoptosis and cell cycle arrest [193, 214, 224]. TQ has been reported to induce apoptosis by either p53-dependent or p53-independent pathways. One study reported the apoptotic effect of TQ in HCT-116 colon cancer cell via upregulation of p53 and p21, leading to a decrease in anti-apoptotic Bcl-2 protein expression (p53-dependent pathway) [215]. TQ was reported to induce apoptosis in HL-60 myeloblastic leukemia cells by

disruption of mitochondrial membrane potential and activation of caspase-8 (p53-independent manner) [222]. Besides p53, other molecular targets have been reported to be involved in TQ-induced anti-cancer and apoptotic effects. It has been reported that TQ-induced apoptotic effects in MCF-7/DOX breast cancer cells were due to upregulation of PTEN with suppression of p-Akt [225]. In addition, TQ was found to upregulate p73 with a decrease in UHRF1 in p53 mutant acute lymphoblastic leukemia Jurkat cells [226]. TQ also caused inhibition of STAT3 and NF-kB and their regulatory gene products in U266 multiple myeloma cells and KBM-5 human myeloid cells, respectively [227-229]. These regulatory gene products include COX-2, VEGF, cyclin D1, Bcl-2, Bcl-xL, c-Myc, MMP-9, IAP1, IAP2, Mcl-1 and survivin . In M059J and M059K glioblastoma cells, TQ damaged DNA by inhibition of telomerase, resulting in telomere attrition [220]. TQ also induced apoptosis in acute lymphoblastic leukemia Jurkat cells through production of reactive oxygen species (ROS), resulting in loss of mitochondrial membrane potential [230]. Nevertheless, activation of JNK and p38 MAPK pathways, with downregulation of mucin-4, was induced by TQ and led to apoptosis in FG/COLO357 pancreatic cancer cells [231].

TQ has also been reported to suppress growth by arresting cells in different phases of cell cycle. Several studies demonstrated the cell cycle arresting activity of TQ at different stages of the cell cycle in different tumours including mouse papilloma carcinoma cells (SP-1) and mouse spindle carcinoma cells (I7) [218], LNCaP prostate cancer cells [219], HCT116 human colorectal carcinoma cells [215], acute lymphoblastic leukemia Jurkat cell lines [226], MNNG/HOS human osteosarcoma cells [213] and MCF-7/DOX doxorubicin-resistant breast cancer cells [225].

**Table 3. The anti-proliferative effect of TQ on different cancer cell lines.**

| Type of cancer | Cell lines | References |
|---|---|---|
| Glioma/Glioblastoma | U87 MG, T98G, M059K and M059J | [220, 232] |
| Breast Adenocarcinoma | Multi-drug-resistant MCF-7/ TOPO, MCF-7, MDA-MB-231, MDA-MB-468, T-47D and BT-474 | [233-235] |
| Leukemia | HL-60 and Jurkat | [222, 226, 233] |
| Lung Cancer | NCI-H460 and A549 | [236, 237] |
| Colorectal Carcinoma | HT-29, HCT-116, DLD-1, Lovo and Caco-2 | [237, 238] |
| Pancreatic Cancer | MIA PaCa-2, HPAC and BxPC-3 | [237, 239] |
| Osteosarcoma | MG63 and MNNG/HOS | [213] |
| Prostate Cancer | LNCaP, C4-2B, DU145 and PC-3 | [219, 221, 240] |
| Colon cancer | HT-29, HCT-116, DLD-1, Lovo and Caco-2 | [238] |
| Primary effusion lymphoma (PEL) | BC1, BC3, BCBL1, and HBL6 | [230] |
| Renal cancer | ACHN | [241] |

#### *1.6.1.2. Combination of TQ with chemotherapeutic drugs:*

Several studies have reported a powerful effect of TQ in improving the therapeutic indices of certain anti-tumour drugs. Examples of *in vitro* studies include NC1-H460 non-small lung carcinoma cell lines, where the combination of TQ and cisplatin showed greater cytotoxicity [236]. In HPAC and BxPC-3 human pancreatic carcinoma cell lines, the addition of TQ to oxaliplatin and gemcitabine improved their anti-tumour effect [239]. TQ also enhanced the cytotoxic effect of paclitaxel and doxorubicin against human chronic myeloid leukemia KBM-5 cells [228], and the apoptotic activity of thalidomide and bortezomib in human multiple myeloma U266 cell lines [227]. Finally, TQ has been demonstrated to sensitize the human breast carcinoma cells MCF7 and T47D to the cytotoxic effect of ionizing radiation [242].

#### *1.6.1.3. In vivo chemopreventative and chemoprotective effects of TQ*

The chemotherapeutic potential of TQ has been investigated in animal models with different types of cancer and was shown to prevent carcinogenesis [193, 200]. TQ in conjunction with other clinically available anti-cancer drugs also exerted protection against toxicities caused by these conventional chemotherapies, without affecting their therapeutic efficacy.

TQ reduced the size and number of aberrant crypt foci (ACF) in mice with colon cancer induced by 1,2-dimethyl hydrazine [243] and inhibited the incidence of benzo(a)pyrene (BP)-induced forestomach tumours in Swiss albino mice [244]. Administration of TQ to rats with N-nitrosodiethylamine (NDEA)-induced hepatic cancer reduced liver injury and tumor markers, inhibited the formation of hepatic nodules and diminished tumor multiplicity [245]. TQ reduced ifosfamide-induced renal toxicity in Ehrlich ascites carcinoma-bearing mice. Administration of TQ enhanced the efficacy of ifosfamide and resulted in lower mortality and decreased body weight loss in mice [246]. Another study carried out by Badary and Gamal El-Din [247] reported the anti-tumour effect of TQ against fibrosarcoma tumours in mice induced by 20-methylcholanthrene. TQ also enhanced the anti-tumour effect of cisplatin in mice and rats and averted cisplatin-induced nephrotoxicity [248]. TQ also enhanced the efficacy of doxorubicin and prevented cardiotoxicity caused by doxorubicin [249, 250]. Another study carried out by Mansour [251] demonstrated the protective effect of TQ against hepatotoxicity induced by carbon tetrachloride in mice. Finally, the addition of TQ to gemcitabine and oxaliplatin has been shown to enhance the anti-tumour efficacy of these two anti-cancer drugs in an orthotopic model of pancreatic cancer [239].

The growth inhibitory effect of TQ has also been reported in other models such as xenograft models of C4-2B prostatic cancer cells [219], NCI-H460 lung cancer cells [236], HPAC pancreatic cancer cells [239] and HCT-116 colon cancer cells [243].

#### *1.6.1.4. Inhibition of tumour metastasis and angiogenesis by TQ*

Several studies reported the potential effect of TQ in inhibiting tumour metastasis and angiogenesis. Yi, Cho [252] reported the inhibition of human umbilical vein endothelial cell migration, invasion and tube formation by TQ. In the same study, TQ was found to inhibit angiogenesis in a xenograft mouse model of PC-3 prostate cancer cells, through a reduction in the number of blood vessels present in tumors. TQ was also found to suppress migration and invasion of MDA-MB-231 breast cancer cells in a concentration-dependent manner [234] as well as in C26 colon cancer cells, at sub-cytotoxicity dose [243]. Another study reported the inhibitory effect of TQ on FG/COLO357 pancreatic cancer cells, where TQ suppressed the migration of cells in a dose-dependent manner [231]. TQ also inhibited the invasion of non-small cell lung cancer NCIH460 cells [236].

#### *1.6.1.5. Clinical studies of TQ*

To date, there are a limited number of clinical studies of TQ that have been conducted in humans. This limitation could be due to the lack of reported maximum safe and tolerated dose, and lack of known toxicity of TQ to humans. Due to the hydrophobic nature of TQ, it has poor water solubility and bioavailability resulting in a reduced absorption, which could prevents TQ from reaching tumour site especially in aggressive tumours. Additionally, lack of human pharmacokinetics and pharmacodynamics limited the use of TQ in clinical studies [253]. TQ has been observed to interact with some constituents of blood that affects its anti-cancer properties; as study carried out by El-Najjar et al. (2011) [254] reported extensive interaction between TQ and two major plasma proteins, bovine serum albumin (BSA) and alpha-1 acid glycoprotein (AGP), which have essential roles in the delivery and detoxification of drugs [255, 256]. In this study, TQ-BSA binding was shown to suppress cell death induced by TQ against DLD-1 and HCT-116 colon cancer cells, whereas TQ–AGP binding had no effect on TQ activity against these cell lines. One study of pharmacokinetic parameters of TQ in plasma was carried out in rabbit models. In this study, TQ was administrated orally (20 mg/kg) and intravenously (5

mg/kg). The elimination half-life time ($T_{1/2}$) of orally and intravenously administered TQ were 63.43 ± 10.69 and 274.61 ± 8.48 minutes, respectively, with absorption $T_{1/2}$ of 217 minutes. The bioavailability of TQ was reported to be 58%. These data support the rapid elimination of TQ when given orally and confirm its slow absorption [257]. Another study in rabbits showed that oral intake of TQ-loaded nanostructured lipid carriers improved the pharmacokinetics of TQ. The elimination half-life (T1/2) of TQ was 4.49 ± 0.015 h [258].

To enhance bioavailability, modifications to TQ structure and formation by encapsulated or nano-particulated TQ have been employed to synthesize more soluble form. Some reported TQ analogs include Poloxin, 4-acylhydrazones, 6-Alkyl, TQ-2G, TQ-4A1 andTQ-5A1 [259-261]. These TQ analogs have been shown to be more effective in treating cancer cells than TQ. For example, conjugation of TQ to fatty acid groups was reported to enhance the anti-cancer potential of TQ. Attachment of TQ to triterpene betulinic acid was reported to exert greater anti-tumor activity against HL-60 leukemia cell lines [262]. The encapsulation of TQ in nanoparticles was also found to improve TQ's bioavailability. Several nanoparticles of TQ have been reported including poly (lactide-co-glycolide) nanoparticles and PLGA-PEG 5000 nanoparticles [263, 264].

There are few clinical trials of TQ have been carried out. Al-Amri and Bamosa [265], conducted a phase I trial of TQ in adult patients with solid tumors or hematological malignancies. They reported that TQ induced neither anti-tumor nor toxic effects in patients. In addition, these authors established the safety of a dose of 2600 mg/day of TQ in humans. Another double-blind crossover clinical study of TQ was conducted in children with refractory epilepsy. TQ exerted anti-epileptic activity in children treated with TQ [266]. These two clinical studies suggest the potential beneficial and therapeutic use of TQ without any adverse effect in humans.

### 1.7. Hypothesis and objectives of thesis

Despite several studies investigating the anti-tumour activities of TQ on different types of cancers, the anti-tumour effect of TQ on hepatocellular carcinoma and cholangiocarcinoma cells and the mechanisms of actions involved remain poorly explained. The hypothesis of the present study was that TQ, the major active constituent of *Nigella sativa* seed extract, induces apoptosis in HCC and CCA cell lines. To test this hypothesis, the following objectives were carried out:

- To evaluate the anti-proliferative and cytotoxic activities of TQ in HCC and CCA cell lines.
- To investigate the underlying mechanisms of the growth inhibitory effect of TQ.
- To determine the mode of death of tumour cells treated with TQ (apoptosis or necrosis).
- To determine the subcellular alterations induced by TQ that lead to tumour cell death.

# Chapter 2: Materials And Methods

## 2.1. Cell lines and culture conditions

Hepatocellular carcinoma HepG2 cells were purchased from the American Type Cultural Collection (ATCC) (Manassas, VA, USA) and were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Canada Inc, Burlington, ON, Canada) supplemented with 10% Fetal Bovine Serum (FBS) (PAA Laboratories Inc., Etobicoke, ON, Canada) amd 1 ml gentamicin (Gibco).

Cholangiocarcinoma HuCCT1 cells were obtained from the cell culture bank of the Japan Health Sciences Foundation and were grown in Roswell Park Memorial Institute (RPMI-1680) medium (Invitrogen Canada Inc., Burlington, ON, Canada) and supplemented with 10% FBS and 1 ml gentamicin.

The human embryonic kidney HEK293T cells were gifted from Dr. Judith Hugh Lab and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Canada Inc., Burlington, ON, Canada) supplemented with 10% FBS and 5 ml of penicillin-streptomycin (PAA Laboratories).

The immortalized human liver THLE-3 cells were purchased from the American Type Cultural Collection (ATCC) (Manassas, VA, USA) and cultured in previously coated flasks with 0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen type 1 and 0.01 mg/ml bovine serum albumin dissolved in Bronchial Epithelial Cell Growth Medium (BEGM) for 24h. The cells were cultivated in BEGM supplemented with BEGM SingleQuot kit except GA-1000 and Epinephrine (Lonza Group Ltd., Basel, Switzerland), 10% FBS and 1 ml gentamicin.

All cells were grown as monolayer culture in their proper media and maintained in a humidified atmosphere in a 5% CO2 incubator at 37C. They were passaged when reached 70-90% confluency using trypsin plus 0.25% EDTA for 5 min in CO2 incubator.

## 2.2. Preparation of thymoquinone

A 1M stock solution of TQ was prepared by dissolving TQ powder (0.1642 g) in cell-culture tested, sterile 100% dimethyl sulfoxide (DMSO) (1ml) (Sigma-Aldrich, St. Louis, Mo, USA). A 2X stock solutions [20-400 μM] were prepared in 100% DMSO (1ml). All stocks were divided into aliquots, covered with aluminum foil and stored at -20°C until use. Appropriate

working concentrations of TQ [10-200 μM] were prepared by dilution in complete cell culture medium freshly before use. The final DMSO concentration added on cells was 0.05%.

### 2.3. Experimental methods

#### 2.3.1. Cell proliferation assay

A commercially available colorimetric kit, CellTiter 96 AQueous One Solution Cell Proliferation Kit (MTS) (Promega, USA), was used to determine the number of viable cells. This method is based on the bio-reduction of the MTS tetrazolium compound by NADPH or NADH produced by dehydrogenase enzymes of active cells into a purple Formazan product which can be detected by measuring absorbance at 490 nm. The amount of Formazan produced is directly proportional to number of metabolically active cells. The kit was used according to manufacturer's protocol. Briefly, HuccT1, HepG2 and THLE-3 cell lines were seeded at density of 6000 cells/well in quadruplicates wells of a 96-well tissue culture plates with 50 μL of complete media. Cells were allowed to attach and grow overnight at 37°C in a 5% CO2 humidified atmosphere. Next day, 50 μL of fresh complete media containing thymoquinone [10-200 μM] were added into each well and cultured in humidified incubator for 12, 24 and 48 hours. A Colorimetric 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was added (20 μl/well) into each well and incubated for 4 hours. Absorbance at 490 nm was recorded using SpecraMax M3 Microplate Reader (Molecular Devices, CA, USA). The background absorbance of sample wells was normalized to that of negative control wells, which contain only Media. The percentage of cell proliferation was calculated using the following formula:

$$\%\ \text{Proliferation} = \frac{\text{Absorbance of treated wells}}{\text{Absorbance of control wells}} \times 100$$

Three independent experiments in quadruplicates were done for HepG2 and HuCCT1 cell lines and only one individual experiment in quadruplicates was done for THLE-3 cell lines.

From the obtained MTS results, the $IC_{50}$ value, which is defined as the concentration of TQ that causes 50% cell death on these cell lines, was calculated for each concentration by plotting a dose-response curve (**Appendix A**).

### 2.3.2. Cell cycle analysis

To determine the distribution of cell lines in each phase of cell cycle, the propidium iodide (PI) (Sigma-Aldrich, St. Louis, Mo, USA) was used to stain the DNA content of each cell line. At a density of $1 \times 10^6 - 3 \times 10^6$ cells/dish, the HuccT1 and HepG2 cell lines were seeded in 30mm tissue culture plates in 5 ml of complete medium. Cells were incubated and allowed to adhere in $CO_2$ atmosphere. After 24 hours adherence, cells were incubated with different concentration of TQ [10−100 μM] in 5 ml of fresh complete medium and incubated for 24 h. Thereafter, both adherent and non-adherent cells were collected and centrifuged at 300 × g for 5 minutes. Then cell pellets were washed twice with FACS washing buffer and fixed with 70% Ice-cold ethanol for a minimum of 24 hours at -20 °C. Fixed cells were centrifuged at a higher speed and washed with FCS washing buffer twice. Cell pellets were stained with 1 ml of Propidium iodide solution [50 μg/ml propidium iodide and 3.8 mM sodium citrate in PBS] with the addition of 50 μl of RNase A (10 μg/ml), mixed well and placed in 4 °C protected from light until use. The distribution of PI-stained cells across cell cycle was analyzed with fluorescence activated cell sorter (BD FACS Calibure) flow cytometer and the data were processed using CellQuest Pro 6.0 software. Data were collected from three individual experiments.

### 2.3.3. Apoptosis and necrosis assay

Annexin V- FITC apoptosis detection kit (BD biosciences) was used to quantify the percentage of cells undergoing apoptosis and to determine the mode of cell death whether by apoptosis or necrosis in the presence or absence of TQ. The experiment was carried out according to the manufacturer's protocol. Briefly, cells were seeded ($1 \times 10^6 - 3 \times 10^6$) per dish and allowed to adhere overnight in $CO_2$ incubator. Following 24 hours incubation, TQ treatments [10-100 μM] were added and plates were incubated for another 24 hours in $CO_2$ atmosphere. Both adherent and nonadherent cells were trypsinized, collected and centrifuged for 5 minutes at 300g. Cell pellets were washed with 2 ml of cold PBS twice, re-suspended in 100 μl of 1X binding buffer and stained with 5 μl of FITC Annexin V and 5 μl of PI for 15 minutes in the dark at room temperature. Following incubation, 1 ml of 1X binding buffer was added and the analysis was done using BD FACS Calibure flow cytometer within an hour. Data was collected from three individual Experiments.

### 2.3.4. Morphological assessment

Morphological changes in different cell lines after treatment with increasing concentrations of TQ were observed. Briefly, HuccT1, HepG2 and THLE-3 cell lines (4000 cells/well) plated in 8-well glass chambered slides in 200 µl of complete medium and left to grow overnight in $CO_2$ incubator. Then 200 µl of fresh complete medium containing TQ treatments [10-200 µM] were added into each well and incubated for 24 hours. HepG2, HuCCT1 and THLE-3 cells were visualized using the Zeiss AxioObserver Z1 (inverted) confocal microscopy (Zeiss 710 LSM). HEK293T cell lines were seeded at a density of $0.3 \times 10^6$ cells/well in 6-well tissue culture plates and allowed to adhere over night. Cells were then treated with TQ for 24 hours and visualized using digital microscopy. All images were processed using ZEN 2009 software (Carl Zeiss, Canada). One experiment was performed for each cell line.

### 2.3.5. Mitochondrial membrane potential using JC-1 (5, 5′, 6, 6′-teterachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide)

Changes in mitochondrial membrane potential (MMP) were detected by using the fluorescent JC-1 dye (Molecular Probes, Invitrogen, Germany) that fluoresces red/orange fluorescence in intact and healthy mitochondria and fluoresces green fluorescence in the cytoplasm in disrupted mitochondria. Between 10,000 and 15,000 cells/well of HuccT1 and HepG2 cell lines were cultured in 8-well glass chambered slides and incubated in $CO_2$ incubator for 24 hours. Increasing concentrations of TQ [10 - 200 µM] were added into each well and incubated in $CO_2$ incubator for 24 hours. JC-1 stock solution (1 mg/ml) was prepared in DMSO. The JC-1 working solution with a final concentration of 0.1 µg/ml was prepared in complete media and 40 µl was added into each well and incubated for 30 minutes in $CO_2$ incubator. Cells were visualized using the Zeiss AxioObserver Z1 (inverted) confocal microscopy (Zeiss 710 LSM). All images were processed using ZEN 2009 software (Carl Zeiss, Canada). One experiment was performed for each cell line.

### 2.3.6. Statistical analysis

All obtained data were presented as mean±S.E.M of three or four replicates from three individual experiments and analyzed statistically using Statistical Package for Social Sciences SPSS software (version 21.0). The statistical analysis for anti-proliferative assay was conducted using one-way analysis of variance (ANOVA) test to determine the significant differences

between different concentrations followed by Dunnett's post hoc test to compare the significant difference among concentrations in comparison to non-treated controls. For cell cycle and apoptotic assays, non-parametric Kruskal-Wallis test was used to determine significant changes across the different groups. A p-value of $<0.05$ was considered to be statistically significant.

# Chapter 3: Results

## 3.1. Inhibition of cell proliferation of tumor cell lines by TQ:

The anti-proliferative effect of TQ on HCC cell lines, CCA cell lines and normal THLE-3 cell lines following treatment with different concentrations of TQ was measured using the colorimetric MTS assay. All cell lines were exposed to different concentrations of TQ (10-200 μM) for varying durations (12, 24 and 48 h). Negative controls (cells not exposed to TQ) and vehicle controls (cells treated with 0.05% DMSO) were included. No positive controls were included in this experiment.

A concentration- and time-related inhibition of cell growth was observed in TQ-treated cells compared to non-treated controls. In HepG2 cells a significant reduction in the percentage of cell growth was observed at 25 μM TQ and higher after 12 h incubation ($P<0.05$, **Figure 5.a**). At 24 h, the percentage of cell viability after treatment with 25 μM TQ and higher was statistically significantly lower than control. At 48 h, significant increase in cell viability was observed at 10 μM and 25 μM, while concentrations from 50 μM and higher resulted in significantly decreased cell viability. In HuCCT1 cell lines, cell proliferation was significantly reduced at 75 μM TQ and higher, at 12 h incubation ($P<0.05$, **Figure 5.b**). At 24 h, 50 μM TQ and higher resulted in a significant decrease in the number of viable cells. Incubation for 48 h at 25 μM and higher also resulted in a significant suppression of growth. In both cell lines, DMSO (0.05%) treatment did not result in observable cytotoxic effects.

In contrast, TQ did not exert a cytotoxic effect toward normal THLE-3 cell lines (**Figure 5.c)**.

The $IC_{50}$ values of each cell lines for the respective durations are listed in **Table 4**. The differences in these values indicate that TQ cytotoxicity might be related to the type of cancer and could be cell line specific.

Since substantial variability in cell viability was observed at the 12 h incubation time point, an incubation period of 24 h was chosen for further experiments, representing the shortest time when cells exhibited full biochemical response to the TQ. Additionally, $IC_{50}$ values for TQ were 100 μM or less. Therefore, 10-100 μM concentrations of TQ were selected for the subsequent studies.

**Table 4. The IC50 values of TQ in different cell lines after 12, 24 and 48 hr of treatment**

| Cell lines | 12 h | 24 h | 48 h |
|---|---|---|---|
| **HepG2** | 50.74 | 34.23 | 50.84 |
| **HuCCT1** | 155.1 | 63.12 | 61.59 |
| **THLE-3** | - | 200 | - |

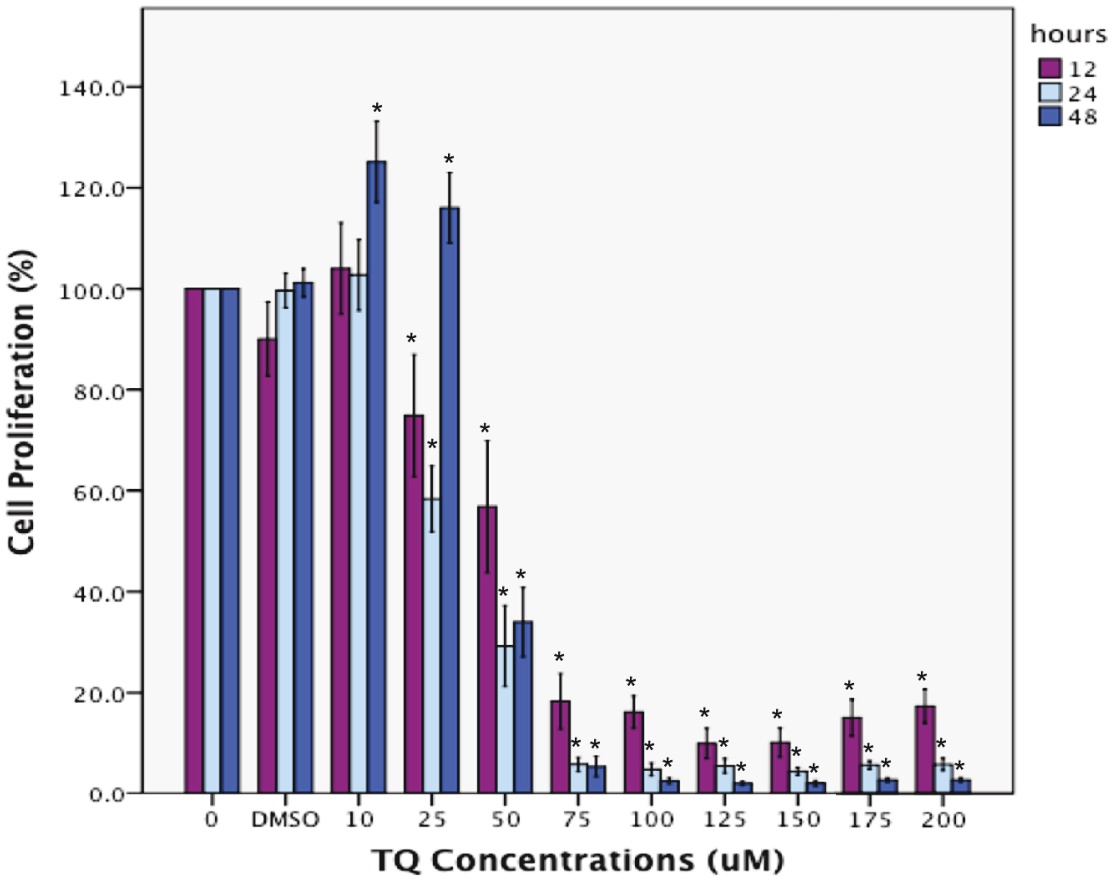



**Figure 5.a. Anti-proliferative effect of TQ on HepG2 cell lines. Values are presented as mean of 3 individual experiments ± SEM**

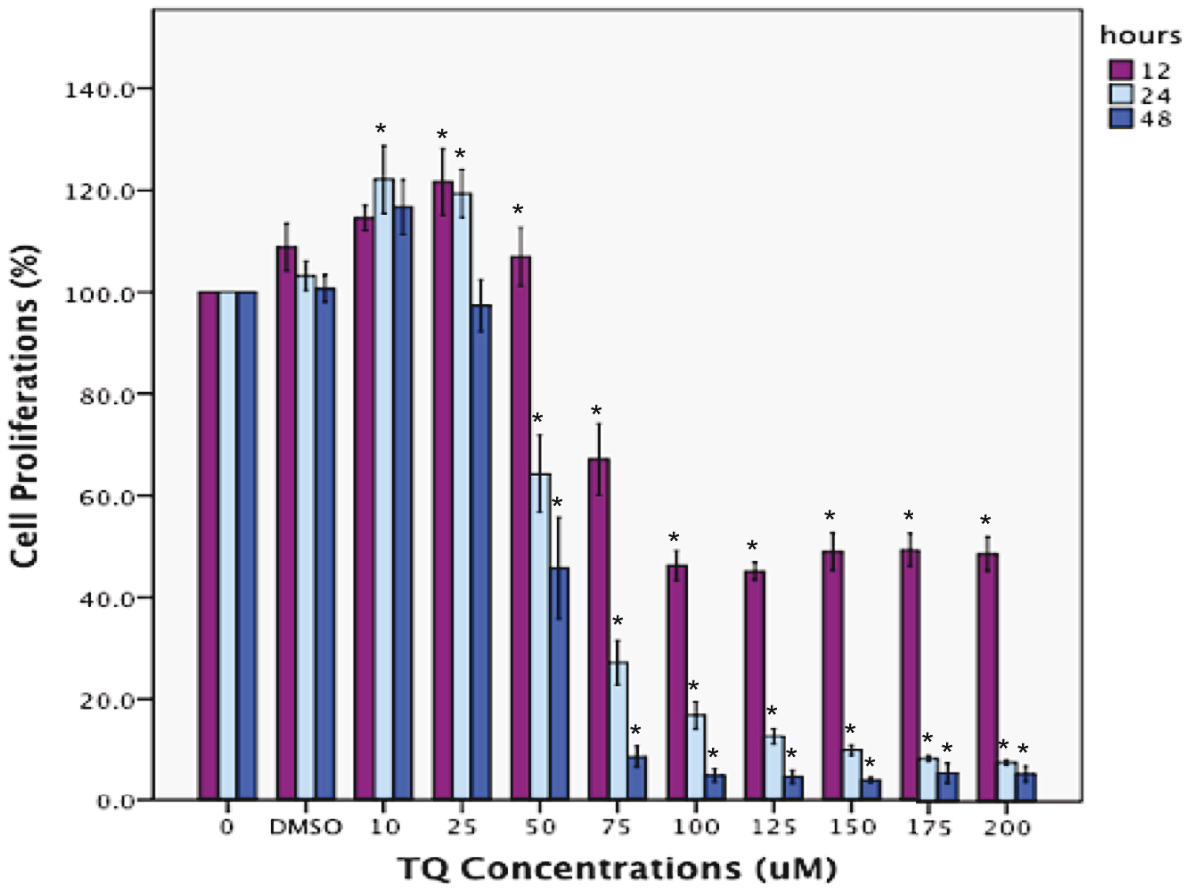



**Figure 5.b. Anti-proliferative effect of TQ on HuccT1 cell lines. Values are presented as mean of 3 individual experiments ± SEM**

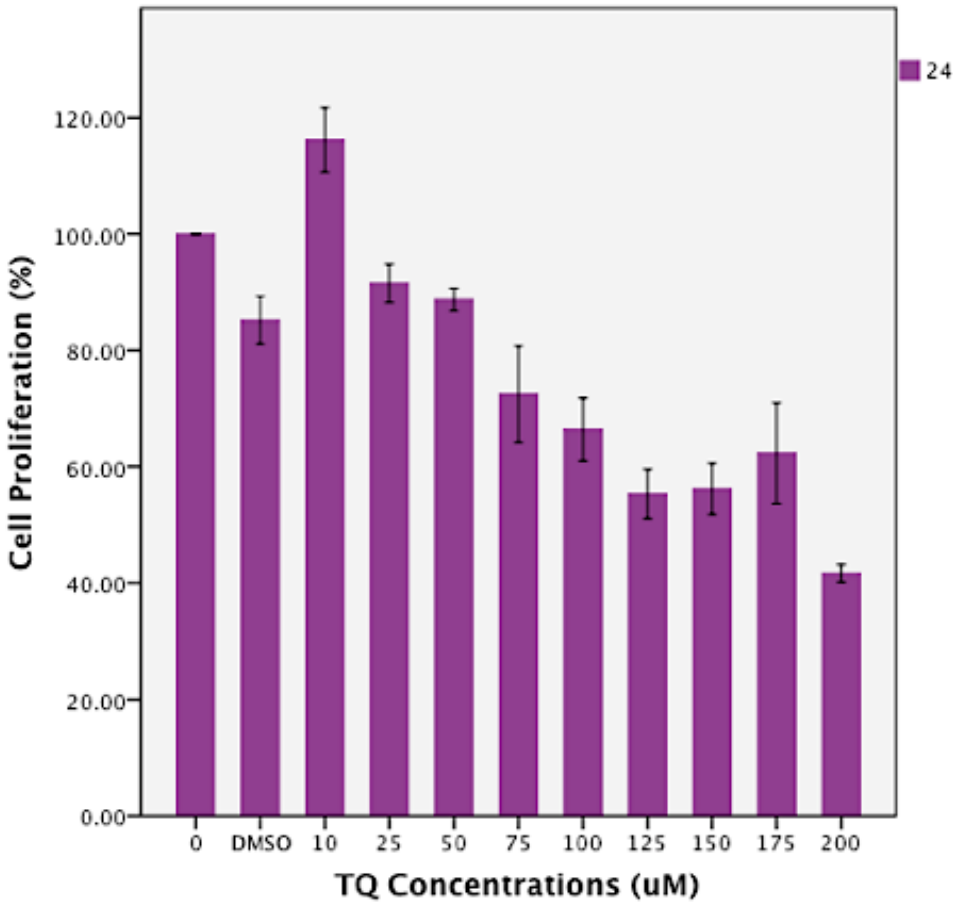



**Figure 5.c. Anti-proliferative effect of TQ on THLE-3 cell lines. Data shown are from one experiment done in quadruplicates.**

### 3.2. Effect of TQ on cell cycle distribution

To determine if the cytotoxic effect of TQ is associated with disruption of cell cycle, changes in cell cycle progression of HepG2 and HuCCT1 cell lines were investigated. Cells were treated with increasing doses of TQ for 24 h followed by PI staining of DNA content. Flow cytometry was employed to quantify cell populations in different cell cycle phases (sub-G1, G1, S and G2/M phases).

Treatment of HepG2 cell lines with TQ did not result in any significant accumulation of cells in cell cycle phases G1, S or G2/M (**Figure 6.a, 6.b**). The number of cells in G1 and S phases decreased with higher doses of TQ. The percentage of cells in the G2/M phase increased after incubation with 10, 25 and 50 μM, and decreased after incubation with 75 and 100 μM TQ.

In HuCCT1 cells, the percentage of cells in the G1 phase significantly increased in a dose-dependent manner, compared to non-treated control ($P<0.05$, **Figure 7.a, 7.b**). Number of cells in the G1 phase increased from 46.8% under control conditions to 60.3% after incubation with 100 μM TQ. A significant decrease of G2/M phase was observed ($P<0.05$) but no significant changes were observed in S phase.

For both cell lines, the percentage of cells in the subG1 phase, which represents the apoptotic population, were significantly increased in a dose-related manner ($P<0.05$, **Figure 8**)(**Appendix B**).

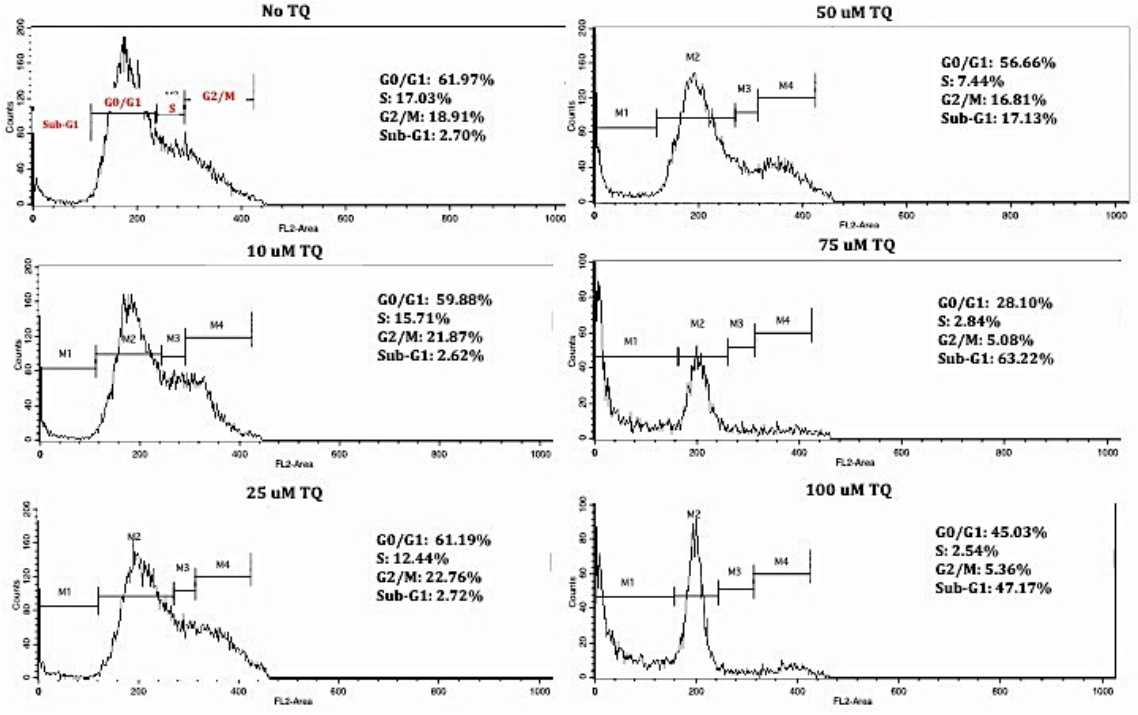



**Figure 6.a. Histogram of Cell Cycle distribution of HepG2 cell lines. The results shown are one representative of three individual experiments.**

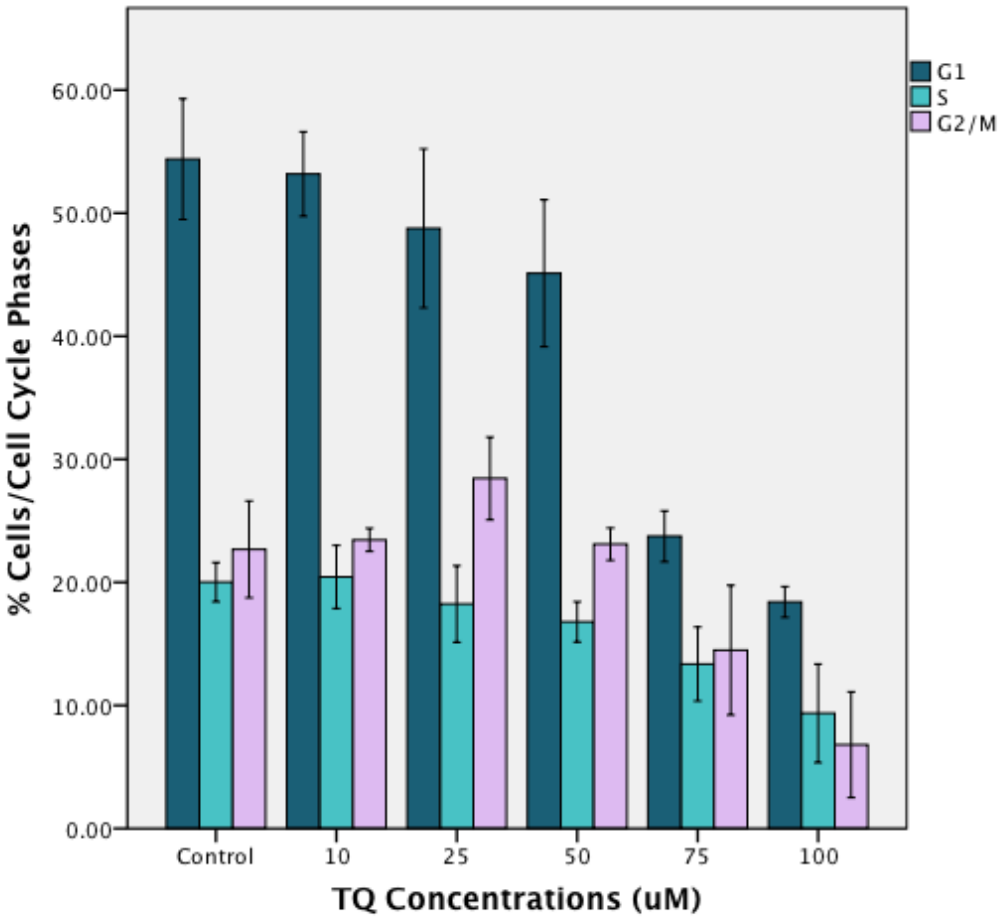



**Figure 6.b. Distribution of Cell Cycle Phases in HepG2 cell lines. Values are presented as mean of 3 individual experiments ± SEM**

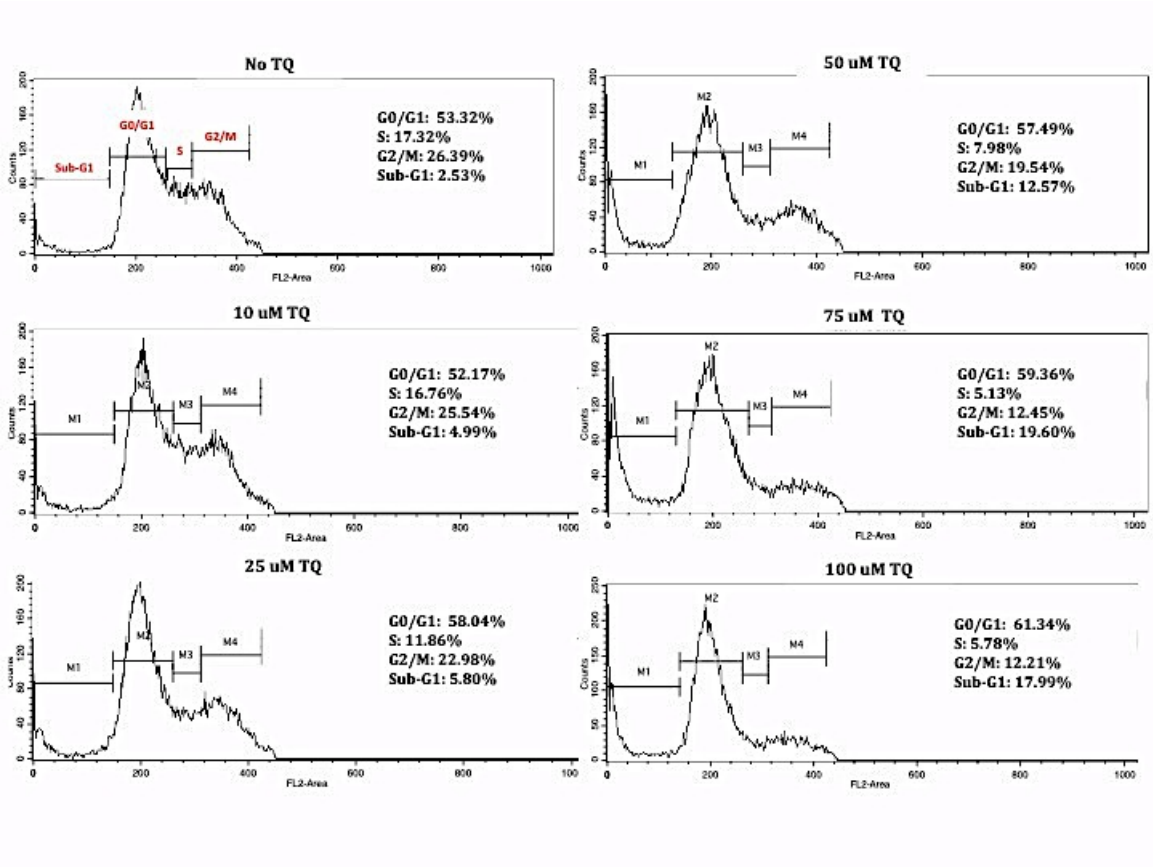



**Figure 7.a. Histogram of Cell Cycle distribution of HuCCT1 Cell Lines. The results shown are one representative of three individual experiments.**

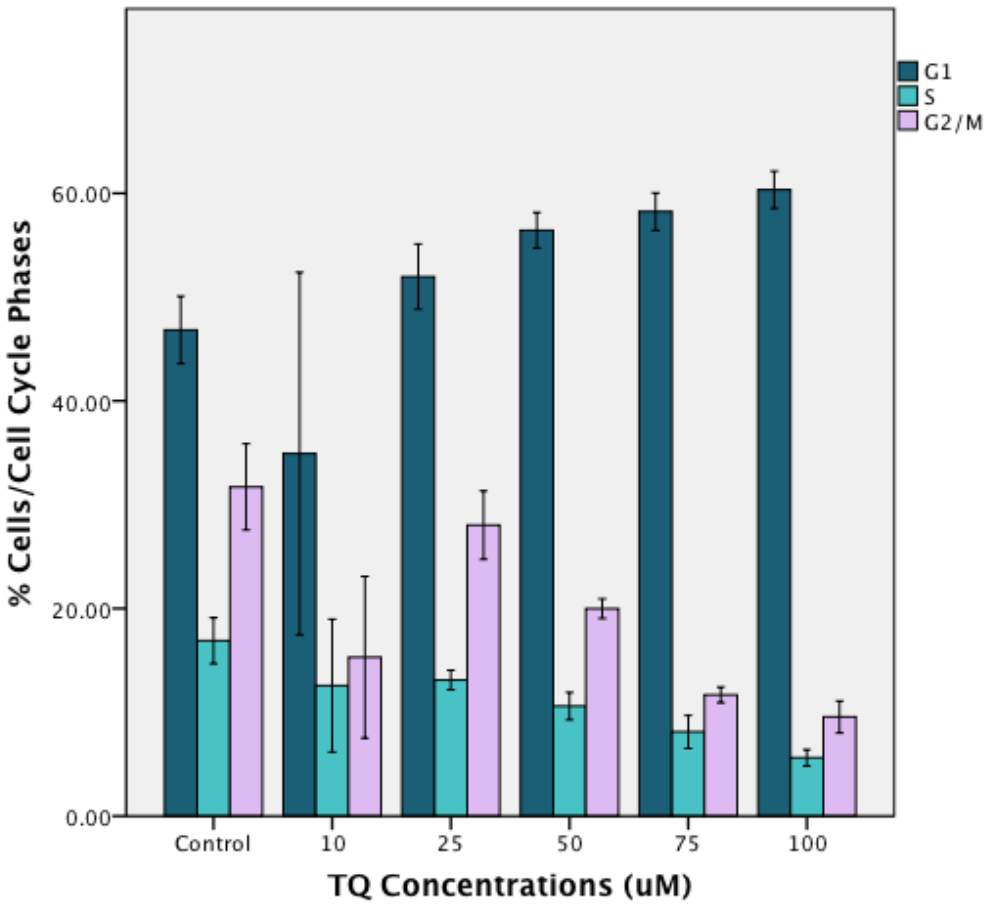



**Figure 7.b. Distribution of Cell Cycle Phases in HuCCT1 cell lines. Values are presented as mean of 3 individual experiments ± SEM**

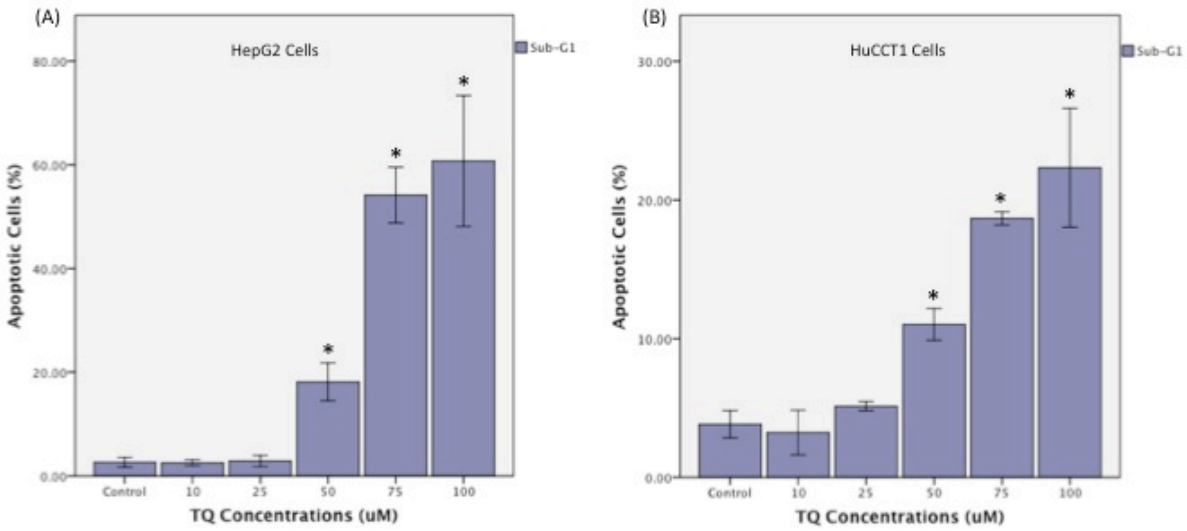



**Figure 8. The Induction of Apoptosis in both (A) HepG2 cell lines and (B) HuCCT1 cell lines. Values are presented as mean of 3 individual experiments ± SEM**

### 3.3. Determination of mode of cell death using Annexin V/PI staining

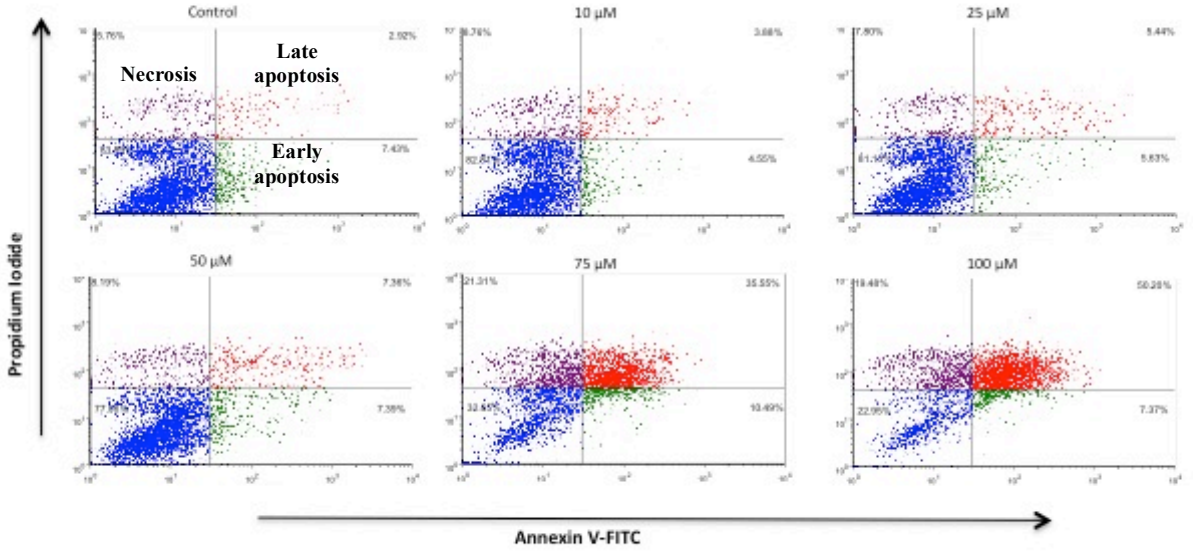
The mode of cell death induced by TQ was further investigated to determine whether death is due to apoptosis or necrosis. This was elucidated using Annexin V/PI assay. As described in the Materials and Methods section, HepG2 and HuCCT1 cell lines were treated with increasing concentrations of TQ for 24 h followed by Annexin V/PI staining.

The representative dot plots of flow cytometric analyses of HepG2 and HuCCT1 cells demonstrated four different distributions: live or healthy cells (Lower Left; Annexin V and PI negative), cells in early apoptosis (Lower Right; Annexin V positive and PI negative), cells in late apoptosis (Upper Right; Annexin V and PI positive) and dead or necrotic cells (Upper Left; Annexin V negative and PI positive).

The results indicated that treating cells with TQ resulted in a significant, dose-related increase in the percentage of apoptotic cells compared to non-treated controls. Additionally, a slight increase in number of necrotic cells was seen.

The percentage of live cells was significantly reduced. The apoptotic-inducing effect of TQ was demonstrated by the shift of HepG2 and HuCCT1 cells toward lower and upper right quadrants (**Figure 9**). The total number of early and late apoptotic cells is higher compared to the number of necrotic cells, indicating that apoptosis is the main mechanism by which TQ causes cell death, in both cell lines (**Figure 10**).

A)




B)

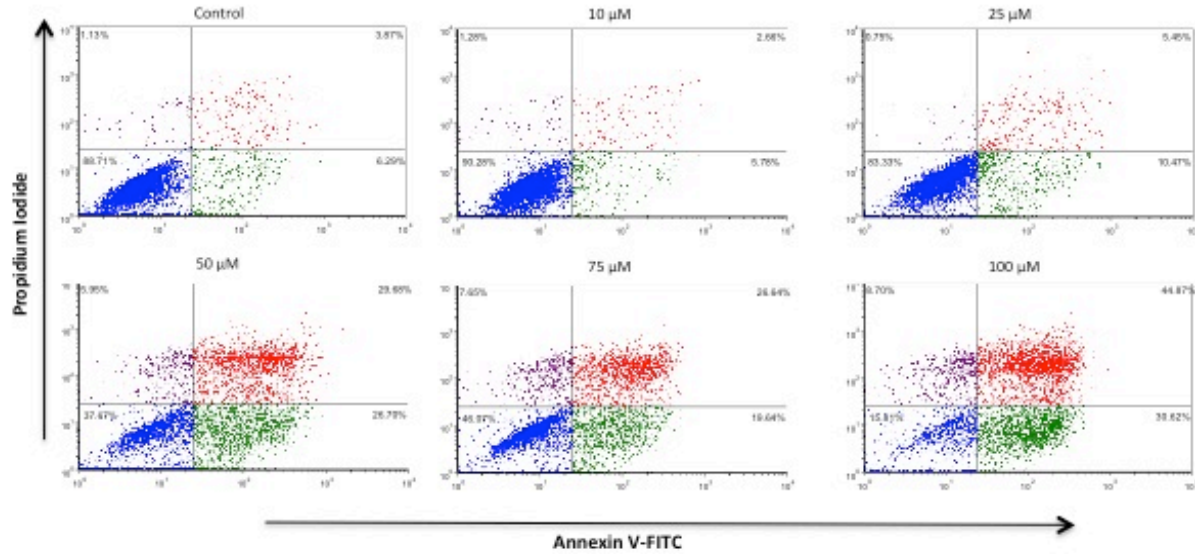
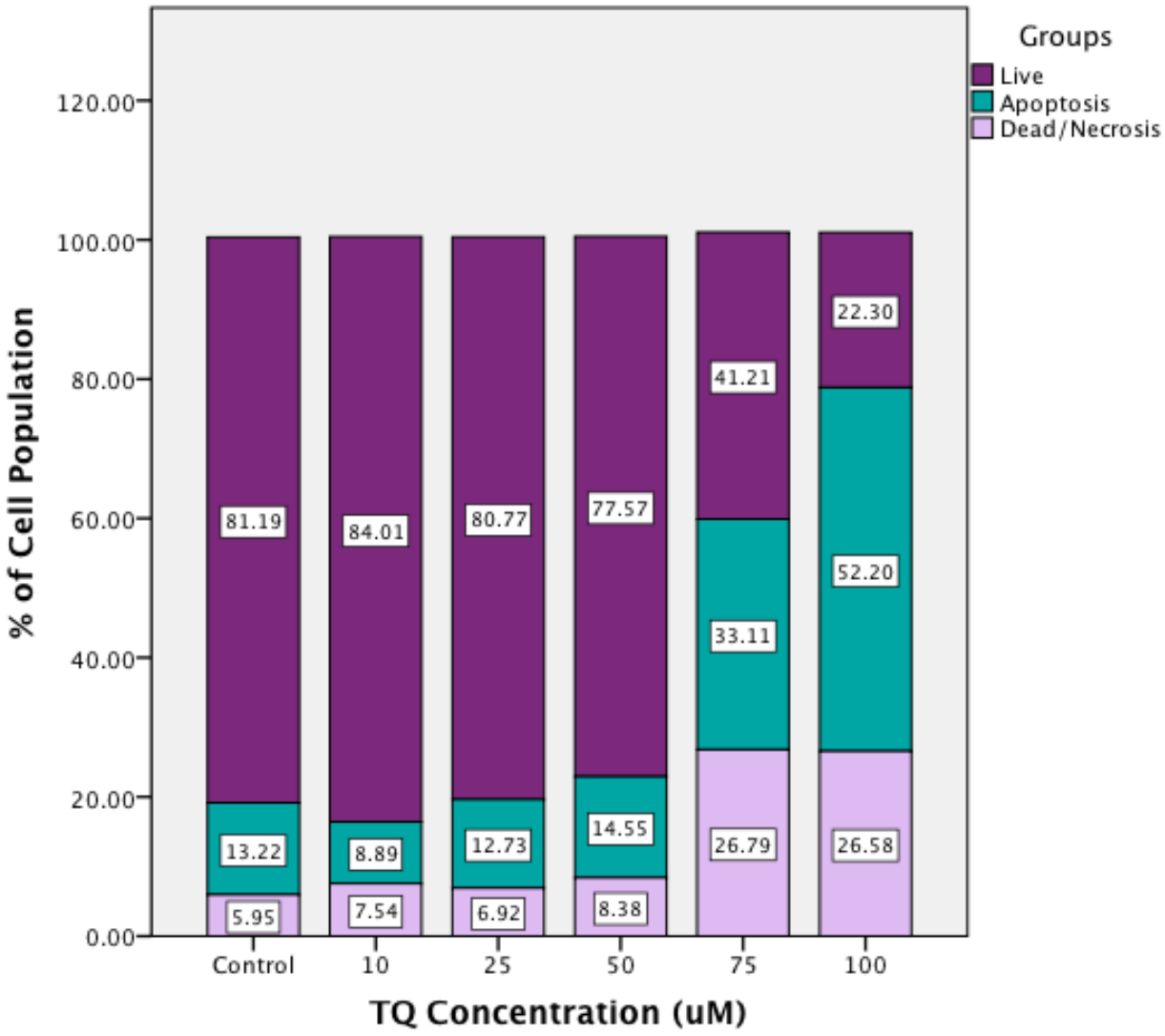



**Figure 9. Flow cytometric plots of FITC Annexin V/PI staining of (A) HepG2 and (B) HuCCT1 cell lines. Results shown are one representative of three independent experiments.**

A)


Groups
Live
Apoptosis
Dead/Necrosis
% of Cell Population
120.00
100.00
80.00
60.00
40.00
20.00
0.00
81.19
84.01
80.77
77.57
41.21
22.30
52.20
33.11
13.22
8.89
12.73
14.55
26.79
26.58
5.95
7.54
6.92
8.38
Control
10
25
50
75
100
TQ Concentration (uM)

B)

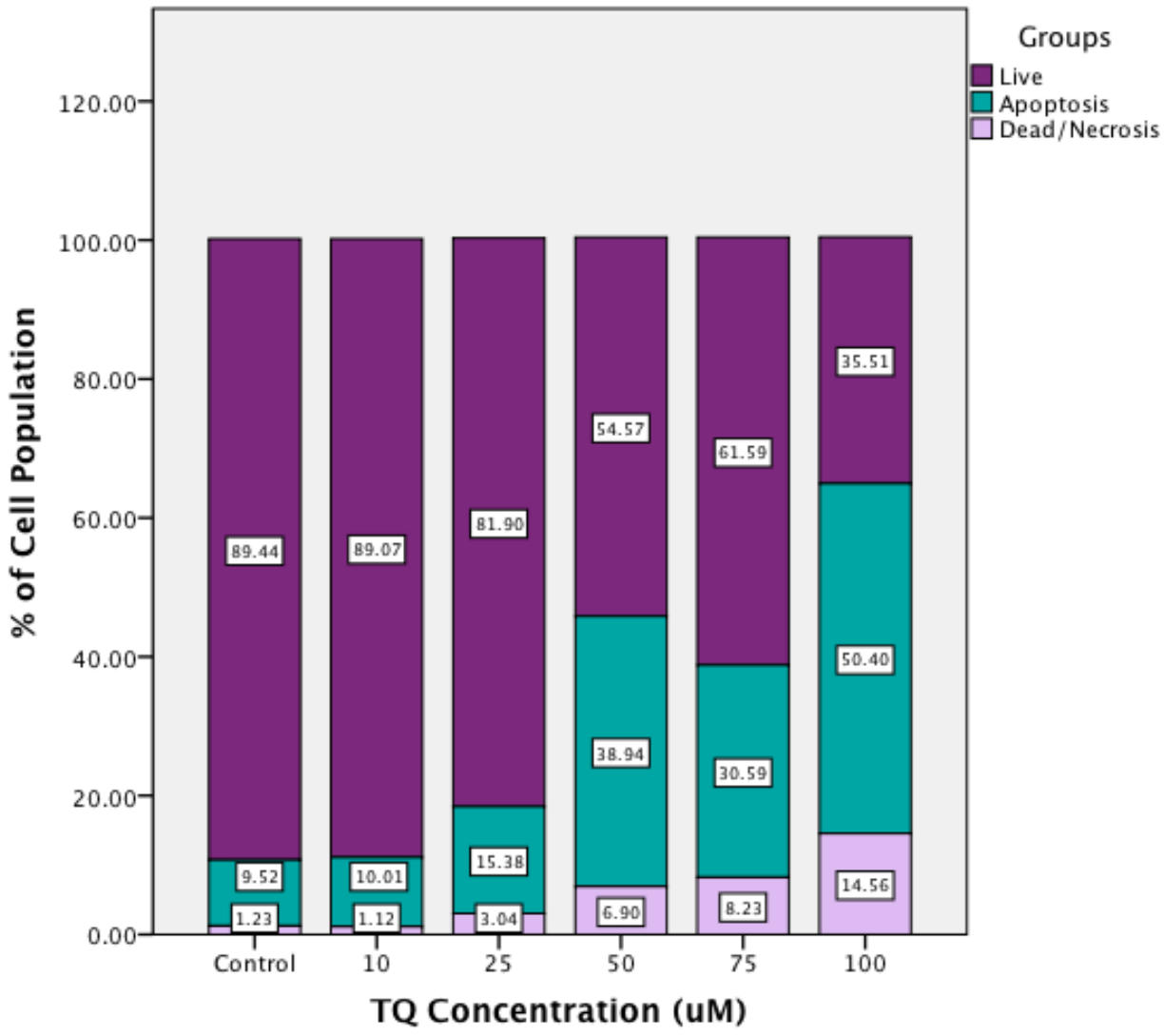



**Figure 10. Flow cytometric Analysis of FITC Annexin V/PI staining of (A) HepG2 and (B) HuCCT1 cell lines. Values are presented as mean of 3 individual experiments**

### 3.4. Determination of morphological changes of cells treated with TQ

Confocal microscopy was used to assess morphological alterations of HepG2, HuCCT1 and normal THLE-3 cell lines following exposure to different concentrations of TQ for 24 h. HepG2 and HuCCT1 cell lines treated with TQ showed a noticeable reduction in cell number, which was more obvious at higher concentrations of TQ (**Figures 11a, 11b**). Cells also began to lose cell-cell contact and to shrink and round up, and were clustered together and detached from the surface of culture dishes. Some cells exhibited chromatin condensation and membrane blebbing.

All of these observed changes are indications of apoptosis-mediated cell death. As concentration of TQ increases, more effects were observed in cells, such as formation of apoptotic bodies with fragmented nuclei. In contrast, untreated cells and vehicle control cells treated with DMSO displayed no observable effects. They were well spread and attached to the surface of the culture dish, with prominent and intact nuclei.

Similarly, no effect on the normal HEK293T cell lines was observed after TQ treatment, as confirmed by light microscopic examination (**Figure 12**). Thus confirms that TQ had no toxicity on HEK293T cell lines. In contrast, normal THLE-3 normal cell lines displayed slight changes in the morphological appearance in response to TQ exposure (**Figure 13**).

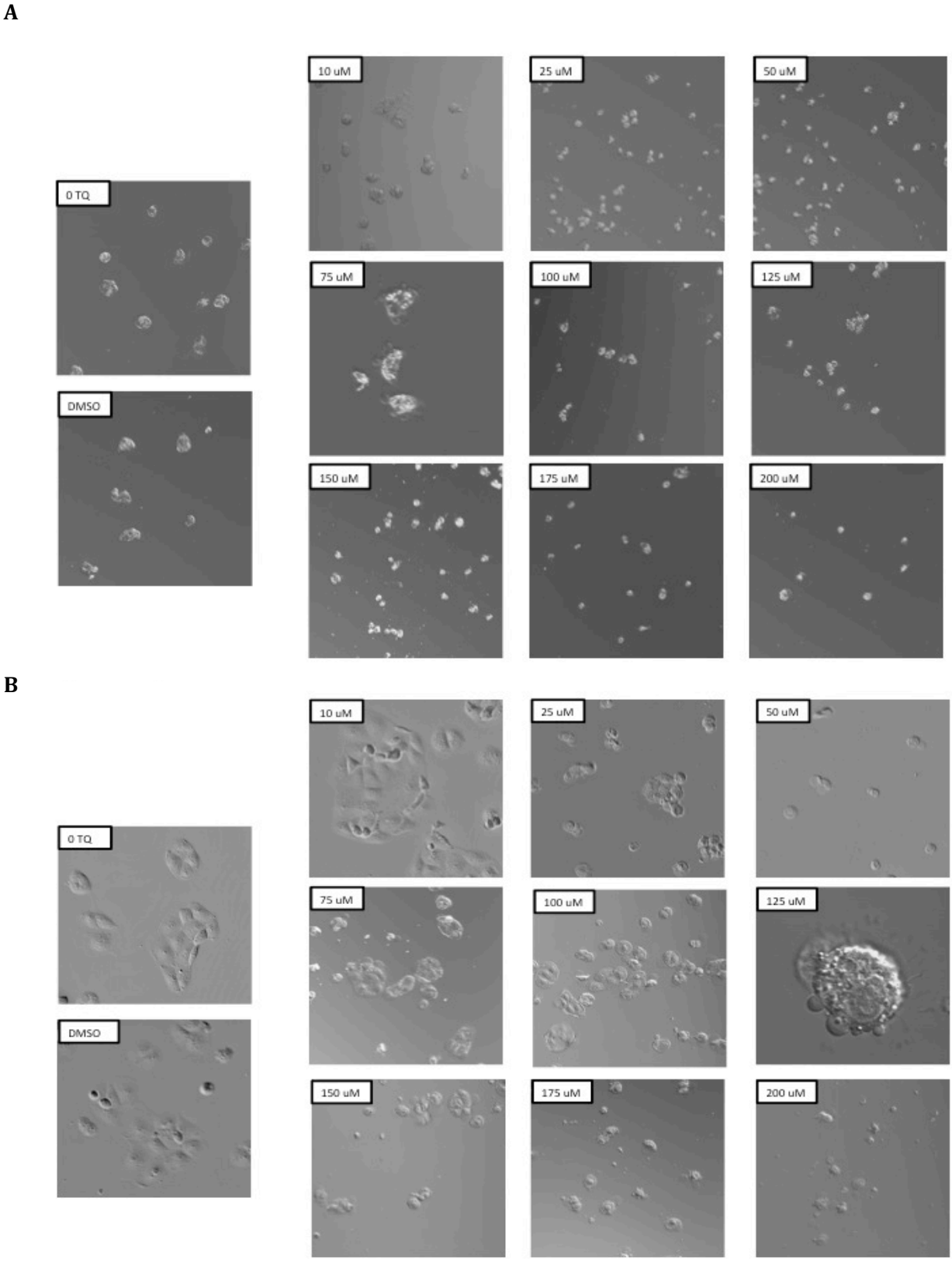



**Figure 11. Morphological Alterations Associated with TQ treatments in (A) HepG2 cells and (B) HuCCT1 cells as seen by confocal microscopy**

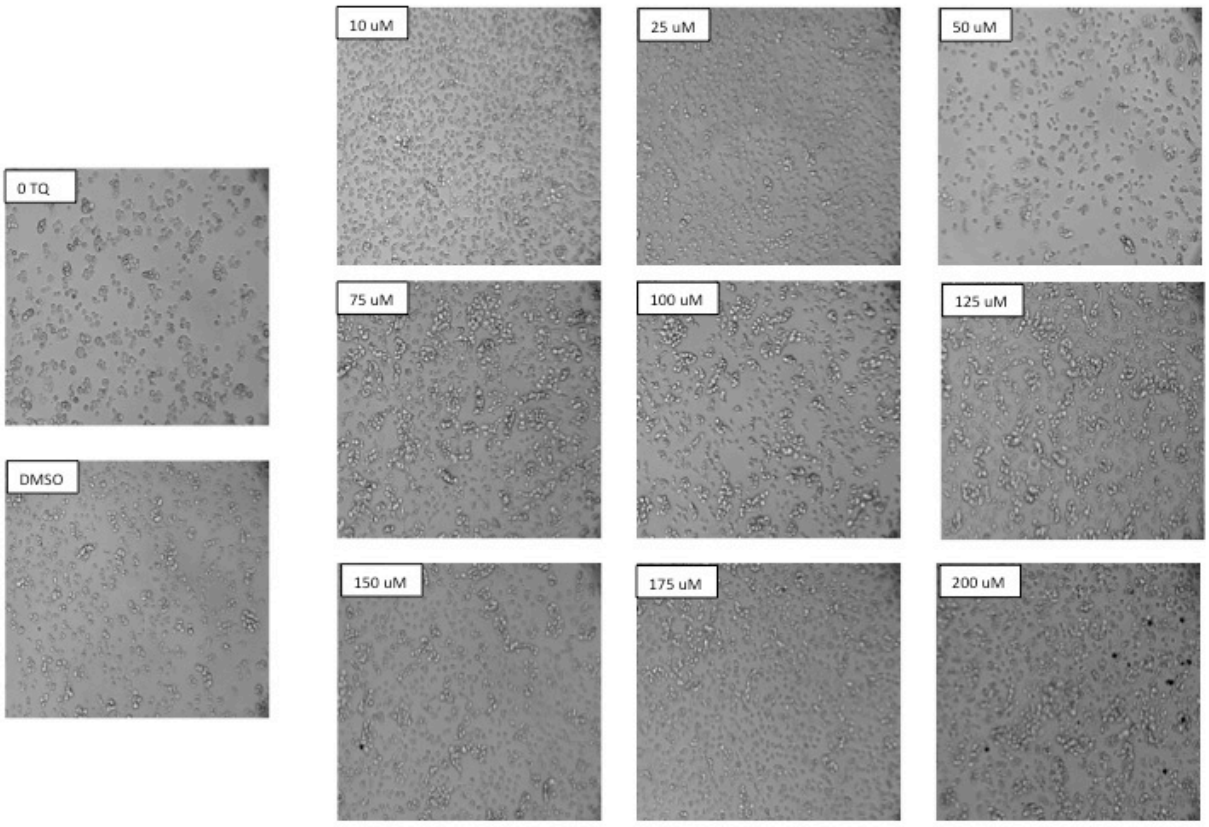



**Figure 12. Digital Images of Normal HEK293T cell lines following exposure to TQ concentrations.**

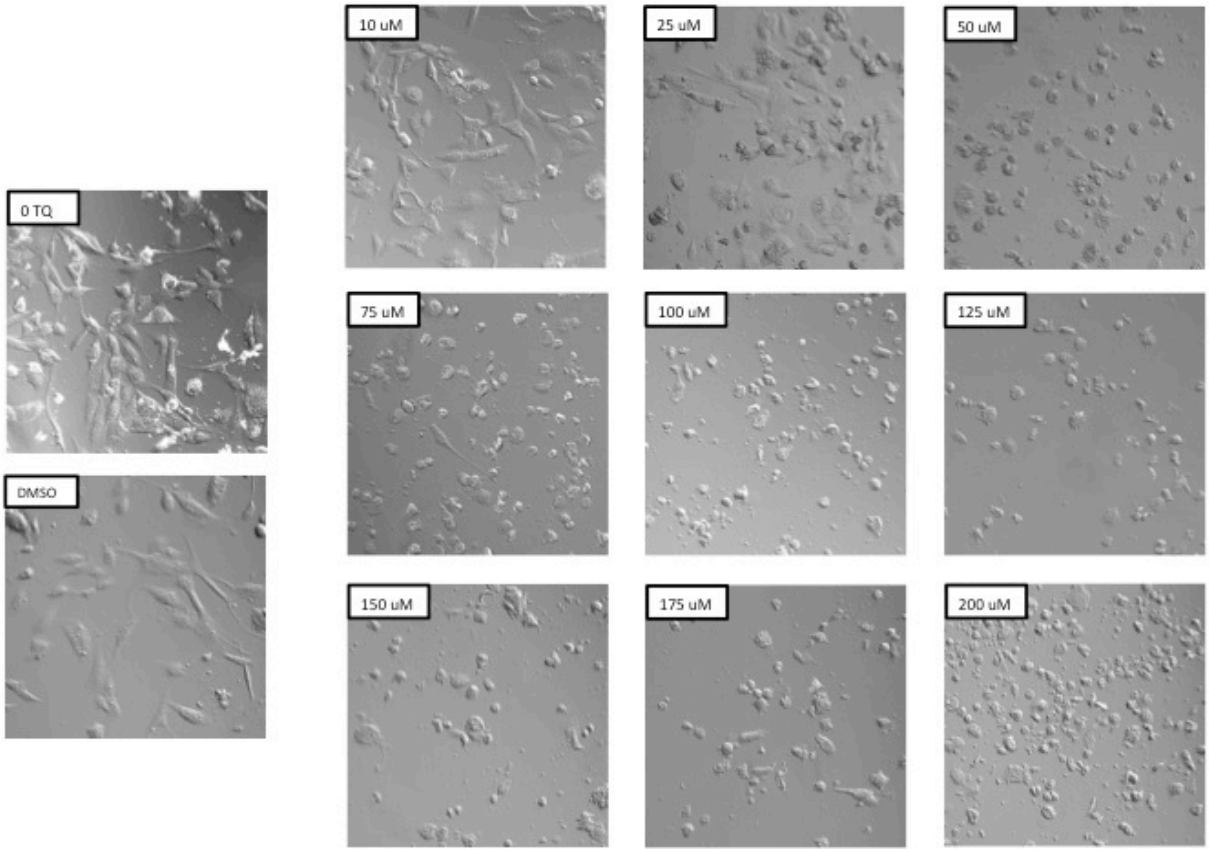



**Figure 13. Confocal Images of Normal THLE-3 cell lines following exposure to TQ concentrations**

### 3.5. Disruption of mitochondrial membrane potential (MMP) by TQ

Mitochondria play an essential role in cellular apoptosis. Hence, the changes in MMP of TQ-treated cells were detected using the fluorescent cationic JC-1 dye. In the normal polarized mitochondrial membrane, the JC-1 dye emits red fluorescence (dimer) and in the depolarized mitochondrial membrane it emits green fluorescence (monomer).

The exposure of cell lines to TQ resulted in a decrease in MMP in a dose-dependent manner in both HuCCT1 and HepG2 cell lines compared to non-treated controls. Due to altered MMP in TQ-treated cells, the JC-1 dye did not accumulate in the mitochondria and persisted in its monomeric form emitting green fluorescence.

A gradual reduction in MMP was observed until it was completely disappeared at 100 μM for HepG2 cells and at 175 μM for HuCCT1 cells (**Figures 14a, 14b**). In untreated controls, the JC-1 dye aggregated in the mitochondria emitting a bright red fluorescence.

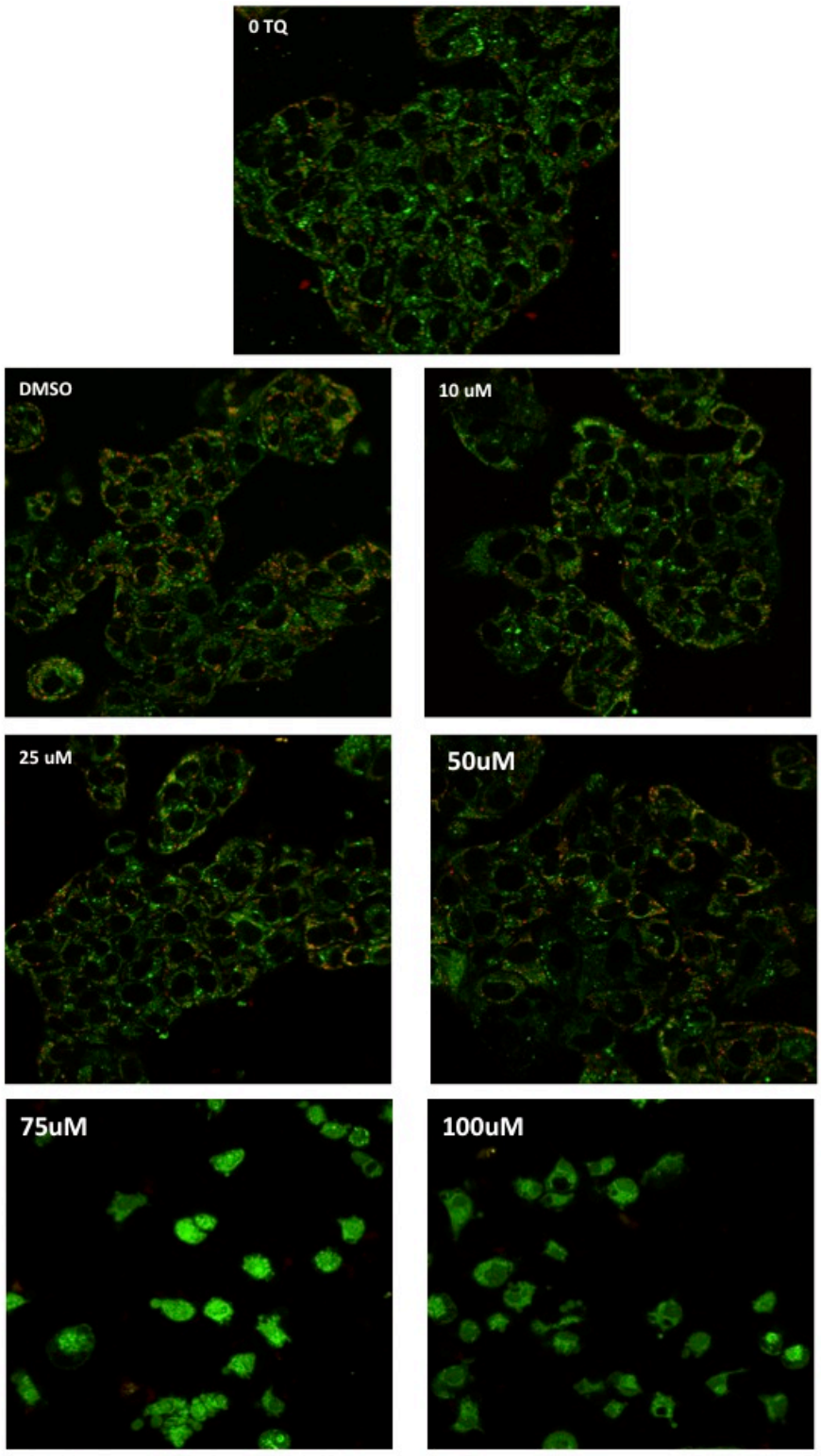



**Figure 14.a. The disruption of MMP in HepG2 cells following exposure to TQ concentration for 24 hr**

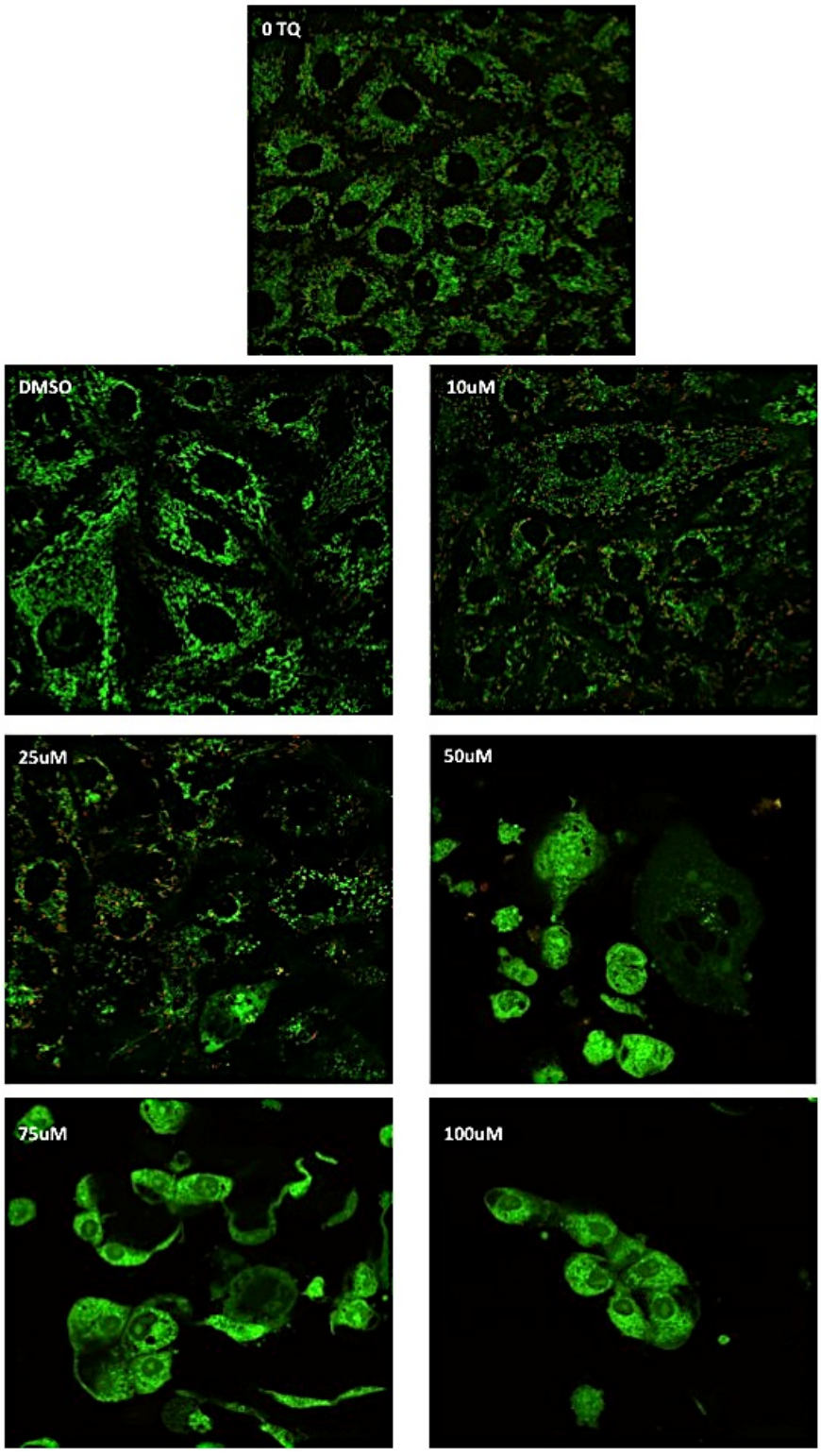



**Figure 14.b. The disruption of MMP in HuCCT1 cells following exposure to TQ concentration for 24 hr**

# Chapter 4: Discussion

## 4.1. General discussion

Cancer is a multi-complex and multi-factorial disease characterized by uncontrolled proliferation and dedifferentiation of cells. It usually arises from alterations in several signaling pathways and multiple DNA affecting the survival and development of cells [267, 268]. Hepatocellular carcinoma and cholangiocarcinoma are the two most common primary malignancies of liver in adults with an annual increase in their incidence over the past decade [269, 270].

Patients with these cancers are often diagnosed at advanced stage of pathogenesis and have poor prognosis with low survival rate [6, 72, 271]. The treatment options for cancerous tumors, which have been shown to prolong life, involve conventional therapy like chemotherapy with or without the combination of radiotherapy and/or surgery and immunotherapy that unfortunately has narrow therapeutic effect. These treatment options are expensive, not easily available and associated with multiple side effects. For example, the use of chemotherapy has been linked with the inhibition of proliferation of bone marrow stem cells result in immune suppression [272]. Additionally, radiotherapy damage DNA and suppress its duplication in order to kill the cancerous cells, but also targets normal cells. Cancer patients also experience other undesirable side effects like bone necrosis, lung fibrosis, renal damage, alopecia, nausea, vomiting and ulceration [187]. The toxicity of chemotherapeutic agents against tissues or cells is associated with proliferation rate. Toxic effect of these agents is lower on slowly dividing tissues or cells, resulting in reduced effect on slowly growing tumors. Rapidly dividing tissues or cells, including normal tissues with high proliferation rate, will be more readily afflicted by chemotherapeutic agents. Effects on normal tissues by chemotherapy include the deterioration of gastrointestinal mucosa and the suppression of bone marrow.

The majority of existing anticancer drugs were developed to target a specific signaling pathway responsible for tumorigenesis. However, cancer is a multi-complex disease and using a multi-targeted drug would be a more effective antitumor treatment [267, 273]. Therefore, an ideal anticancer drug should be multi-targeted that targets cancerous cells without harming normal cells.

Natural compounds derived from plants, herbs and spices have garnered substantial interest as an alternative therapy for treating cancer patients, as these frequently have been shown to target multiple signaling pathways with minimal or no side effects [274]. Furthermore, medicinal plants have been traditionally used for centuries as therapeutic agents for treating different diseases and illnesses including cancer and their possible side effects may have already been identified. Thus, natural products provide a promising lead for the discovery of novel chemotherapeutics and pharmacological targets as they provide a wide spectrum of valuable sources with several biological activities [275, 276].

Interestingly, many approved anticancer drugs were derived from constituents discovered from naturally existing sources either as natural or pure compounds or as natural derivatives with some semi-synthetic modifications [276, 277]. A review by Gurib-Fakim [278] described many of the marketed anticancer drugs derived from natural sources. Examples of these pharmaceuticals include Paclitaxel (Taxol), a drug used to treat lung cancer, which was isolated from *Taxus brevifolia* normally known as the Pacific Yew tree. Vincristine and Vinblastine are alkaloidal compounds isolated from *Catharanthus roseus*, also known as Periwinkle. Both alkaloids are reported to have anticancer activities. Additionally, two compounds, quassinoid glucosides bruceantinoside-A and B and bruceanic acids, were isolated from *Brucea antidysenterica* and were reported to have antitumor activity against several types of cancers. Another plant-derived anticancer drug is Vepesid, which is made from the semi-synthetic derivative (Etoposide) from podophyllotoxin - the active constituent of *Podophyllum peltatum,* commonly known as May apple or Devil's apple.

In this thesis, thymoquinone, a phytochemical extracted from seeds of *Nigella Sativa* plant, was screened for the possible anticancer activity in HCC and CCA cell lines. The results demonstrated that thymoquinone inhibited the proliferation of cancer cell lines and induced cell cycle disruption and apoptosis through the mitochondrial pathway.

### 4.2. Reduction of cell viability after exposure to TQ

The anti-proliferative activity of TQ was tested using the colorimetric MTS assay. Briefly, all cell lines were seeded overnight and exposed to different concentrations of TQ [10-200 μM] for 12, 24 and 48 h. The experimental findings showed a concentration- and time-dependent reduction in cell viability of TQ-treated cells compared to non-treated controls (**Figure**

**5a, 5b, 5c**). The $IC_{50}$ values for TQ were calculated (the concentration that results in 50% cell death).

The results demonstrated a dose- and time-dependent decrease in cell proliferation of TQ-treated HepG2 and HuCCT1 cells. However, a slight paradoxical increase in cell proliferation at concentrations of 10 and 25 μM at 48 h of incubation were observed in HepG2 cells. This could be due to initial co-activation of proliferative genes of cancer cells such as VEGF, MAPK proteins like JNK and ERKs and AKT/PKB before the concentration of TQ needed to kill cells is achieved.

TQ was found to be cytotoxic to both HepG2 with $IC_{50}$ values of 50.74 μM, 34.23 μM and 50.84 μM, for 12, 24 and 48 hours and HuCCT1 cell lines with $IC_{50}$ values of 155.1 μM, 63.12 μM and 61.59 μM, for 12, 24 and 48 hours, respectively. HepG2 cell lines showed to be more sensitive to TQ treatment.

These results support the findings of a previous study that reported a significant inhibition of growth of HepG2 cell lines corresponding to TQ exposure, in a concentration dependent manner [279]. In that study, the $IC_{50}$ value of TQ after 24 h was reported to be 350 μM. However, a similar study by Attoub et al. (2013) confirmed the dose-dependent growth inhibition of TQ on several cell lines including HepG2 but reported an $IC_{50}$ value of 34 μM [280]. Additionally, TQ was reported to reduce the cell viability of two HCC cell lines -HepG2 and Huh-7- in a dose dependent manner [281].

Only one study has reported the anti-proliferative effect of TQ on HuCCT1 cells [282]. The results indicated that TQ caused a concentration- and time-dependent reduction in the number of viable cells. We observed similar results where TQ reduced cell proliferation *in vitro* in different cancerous cell lines, which are summarized in **Table 3**.

The anti-proliferative effect of TQ on normal THLE-3 cell lines was also tested in the current study. However, due to a limited number of THLE-3 cell lines in the lab and difficulties in culturing them, only one experiment was carried out, in quadruplicate. The results indicated that lower concentrations of TQ exert no effect on normal cells. The $IC_{50}$ after 24 hours exposure to TQ was 200 μM, which is the highest concentration used in this study. Our results indicate that TQ has limited cytotoxic effect on non-cancerous cell lines, which correlate with the findings of other studies. For example, TQ has been reported to have some cytotoxic effect on normal human

cell lines including lung fibroblast cells (IMR90) [220], intestinal cells (FHs74Int) [238] and prostate epithelial cells (BPH-1) [219].

One limitation of this experiment is that no positive control was used like using a cytotoxic drug (saponin) or lytic detergent. According to previously published studies on TQ and manufacturer's protocol, positive controls were not used, as they are not required.

**4.3. Disruption of cell cycle distribution in TQ-treated cells**

Cell cycle checkpoints play an essential role in ensuring the accuracy of the replication and division of DNA [174, 283]. Cell proliferation results from completion of the cell cycle, which is comprised of four phases. These phases include the G1 phase (the interval prior to replication of DNA), S phase (the synthesis of DNA phase), G2/M phase (the gap after the replication of DNA) and M phase (the mitotic phase) [173, 174]. In the event that DNA damage occurs, cell cycle arrest ensues to permit time for DNA repair [284]. This repair pathway consists of three checkpoints. The first checkpoint takes place at the end of the G1 phase and is regulated by P16, a cycline dependent kinase inhibitor (CDKI). The second checkpoint occurs at end of the G2 phase and is controlled by cycline dependent kinases (CDK1). The last checkpoint is called the spindle assembly checkpoint (SAC), which maintains accurate distribution of chromosomes to daughter cells [171, 285, 286]. Cell cycle arrest checkpoints provide possible targets for therapeutic agents to treat cancers and quinones are reported to be one of drugs that cause cell cycle arrest [180, 287].

Accordingly, the capacity of TQ to induce cell cycle arrest in HCC and CCA cell lines was investigated. Briefly, HepG2 and HuCCT1 cell lines were seeded overnight and then treated with different concentrations of TQ for 24 hours. Cells were harvested and fixed with 70% ethanol. The DNA contents of cells were stained with PI and analyzed using flow cytometry.

HepG2 cell lines did not show accumulation of cells in any cell cycle phase. However, the number of cells in the G2/M phase increased with lower concentrations (10 μM, 25 μM and 50 μM ) and decreased with 75 μM and 100 μM (**Figure 6b**). The decrease in cells in the G2/M phase at high concentrations may be due to cell loss during cellular preparations, as the majority of cells were dead and were floating in the media. This resulted in low cell numbers when analyzed by flow cytometry; typically 20,000 events are needed and in these samples only around 10,000 events were available. If 20,000 events were captured, the percentage of cells would be increased with 75 μM and 100 μM TQ, confirming the cell cycle arrest of HepG2 cell lines in

G2/M phase. This result corresponds to findings reported in a similar study, where TQ was found to arrest HepG2 cells in G2/M phase of cell cycle [281]. Contrary to the observations of the current study, another study reported that exposure of HepG2 cells to TQ induced cell cycle arrest at the G1 phase [279]. These variations in the ability of TQ to arrest HepG2 cells in G1 and G2/M phases could be due to different concentrations of TQ used in each study as well as different duration periods. Another possibility is type of solvent used to dissolve TQ. In the first study done by Ashour et al. (2014), TQ was prepared in ethanol and different concentrations used were 6, 12.5, 25, 50 and 100 μM. Cells were exposed to TQ for 6, 12 and 18 hours [281]. Whereas in Hassan et al. (2008), researchers dissolved TQ in methanol and further concentrations (25, 50, 100, 200 and 400 μM) were prepared in medium [279]. However, 350 μM TQ concentration was the only dose tested for cell cycle analysis with incubation periods of 6 and 12 hours.

Similar to the findings of the current study, other studies investigating the cell cycle arrest activity of TQ have demonstrated the G2/M arrest in other cell lines involving I7 mouse spindle carcinoma cells via increase in p53 and decrease in cycline B [288], MNNG/HOS human osteosarcoma cells due to $p21^{WAF1}$ up-regulation [213], MCF-7/DOX doxorubicin-resistant breast cancer cells through increase in p53 and p21 proteins [225], MNNG/HOS osteosarcoma cell lines by the up-regulation of $p21^{Waf1}$ [213].

Moreover, as shown in **Figure 7b**, the results indicate that TQ induced cell cycle arrest in G1 phase in HuCCT1 cell lines, made evident by the increased G1 population. However, these findings are opposing to a previous study where TQ caused G2/M arrest in HuCCT1 cells [282]. In this study, researchers used TQ at concentrations of 20-60 μM and incubated cells with treatments for 48 hours. Whereas in my experiment, TQ concentrations were 10-100 μM and the incubation period was 24 hours. The prolong exposure to TQ for 48 hours could be the explanation of the G2/M arrest seen in Xu, Ma [282].

Nevertheless, several studies have demonstrated the G1-arresting property of TQ in different cancer cell lines. For instance, TQ was shown to induce G1 arrest in HCT 116 human colorectal carcinoma cells through regulation of p53 [215], acute lymphoblastic leukemia Jurkat cell lines via a p73-dependent pathway as evident by p73 SiRNA [226], LNCaP prostatic cancer cells by an increase in p21 and p27 and a decrease in E2 transcriptional factor [219], SP-1 mouse papilloma carcinoma cells through an increase in p16 and decrease in cyclin D1 [288], COS31

canine osteosarcoma cell lines [214], and MDA-MB-468 and MDA-MB-231 triple-negative breast carcinoma cells with mutant p53 [289].

Cumulatively, the results of the current and previous studies confirm the ability of TQ to cause a cell-line-dependent cell cycle arrest at different phases, resulting in suppression of growth. Additionally, in both cell lines studied in the present study, TQ-mediated cell cycle arrest was associated with an increase in the apoptotic cell population (Sub-G1) as seen in **Figure 4**.

Derivatives of TQ like TQ-H-10 (thymoquinone-4-α-linolenoylhydrazone) and TQ-H-11 (thymoquinone-4-palmitoylhydrazone) have been reported to cause cell cycle arrest in the S/G2 phase in HCT116 human colon cancer cells [290].

The mechanism involved in TQ-induced cell cycle disruption in cancer cells has been reported to be due to effects of TQ on CDKs, their inhibitors and cyclines. These are the regulators of transitions between cell cycle phases [291]. It has also been reported that the main mechanism of TQ-induced cell cycle arrest was up-regulation of tumor suppressor p53 and p21 proteins, the latter of which is the transcriptional target of p53 [291]. Additionally, TQ was found to cause cell cycle arrest by targeting other proteins like p16, p73 and cyclin B as described earlier.

### 4.4. Induction of apoptosis in cell lines following TQ treatment

In addition to anti-proliferative and cell cycle arrest effects, the apoptotic effects of TQ were investigated. Both HepG2 and HuCCT1 cell lines were seeded and treated with different doses of TQ for 24 h. Following the treatment, cells were stained with Annexin V/PI and analyzed using flow cytometry. Annexin V is a $Ca^{+2}$ dependent phospholipid binding protein, which has the ability to bind to the phosphatidylserine (PS) protein that externalizes from the inner membrane to the outer surface of plasma membrane of cells undergoing apoptosis [148, 292-294]. Annexin V is used in combination with propidium iodide (PI) dye to distinguish early from late apoptotic cells. Live cells have intact plasma membranes that exclude PI, whereas membranes of damaged cells are permeable to PI [145, 292]. .

The results obtained indicated that TQ treatments of HepG2 and HuCCT1 cell lines increased the percentage of cells in the early and late stage of apoptosis in a concentration-dependent manner (**Figures 9, 10**). These findings suggest that TQ-mediated cell death occurs via induction of apoptosis in these cell lines, which is consistent with data from previous studies showing the apoptotic effect of TQ in several cancer cell lines. Similar studies investigating the

effect of TQ on HepG2 cells have reported that TQ increased the percentage of cells in early and late apoptosis in a concentration-dependent manner [281]. Another study reported that treatment of HepG2 cells with TQ caused 57% of cells to undergo apoptosis [279]. Additionally, the presented apoptotic effect of TQ on HuCCT1 was consistent with a previous study reporting the apoptosis-inducing ability of TQ in HuCCT1 cell lines [282].

These results are in agreement with earlier studies that tested the apoptotic activity of TQ on various cancer cell lines. One study showed that 100 μM TQ induces apoptosis in HCT-116 colorectal carcinoma cells [215]. Moreover, TQ was found to mediate apoptosis in p53-null HL-60 cells in a dose- and time-dependent manner [222]. The induction of apoptosis was also seen in TQ-treated SP-1 and I7 cells [288]. Furthermore, the apoptotic effect of TQ was reported in primary effusion lymphoma (PEL) cell lines including BC-1, BC-3, BCBL-1, and HBL-6 where TQ caused a dose-dependent apoptosis [230]. TQ-mediated apoptosis has been observed in other cancer cells including DLD-1 colon cancer cell lines [238], HUVEC human umbilical vein endothelial cells [252], NCI-H460 and NCI-H146 lung cancer cells [236], M059J, ACHN human renal cell carcinoma [241] and M059K human glioblastoma cell lines [220].

The induction of apoptosis in different cancer cells following treatment of TQ found to be mediated by p53-dependent and p53-independent pathways [215, 222, 241]. Additionally, p73, NF-kB, STAT3 and PTEN pathways were also involved in TQ-mediated apoptosis in different cell models [194]. Finally, oxidative stress via generation of ROS may mediate TQ-inducing apoptosis [238].

Moreover, prolonged exposure to TQ found to induce necrosis in cancerous cell lines. For example, TQ induced 43% necrosis in COS31 canine osteosarcoma cells [214]. This suggests that TQ induces apoptosis in the majority of cancer cells but with prolonged exposure and at higher concentrations of TQ, necrosis was also observed. The mode of cell death after TQ treatment varies depending on type of cancerous cell lines [237].

### 4.6. Morphological alterations in TQ-treated tumour cells

The induction of apoptosis in HepG2 and HuCCT1 cell lines was further supported by the examination of morphological changes of cells following exposure to increasing concentrations of TQ for 24 h.

The results indicated that treatment of cells with TQ caused significant alterations in cellular morphology comparing to non-treated control as shown in **Figure 11a, 11b**. After a 24 h

treatment with TQ, HepG2 and HuCCT1 cells displayed noticeable alterations and distress such as cell shrinkage, reduced cytoplasm, and reduced nuclear size. Additionally, TQ treatment caused cells to round-up, reduce adhesion and detach from the surface of plates. With higher concentrations of TQ, the reduction in cell number was more obvious and most cells were detached and aggregated together, forming clusters. Moreover, the majority of cells were dead, evidenced by floating cells in media, fragmentation of cells and presence of background debri (apoptotic bodies). All of these features are hallmarks and signs of apoptosis [145].

In contrast, untreated control cells in all cell lines exhibited a high proliferation rate with around 90% confluency; strong adhesion to the surface of the culture dish was observed.

The morphological assessment of normal HEK293T cell lines was performed using light microscopy. As shown in **Figure 12**, TQ-treated cells remained similar in shape to untreated control cells. The proliferation rate of cells was consistent over the concentration ranges of TQ employed in this study. These findings confirmed that TQ has no cytotoxic effect toward non-cancerous cell lines. One study done on HEK293T cell lines showed that pretreatment of these cell lines with TQ had no cytotoxic effect and IC50 value of TQ was > 100 μg/ml [295].

Alterations in morphology of TQ-treated normal THLE-3 cell lines were also examined using confocal microscopy. The results revealed some changes in cellular appearance of cells as a result of TQ treatment. As shown in **Figure 13**, the presence of background debri and detached cells occurred even under untreated control conditions. At the time of experiment, there were difficulties culturing the THLE-3 cell lines and cells were not attaching probably to the surface of culture plates. The experiment could not be repeated because cells were unavailable. This explains the unexpected results of morphological assessment as they contradict the results obtained by MTS assay. It was difficult to conclude whether the morphological changes observed related to TQ treatment.

These results confirmed the significant cytotoxic effect of TQ on cancerous cell lines and suggest its possible effect to induce cell death in these cell lines through apoptotic pathway.

### 4.7. Alteration of MMP in cells treated with TQ

Mitochondria are essential organelles and key regulators of cell function that contribute to the regulation of cell survival and death. Its main function is the generation of energy (ATP) by transport of electrons across membrane and oxidative phosphorylation. Electrochemical proton

gradient, generated within inner membrane of mitochondria, create differences in electrical potential leading to MMP.

MMP is important for the production of ATP, that is driven by Electron transport chain that consists of complexes I, II, III, IV and V [296]. MMP also play a critical role in homeostasis of $Ca^{2+}$, importation of proteins and transportation of metabolites. High level of $Ca^{2+}$ in cytoplasm will cause accumulation of $Ca^{2+}$ in mitochondria leading to reduction of MMP and hence reduction of ATP synthesis [297].

Loss of MMP with high levels of $Ca^{2+}$ will cause irreversible opening of mitochondrial permeability transition (MPT) pores allowing free exchange of proteins between inner and outer mitochondrial membrane [298]. Consequently, swelling of mitochondria and disruption of outer membrane occurs leading to release of cytochrome c into cytoplasm. In cytoplasm, cytochrome c binds to apoptosis protease-activating factor (Apaf-l) resulting in the caspase activation responsible for apoptosis [299-303]. Several factors have been involved in opening and closer of MPT pores including anti-apoptotic Bcl-2 proteins, cyclosporine A, reactive oxygen species and glutathione [304, 305].

In cancer cells, the MMP is mainly elevated. Determining loss of MMP is an essential indicator of mitochondrial disruption and apoptosis induction. This can be conducted by using fluorescent dyes that distribute across mitochondria and aggregate in negatively charged area.

The effect of TQ on MMP of HepG2 and HuCCT1 cells was investigated to determine whether TQ-induced apoptosis was mediated by mitochondrial disruption or not. Cells were treated with different doses of TQ for 24 h. Following treatment, cells were stained with JC-1 stain to permit quantification of changes in MMP and visualized using fluorescent microscopy.

As shown in **Figure 14a and 14b**, TQ-treated HepG2 and HuCCT1 cell lines exhibited loss of MMP in a dose-related manner, evidenced by the reduction of red fluorescence indicating depolarized mitochondria. In contrast, untreated controls exhibited a marked red fluorescent, indicative of intact and polarized mitochondria.

These findings suggest TQ-induced apoptosis in HepG2 and HuCCT1 cell lines was mediated via the mitochondrial pathway demonstrated by dissipation of MMP. This discovery is in agreement with previous studies on TQ. For example, the apoptotic effect of TQ on MDA-MB-231 and MDA-MB-468 breast cancer cells was reported to be induced by the mitochondrial pathway, shown by the loss of mitochondrial membrane integrity, release of cytochrome c and

cleavage of PARP [235]. Another study by El-Mahdy et al. (2005) reported that TQ-induced apoptosis in HL-60 myeloblastic leukemia was mediated by a p53-independent pathway, evident by the disruption of MMP and activation of caspases including caspase 8, 9 and 3 [222]. Moreover, Banerjee et al. (2009) investigated the apoptotic effect of TQ on HPAC human pancreatic cancer cells showing that TQ-induced apoptosis in HPAC was mitochondrial in origin as indicated by dose-dependent release of cytochrome c [239]. The contribution of mitochondria to apoptosis induction in MG63 osteosarcoma cell lines as indicated by the activation of caspase 9 and caspase 3 has also been reported [213].

### 4.8. Summary and Conclusion

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are the two most common primary tumors of liver. These lethal cancers are characterized by high invasiveness, infiltration and metastatic potential. Diagnosis of patients with HCC or CCA is typically made at advanced stages; therefore, surgical resection is no longer an option, resulting in poor prognosis and low survival rate. Additionally, at this late stage, the treatment options are limited and unsatisfactory; the administration of high doses of chemotherapy such as cisplatin or 5-fluorouracil is required. However, these chemotherapeutic drugs exert significant toxicity towards normal cells when administered at maximum tolerable doses and resistance to such chemotherapeutics often develops.

Substantial attention has been directed toward identification of naturally occurring anti-tumor agents that exhibit minimal or no toxic effect on normal cells. Many natural substances have been used for centuries in treating cancer. Thymoquinone (TQ), the bioactive constituent of volatile oil of *Nigella Sativa*, has been traditionally used as natural therapeutic for treating several illnesses for more than 2000 years. It has been reported that TQ exhibit anti-tumor, anti-oxidant, and anti-inflammatory properties.

The main objective of this research project was to investigate the anti-cancer activity of commercially available TQ against hepatic cancer cell lines (HCC and CCA). To test the hypothesis that TQ would exhibit anti-cancer effects on HCC and CCA, TQ was screened for potential anti-proliferative and cytotoxic effects using a colorimetric MTS assay, cell cycle disruption was investigated using flow cytometric analysis, apoptosis induction measured using an Annexin V/PI detection kit and microscopic examination of morphological changes and its effect on MMP were assessed using JC-1 dye.

In the light of results demonstrated here, TQ was found to inhibit the proliferation and induce cytotoxicity toward HepG2 and HuCCT1 cell lines in a dose- and time-dependent manner. Additionally, TQ was shown to induce cell cycle disruption in both cell lines and caused cell cycle arrest in the G1 phases of HuCCT1. TQ also exhibited apoptotic effects against these cell lines, as shown by increased number of cells in the early and late stages of apoptosis and visualized features of apoptosis under microscopy including cell shrinkage, chromatin condensation, membrane blebbing and the presence of apoptotic bodies. Finally, TQ caused loss of MMP in a dose-dependent manner, confirming TQ-inducing apoptosis was mediated via the mitochondrial pathway.

In conclusion, TQ extracted from *Nigella Sativa* has been used as a therapeutic agent for hundreds of years; however, the biological mechanisms of action of TQ are not fully understood. Investigating the molecular mechanisms by which TQ induces cell death is important to fully elucidate its potential application for the treatment of hepatic cancers. This thesis provides scientific evidence of the anti-tumor activity of TQ and supports the use of this natural compound as an alternative therapy for treating hepatocellular carcinoma and cholangiocarcinoma. Interestingly, this study is the second study examining the anti-cancer effect of TQ against cholangiocarcinoma cell lines (HuCCT1) after the one done by Xu et al. (2014) [282].

### 4.9. Recommendations for Future studies

The data presented in this thesis improve the understanding and knowledge of the possible mechanism responsible for the anti-proliferation effect of TQ against hepatic cancer cell lines. However, further experiments are needed to more thoroughly understand the sub-cellular mechanisms associated with TQ-induced cell death. The following are suggestions for future directions:

- Investigation of apoptotic markers is required to understand the nature of TQ-induced cellular apoptosis, including assessment of proteins involved in mitochondria-mediated apoptosis such as cytochrome c, APAF-1 and caspases including caspase -9, -8 and -3.
- Exploration of the effect of TQ on protein kinases is needed, as these enzymes are frequent targets for drugs.
- Assessment of the safety, efficacy and dosage of TQ to be used *in vivo* is warranted as well as determining the time-dependent influence of TQ.
- Microarray analyses need to be carried out to identify alterations in DNA following TQ treatment.
- TQ used in combination with other chemotherapeutic drugs may result in improved outcomes. The efficacy and safety of TQ in conjunction with other chemotherapeutic drugs for treating hepatic cancers needs to be determined, as drug-drug interaction may occur.
- Determination of anti-tumor effects of TQ on various cancer cell lines is important. For example, studying p53-null cell lines to determine whether apoptosis is mediated by a p53- dependent or independent pathway would provide better insight about whether the effects of TQ are cell-type or target specific.
- Assessment of the effects of TQ in animal models of hepatic cancer is essential to determining the interaction between normal and tumor cells, which is not possible with *in vitro* studies. Also, *in vivo* models will be necessary to determine the optimum dosage and delivery method of the drug to treat tumors.

## 4.10. References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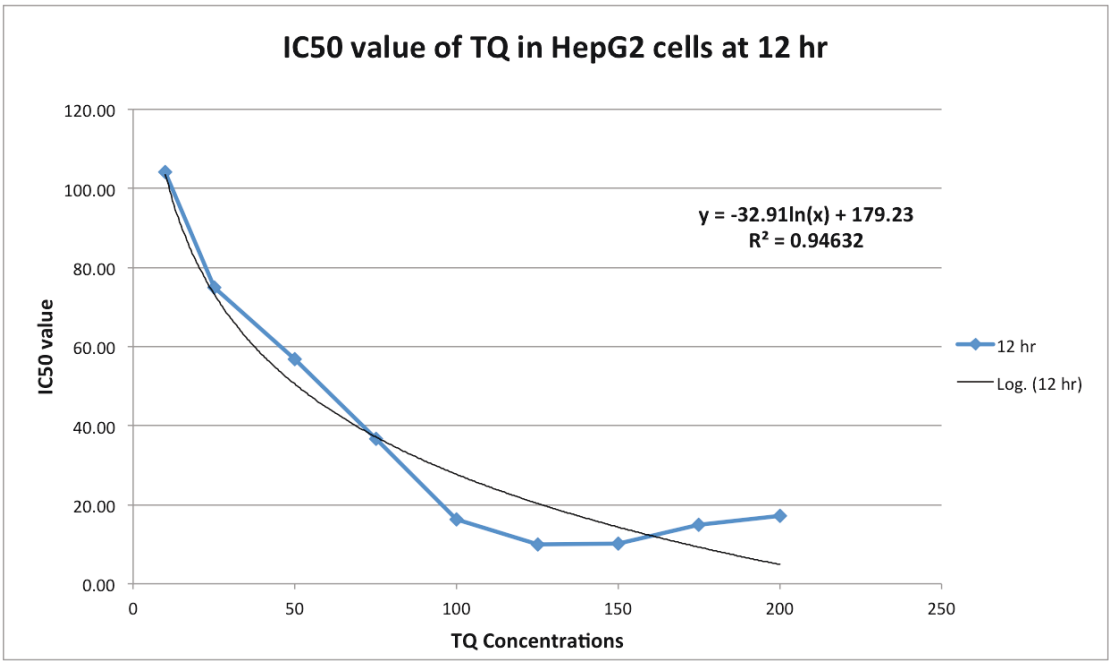
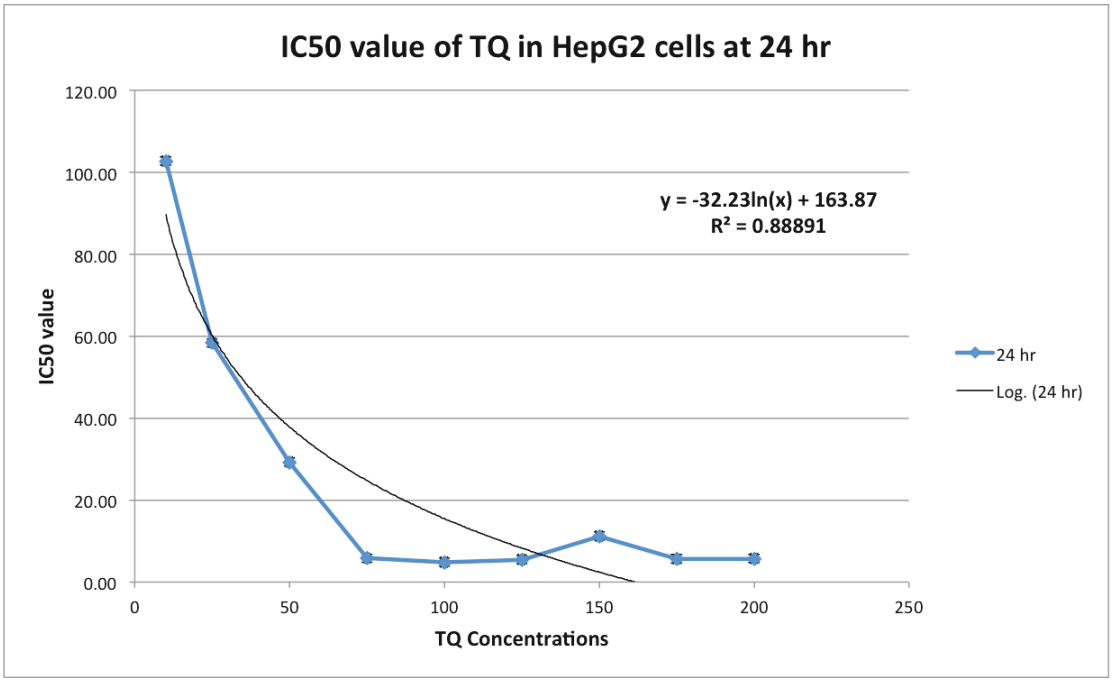
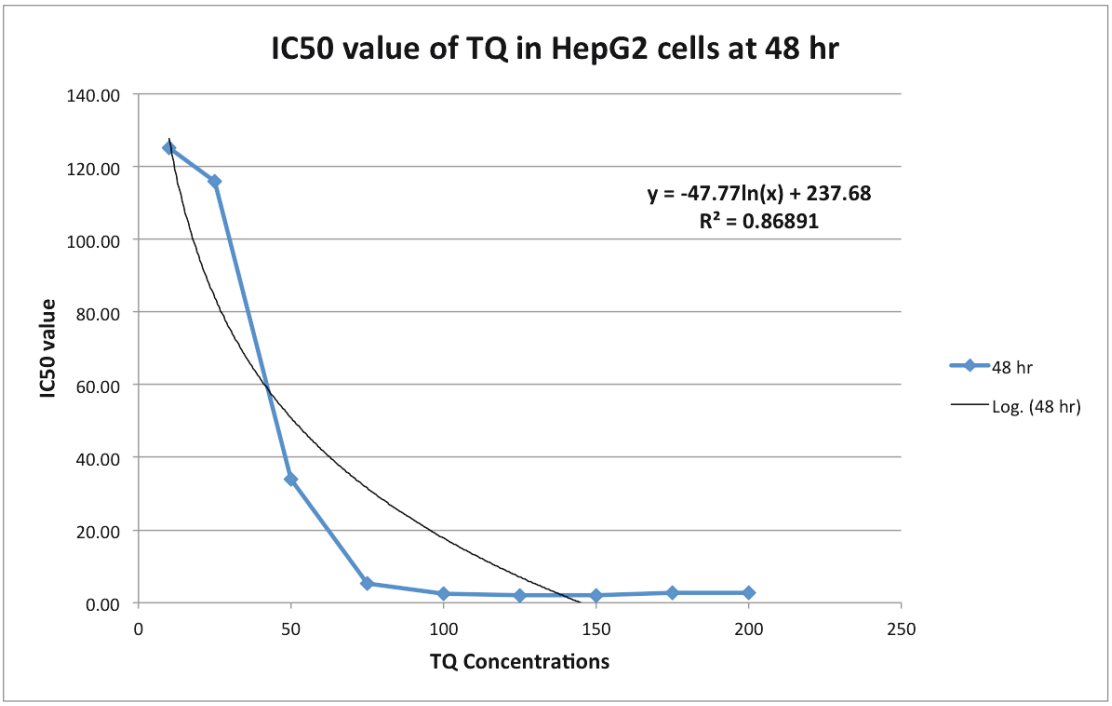
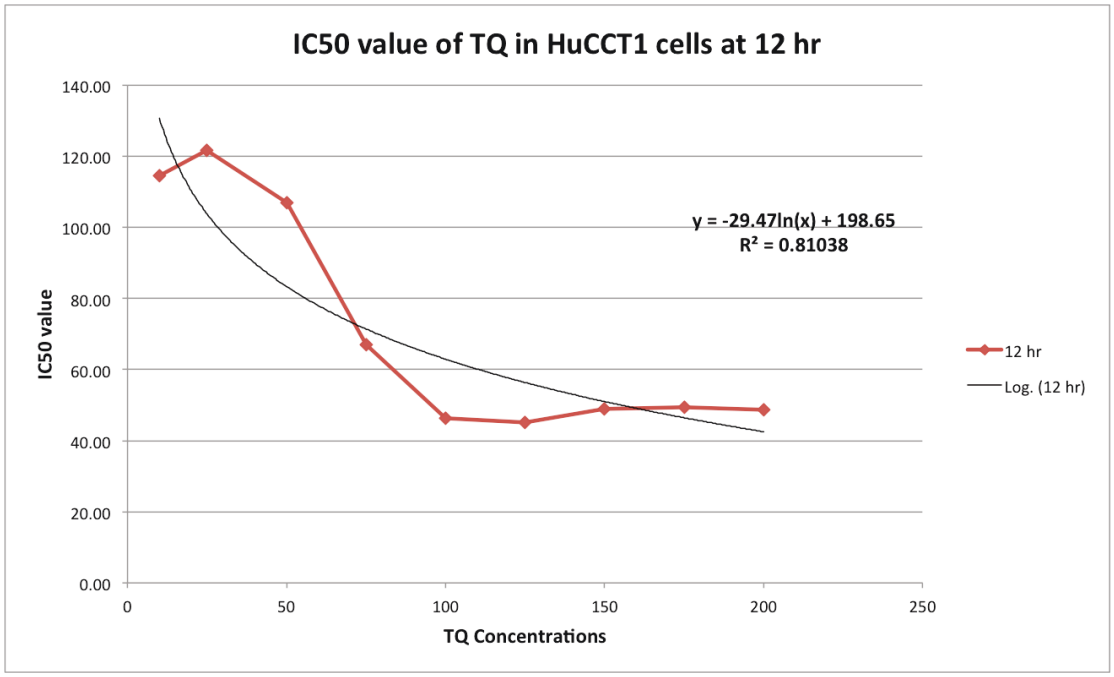
**Appendix A: The calculation of $IC_{50}$ values of TQ.**

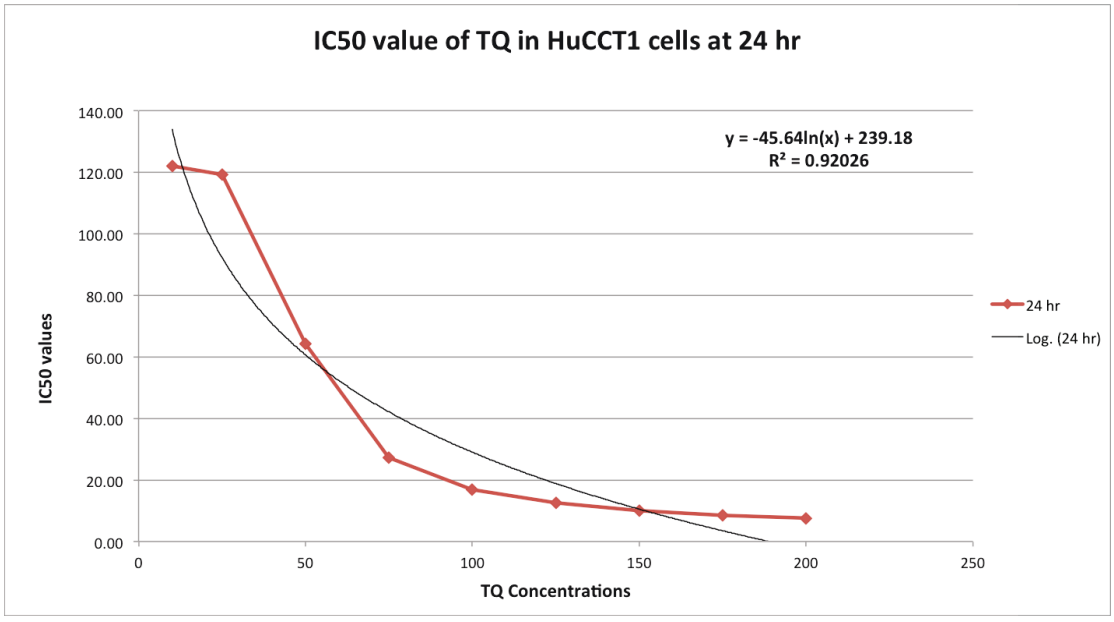
The $IC_{50}$ of TQ in different cell lines at different time points were calculated by using data of MTS assay. Dose response curve was plotted in Excel and logarithmic trend line was added. The $IC_{50}$ was calculated from the formula generated.

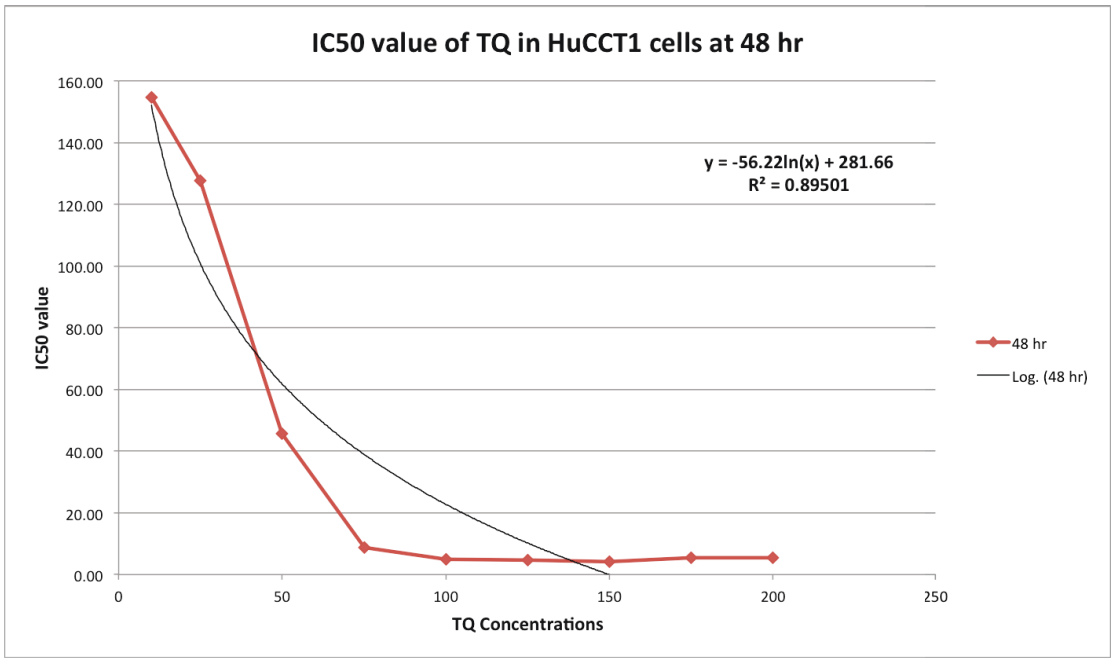



IC50 value of TQ in HepG2 cells at 48 hr
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
IC50 value
0
50
100
150
200
250
TQ Concentrations
y = -47.77ln(x) + 237.68
R² = 0.86891
48 hr
Log. (48 hr)


IC50 value of TQ in HuCCT1 cells at 12 hr
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
IC50 value
0
50
100
150
200
250
TQ Concentrations
y = -29.47ln(x) + 198.65
R² = 0.81038
12 hr
Log. (12 hr)


IC50 value of TQ in HuCCT1 cells at 24 hr
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
IC50 values
0
50
100
150
200
250
TQ Concentrations
y = -45.64ln(x) + 239.18
R² = 0.92026
24 hr
Log. (24 hr)


IC50 value of TQ in HuCCT1 cells at 48 hr
y = -56.22ln(x) + 281.66
R² = 0.89501
IC50 value
TQ Concentrations
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
0
50
100
150
200
250
48 hr
Log. (48 hr)

**Appendix B: Cell Cycle distribution of Cell Lines following Exposure to TQ treatments.** Results shown are one representative of three independent experiments.

| Cell lines | Cell cycle phases | Control group (%) | TQ-treated group (%) | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 TQ | 10 uM | 25 uM | 50 uM | 75 uM | 100 uM |
| **HepG2** | **G1** | 54.3 | 53.1 | 48.7 | 45.1 | 23.7 | 18.4 |
| | **S** | 20.0 | 20.4 | 18.2 | 16.8 | 13.3 | 9.3 |
| | **G2/M** | 22.6 | 23.4 | 28.4 | 23.1 | 14.5 | 6.8 |
| | **SubG1** | 2.6 | 2.4 | 2.8 | 18.1 | 54.1 | 60.7 |
| **HuCCT1** | **G1** | 46.8 | 34.9 | 51.9 | 56.4 | 58.2 | 60.3 |
| | **S** | 16.9 | 12.5 | 13.1 | 10.5 | 8.1 | 5.6 |
| | **G2/M** | 31.7 | 15.3 | 28.0 | 19.9 | 11.8 | 9.5 |
| | **SubG1** | 3.8 | 3.2 | 5.1 | 11.0 | 18.7 | 22.3 |