The University of Nottingham

EVALUATION OF A CELLULAR MODEL FOR ASSESSING CYTOPROTECTION BY NATURAL CHEMICALS

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Abstract

There is considerable interest in the ability of plant-derived antioxidants to protect against oxidative damage associated with disease or exposure to toxic agents. In this study, the cytoprotection effect of the direct antioxidants quercetin (Q) and epigallocatechin-3-gallate (EGCG) and the indirect antioxidants, sulforaphane (SF) and indole-3-carbinol (I3C) was assessed in a cellular protection assay. This assay involved two cytoprotection patterns: (a) 20-hour exposure to phytochemical followed by 5-hour exposure to t-BHP; (b) simultaneous exposure to phytochemicals and t-BHP for 5 hours. HepG2 cells were cultured to a confluent monolayer and exposed to phytochemical +/- t-BHP in serum-containing or serum-free medium, after which cell damage mediated by oxidant stress was assessed by neutral red uptake. Results showed that Q, EGCG and I3C were effective while SF was inactive and toxic to the cells by itself at high concentration during long incubation. On the other hand, short time of incubation with Q, EGCG and SF displayed identical results to prolonged exposure. However, I3C was devoid of protection activity. Moreover, results showed that serum has a major impact on antioxidant activity.

The toxicity effects of t-BHP on HepG2 cells in the presence and/or absence of phytochemicals was evaluated. This assay included exposure of HepG2 cells to phytochemicals for 20 hours prior exposure to different concentrations of t-BHP for 5 hours. Results indicated that full cytoprotection was provided by Q and EGCG while partial protection was displayed by I3C and SFN. On the other hand, results showed complete cell death to have occurred at 0.4 and 0.8 mM t-BHP after 5 hours of incubation with oxidant/ in the absence of phytochemicals. Additionally, presence of serum reduces the toxic effect of t-BHP effects. The possible mechanism of the toxicity of t-BHP on HepG2 was studied to ascertain if t-BHP

initiated apoptosis in HepG2 cells, through determination of activated caspase by different techniques. Basically, HepG2 cells were exposed to +/- 0.4 and 0.8 mM t-BHP for specific times. Results showed no strong evidence for apoptosis although caspase-3 activity increased significantly ($p \leq 0.05$) in treated HpG2 cells with 0.8 mM t-BHP at 150 minutes. Similarly, significant increases in ROS and lipid peroxidation in treated HepG2 cells with 0.8 mM t-BHP ($p \leq 0.05$ and 0.01 respectively) at 150 minutes were obtained. Moreover, a (non-significant) decline in GSH amount was reported.

Treatment of the cells with Q and I3C under the conditions used in the cytoprotection study prevented the weak activation of caspase-3 identified by western blot.

The possible mechanism (s) of protective properties of the phytochemicals in induction of phase ll detoxifying enzymes was studied by use of an intact-cell assay of quinone reductase based upon duroquinone-mediated reduction of ferricyanide. However, studies demonstrated that Q and EGCG themselves acted as intermediate electron donor in the assay, and so interfered with the assay; I3C and SFN did not share this property.

In conclusion, the work presented in the thesis has indicated that the four selected compounds possess cytoprotection effect against oxidative stress induced by t-BHP under particular conditions, and has provided further insights into mechanism of toxicity of t-BHP.

Dedication

To my dear parents and husband

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Acknowledgment

With the last lines of writing, I really would like to acknowledge The University of Nottingham and School of Biomedical Sciences in the United Kingdom for all scientific support.

It is my pleasure to bow with all respect for my main supervisor, Assoc. Prof. Dr. Jeffrey Fry for all guidance, assistance and patience during my study at The University of Nottingham, UK. I am also grateful to my co-supervisor, Dr. Margaret Pratten, The University of Nottingham, UK for her supervision. I would like to thank Mr. Garry Clarke, Mr. Liaque Latif, my colleagues in study Samia Hassan, Mouhamed Alsaqati, Hamad Aljabri, and Hani Almukhtar at School of Biomedical Sciences in the University of Nottingham/ UK for their help, technical assistance and other needed laboratory works throughout the project.

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Thanks Mama & Baba

Maha

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List of abbreviations

ANOVA	Analysis of variance
ARE	Antioxidant response element
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
EDTA	Ethylenediamine tetra acetic acid
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate
FBS	Foetal bovine serum
EC_{50}	Concentration of compound producing 50% effective effects
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSH	Oxidized glutathione
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HepG2	Human hepatoma cell line
I3C	Indole-3-carbinol
KDa	Kilo Dalton
MEME	Minimum essential medium eagle
MAPK	Mitogen activated protein kinase

NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced
NQO1	Quinone reductase
PBS	Phosphate buffer saline
ROS	Reactive oxygen species
SFN	Sulforaphane
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TC_{50}	Toxic concentration for 50% of the population
t-BHP	Tertiary-butyl hydroperoxide
TNF	Tumour necrosis factor
Q	Quercetin

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Table conversion from µg/ml to molarity

Q (MW= 338.26), SFN (MW=177.29), EGCG (MW=458.37), I3C (MW=147.17)

Concentration in (µg/ml)	**Molar concentration (mM)**			
	Q (mM)	I3C (mM)	EGCG (mM)	SFN (mM)
2	-	-	-	0.15
5	-	-	-	0.29
10	-	-	-	0.57
20	-	-	-	1.13
25	0.075	1.7	0.5	-
50	0.15	3.4	1.1	-
75	0.23	5.1	1.65	-
100	0.3	6.8	2.2	-

Chapter 1

General introduction

Chapter 1

General introduction

The present study aimed to establish the use of a cellular protection assay in the screening of antioxidant behaviour of model phytochemicals which are known to act as direct and/or indirect antioxidants. The cellular protection assay is based on the protection of HepG2 cells against oxidative stress induced by tert-butyl hydroperoxide t-BHP (Alia et al. 2006b). These assays involved two cytoprotection patterns: (a) 20-hour exposure to phytochemical followed by 5-hour exposure to t-BHP; (b) 5-hour exposure to phytochemicals and t-BHP simultaneously. Moreover, we focused on the duration of treatment and the effect of presence or absence of serum on the antioxidant activity.

1.1 Cytoprotection

Cell metabolism constantly produces reactive oxygen species (ROS) as a natural by-product of the normal metabolism of oxygen during cellular requirements such as respiration (Wagner *et al.* 2006). This accrual is usually not a dominant phenomenon because cells are able to compensate and maintain an adequate homeostasis between ROS production and its removal via particular enzymatic pathways such as catalase (CAT), superoxide dismutases (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR) or non-enzymatic endogenous antioxidant defence systems such as ascorbic acid, glutathione (GSH), and uric acid

(Lawless *et al.* 2009; Lima *et al.* 2007; Mittal *et al.* 2001; Palmieri and Sblendorio 2006). On the other hand, if this balance is disturbed by an excessive accumulation of ROS (Jeong *et al.* 2005) due to environmental, life style and pathologic factors this results in a situation called oxidative stress (OS). There is increasing evidence that oxidative stress contributes to stress-related disorders and disease conditions such as cancer, chronic obstructive pulmonary disease, asthma, cardiovascular dysfunction and immunosuppression (Tiwari and Kakkar 2009). In consequence, this accumulation of ROS above the protection system of cells may damage the integrity and function of critical molecules; finally cell death can occur from this damage (Lima *et al.* 2007).

On the other hand, substances present in plants, known as phytochemicals, including polyphenols such as flavonoids and anthocyanins, provide resistance for plants against pathogens. The digestive system of humans and animals is robust enough to enable both of them over millions of years of evolution to get benefits from plants digestion (Gopalakrishnan and Tony Kong 2008). There is growing interest in identifying phytochemicals which possess chemopreventive effects. These effects include inibition of metabolic activation and DNA binding of carcinogens, stimulation of detoxification, DNA damage repair, and suppression of cell proliferation and angiogenesis (Surh 2008). In 2006, data reported 1,399,790 new cancer cases and approximately 565 Americans deaths from cancer are expected in the United States (Jemal *et al.* 2006). These data linked cancer and nutritional aspects, and reported that one-third of these deaths were related to poor nutrition, obesity and physical inactivity. Scientific evidence associating various cancers, such as those of breast (Stoll 1998), colon (Newmark *et al.* 2009) and prostate (Llaverias *et al.* 2010), with Western diet patterns including higher intake of fried food, red and processed meats and desserts, has increased recently. On the other hand, lower risk of cancers has been noted for a prudent dietary

pattern, including higher intake of cereals and whole grains, vegetables and fruits, which result in increased levels of minerals such as selenium, dietary fibres and antioxidants (Gopalakrishnan and Tony Kong 2008). Moreover, epidemiological studies have proposed a correlation between consumption of vegetables and fruits with lower incidence of cancer, believed to be due to presence of direct or indirect antioxidants (Lopaczynski and Zeisel 2001; Wagner *et al.* 2006; Wagner *et al.* 2010). This proposition suggests that antioxidants and other phytochemicals present in the diet may protect cells from ROS-mediated DNA damage, which otherwise can lead to mutation and subsequent carcinogenesis (Lopaczynski and Zeisel 2001), and so protect from cancer. These protections are either or both of direct (free radical scavenging) or indirect (up-regulation of cytoprotection proteins) actions. In different pathological situations, antioxidant properties from various sources of natural polyphenols proved beneficial activity through scavenging of free radical species and so preventing the occurrence of oxidative stress (Jimenez-Lopez and Cederbaum 2004). Therefore, antioxidant therapy has been introduced as a new therapeutic approach to directly scavenge the free radicals through a series of reactions or remove them indirectly in order to minimize ROS accumulation (Chang *et al.* 2009).

In 1970, the term "chemoprevention" was coined to describe the abilities of non-toxic chemical substances or their mixtures to reduce risk of malignancy in humans by interference with the development of neoplasia (Sporn *et al.* 1976). Tumour development includes initiation, promotion and/or progression stages. The initiation stage is classified as an irreversible process and includes several events: uptake of the carcinogenic agent such as chemical constituents of tobacco smoke (Adams *et al.* 1987), its distribution and subsequent transport to tissues or organs, where detoxification and or/metabolic activation process occurs. The latter generates reactive metabolites which react with critical cellular macromolecules (DNA) and

cause genotoxic damage, so leading to genetic mutation. In promotion and progression stages, the initiated altered cell may undergo neoplastic transformation when genetic mutations are not repaired. The fundamental role of chemopreventive phytochemicals in initiation stages are blocking agents for carcinogenic chemicals and preventing them from reaching target sites and interacting with target cell DNA. On the other hand, chemopreventive phytochemicals have a different role in promotion and progression stages as agents suppressing the neoplastic transformation of altered cells (Surh 2008).

1.1.1 Antioxidants

Given the accumulating evidence for the beneficial effects of consumption of fresh vegetables and fruits, many attempts have been made to identify specific components in such foods that have ability to prevent or lower risk of cancer and other diseases. Plant-derived antioxidants appear to be major contributors as preventive agents (Agudo *et al.* 2007). Antioxidants are characterised by their ability to scavenge free radicals and other oxidative species (Wright *et al.* 2004) so they are considered as defenders to cells from ROS-mediated DNA damage, which otherwise can lead to mutation and successive carcinogenesis (Lopaczynski and Zeisel 2001). Therefore, they are recommended for cancer prevention according to the inverse dissociation of high antioxidant intake with low risk of cancer such as lung cancer (Wright *et al.* 2004).

Interestingly it has been found that nitrate, a natural compound of root and green vegetables, has no carcinogenic effects on humans but the ingested form of the nitrate can be reduced to nitrate endogenously, which can react with amines and amides in gastrointestinal tract through nitrosation and form *N*-nitroso compounds

which are considered as potent animal carcinogens (Bogovski and Bogovski 1981; Bruningfann and Kaneene 1993; Walker 1990). On the other hand, formation of N-nitroso compounds can be inhibited by antioxidant vitamins, in particular vitamin C and E (Correa 1988; Mirvish 1986) and thus the association of cancer risk with dietary nitrate intake can be prevented. Kim *et al.* (2007) have introduced the nitrate: antioxidant vitamins consumption ratio regarding to the risk of gastric cancer and reported that the intake of nitrate relative to antioxidant vitamins has more effect on gastric cancer than the absolute intake of nitrate. Moreover and according to the above, a suggestion has been made to lower the ratio of nitrate to antioxidant vitamins in order to reduce the incidence of gastric cancer instead of lowering the absolute nitrate intake or increase the intake of antioxidant vitamins which has been taken alone.

Several epidemiological studies around the world have shown a relationship between mortality and vegetables consumption (Agudo *et al.* 2007). In Greek population for example, intake of 230 g/day of vegetables and 200 g/day of fruits have been linked to lower mortality significantly by 12% and 18%, respectively (Krebs *et al.* 2004). Similarly in three communities in the United States, mortality are reduced with high consumption of vegetables and fruits in white and African American adults (Opas *et al.* 2004). In addition, mortality in British cohorts is inversely associated with significant increase of plasma ascorbic acid (Benlhabib *et al.* 2004; Palacios *et al.* 2004) in spite no differences in mortality having been reported in British populations of vegetarian and non-vegetarian people (Weidemann *et al.* 2004).

Evidence from literatures indicates that polyphenols participate as free radical scavengers (Di Meo *et al.* 2013; Visioli *et al.* 1998; Wagner *et al.* 2006) to prevent pro-oxidative states mediated by the formation of free radicals in various pathologies (Jimenez-Lopez and Cederbaum 2004), reducing agents as well as

antioxidant generators for vitamin E as an example (Wagner *et al.* 2006). Flavanols, the major poyphenolic components and considered a group of flavonoids, are widely distributed in many plant-derived foods and exist in free forms at high concentration of green tea, chocolates and other varieties of food (Lin and Harnly 2012). The consumption of food rich in these and other flavonoids compounds is thought to be effective in delaying or impeding development of neurodegenerative diseases in humans (Aruoma *et al.* 2003). Although many studies reported flavonoids as potent antioxidant, the exact mechanisms defining the protective effect are incompletely understood (Wagner *et al.* 2006). In addition, poor absorption and probable metabolism within the gastrointestinal tract for catechins keep arguments and discussion towards these compounds open (Jimenez-Lopez and Cederbaum 2004).

In this study we used four plant-derived chemicals chosen as examples of direct- and indirect-acting antioxidants. The four chemicals were: quercetin (Q); epigallocatechin-3-gallate (EGCG); indol-3-carbinol (I3C); and sulforaphane (SF).

1.1.1.1 Quercetin (Q)

Epidemiological studies have shown a negative correlation between cancer occurrences and flavonoid intake (Gnoni *et al.* 2009), in particularly flavonols such as quercetin (=2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one) (Tan *et al.* 2009) (Fig. 1.1). Quercetin and its derivatives exhibit a wide range of biological activities (Hertog and Hollman 1996) including anti-inflammatory (Loke *et al.* 2008; Shimoda *et al.* 2008), anti-carcinogenic (Williamson *et al.* 1996) and antiviral actions (Fan *et al.* 2011). Moreover, it is regarded as one of the most abundant flavonoids and represents an integral part of human diet. High amounts

of quercetin are found in kale, onions, apples (Hertog *et al.* 1992) as well as in other vegetables, nuts, fruits, tea, seeds and wine (Alia et al. 2006b), with intakes of between 6 and 31 mg/day (Hertog 1995; Kim and Jang 2009). This active flavonoid compound offers a variety of potential therapeutic uses against different human pathologies, including cancer, inflammatory conditions and hypertention (Middleton *et al.* 2000). The protective role of quercetin may be attributed to its characterisation in possessing a dual effect as a direct and indirect antioxidant, acting through different mechanisms. The detoxification of H_2O_2 and scavenging the generated ROS are examples for mechanisms to reduce H_2O_2-induced oxidative stress. Attack of cells by hydroxyl radicals produced from reaction between ROS and transition metal ions such as copper and/or iron results in unavoidable cell damage but quercetin can stop this event by preventing the formation of ROS with those chelating transition metal ions (Kim and Jang 2009). Moreover, quercetin may act on the damaged antioxidant enzyme system of the cell and restore it, as with the endocrine disrupter Aroclor, it restored glutathione content and superoxide dismutase activity (Jeong *et al.* 2005). An additional mechanism and through antioxidant properties of quercetin, generated ROS can be less reactive and less cytopathogenic when it reacts with quercetin and formed oxidized quercetin which is less harmful, and the latter compound interacts With GSH to form 6-glutathionylquercetin and 8-glutathionylquercetin and ultimately return the quercetin to the parent quercetin compound (Kim and Jang 2009).

Quercetin absorption in the intestine can be improved and increased from 24% to 52% when quercitrin (a rhamnose glycoside of quercetin) (Fig. 1.2) is administered. This improvement may be attributed to the physical and chemical properties of quercitrin provided by the sugar portion through binding to aglycone portion of quercetin (Wagner *et al.* 2006).

In malignant cells, chemoprevention by natural antioxidant treatment has been utilized, in particularly polyphenols such as quercetin, to induce apoptosis in cancer cells (Kaufmann and Earnshaw 2000; Ramos 2007) by direct activation of caspase cascade (mitochondrial pathway) such as caspase-3 and -9 as well as Bcl-2 family members (Granado-Serrano *et al.* 2008; Granado-Serrano *et al.* 2006). This kind of treatment has relied on the selective characterisation of an antioxidant, such that the same concentration of quercetin provoked apoptosis (Chowdhury *et al.* 2005) in leukemia (Shen *et al.* 2003), oral (Ong *et al.* 2004), breast (Singhal *et al.* 1995), colon (van Erk *et al.* 2005), lung (van Erk *et al.* 2005) and hepatoma (Chi *et al.* 2001) cancer cells but was without effect in their normal counterparts (Chowdhury *et al.* 2005).

It has recently been found that combination of quercetin with another antioxidant can be better in treatment than individual treatment by quercetin alone. For example, significant effect in all biochemical parameters has been reported when quercetin was combined with alpha-tocopherol. Together, they were able to prevent accumulation of glycoprotein and lipids in myocardial infarction in rats due to their potent anti-lipid peroxidation activities (Punithavathi and Prince 2009). Moreover, importance of co-treatment of antioxidants has also recorded for multi-combinations of quercetin with green tea extract and folic acid to treat and prevent melanocyte oxidative stress related diseases such as vitiligo (Jeong *et al.* 2005).

With the increased life-span of the population, in recent years cancer has become one of the most common diseases in many countries and apoptosis is considered to be a promising strategy for treating tumour (see on later section). As a flavonoid, Q can induce apoptosis in cancer cells by down-regulation of suppressor Bcl2 of apoptosis (Tan *et al.* 2009).

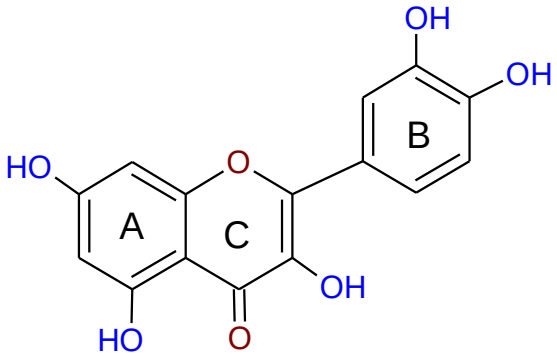

Figure 1.1 Quercetin (Liu *et al.* 2010)

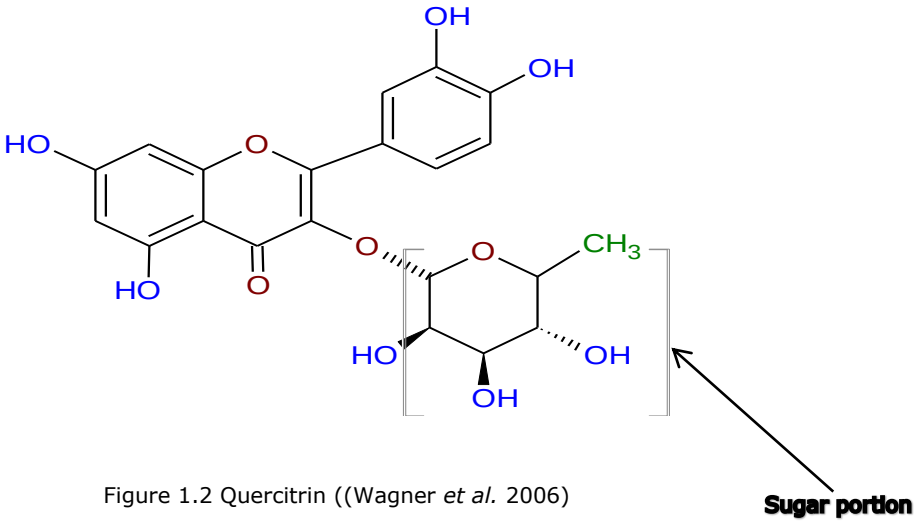

Figure 1.2 Quercitrin ((Wagner *et al.* 2006)

1.1.1.2 Epigallocatechin-3-gallate (EGCG)

Green tea (*Camellia sinesis*) is considered a popular drink in the world having originated from China and now spread all over the world over thousands of years. Its main constituents are vitamins, trace elements, and polyphenols, the latter are

being considered to be the fundamental effective components (Guo *et al.* 1996) due to their antioxidant activities against diseases (Dufresne and Farnworth 2001; Higdon and Frei 2003; Rice-Evans 1999). EGCG can inhibit UVB-induced hydrogen peroxide (H_2O_2) production in human epidermal kerationcytes and attenuate the activation of MAPK signalling pathways resulting in decrease skin disorders (Fig. 1.3) (Katiyar *et al.* 2001).

Green tea polyphenols (GTP), typical flavonoids and commonly known as catechins (RiceEvans *et al.* 1996), are phenolic complex including: (-) epicatechin (EC) (Fig. 1.4), (-) epigallocatechin (EGC) (Fig. 1.5), (-) epicatechin gallate (ECG) (Fig. 1.6) and (-) epigallocatechin-3-gallate (EGCG) (Fig. 1.7) (Guo et al. 1996; Raza and John 2005). They are potent chemopreventive agents protecting against many types of cancer (Raza and John 2005) such as those induced by chemicals or radiation (Cao *et al.* 1996; Dreosti *et al.* 1997; Gensler *et al.* 1996), hepatic injury (Zhong *et al.* 2002), protect myocardium from injuries by ischemia-reperfusion, and protect cells from damage induced by free radicals (Guo *et al.* 1996).

Among all green tea constituents, EGCG, which accounts for about 50-60% of total catechin in green tea (Hu and Kitts 2001), is the main active ingredient due to its direct and indirect antioxidant effects in tumour growth suppression. These effects are mediated by different pathways including direct induction of apoptosis and arrest of the cell cycle (Gupta *et al.* 2003; Hastak *et al.* 2003) and indirect activation of anti-angiogenic and immune functions (Jung and Ellis 2001; Zhu *et al.* 1999). These effects are achieved in a selective manner by EGCG in cancer cells but not in normal cells (Chung *et al.* 2003; Gupta et al. 2004). Thus, EGCG may exert different toxic and/or beneficial effects in malignant and normal cells respectively through its modulation of signal cascades and regulation of cell cycle which may be diverse in both cell types mentioned. In addition, these differences in EGCG actions may also be attributed to the differences between antioxidant defense mechanism

in normal cells and mechanism of oxidative stress in cancer cells (Raza and John 2005). One of the suggested mechanisms for EGCG action in growth suppression in advanced hepatocellular carcinoma (HCC) is that of direct induction of apoptosis through activation of caspases-8,-9 and -3 by down-regulation of Bcl2-alpha through inhibition NF-Kb. Thus, EGCG treatment may be considered useful to improve the prognosis of advanced HCC (Nishikawa *et al.* 2006).

EGCG is involved in preventing the development of liver injury caused by alcohol through free radical scavenging (McDonough 2003). EGCG can be involved in protection of mitochondria against CYP2E1-dependent oxidative stress and prevent dysfunction or damage by stopping directly the effects of toxins on mitochondria membrane permeability transition (Jimenez-Lopez and Cederbaum 2004). Taken together the accessibility properties of EGCG and the direct free radicals scavenging capacity allow identifying it as a potent radical trapping agent in aqueous solution.

Consequently, chain-initiating radical propagation is reduced effectively. More findings have showed that radical scavenging activity would be more potent to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals when EGCG combines with another antioxidant compound such as alpha-tocopherol (Hu and Kitts 2001).

The direct effect of EGCG have been observed in normal hepatic cells treated with a mixture of EGCG and ethanol where EGCG defends potentially against toxicity effect of ethanol and inhibits ethanol-induced apoptosis, oxygen radical formation, lactate dehydrogenase leakage, mitochondria dysfunction and peroxidation of lipids (Kaviarasan *et al.* 2007).

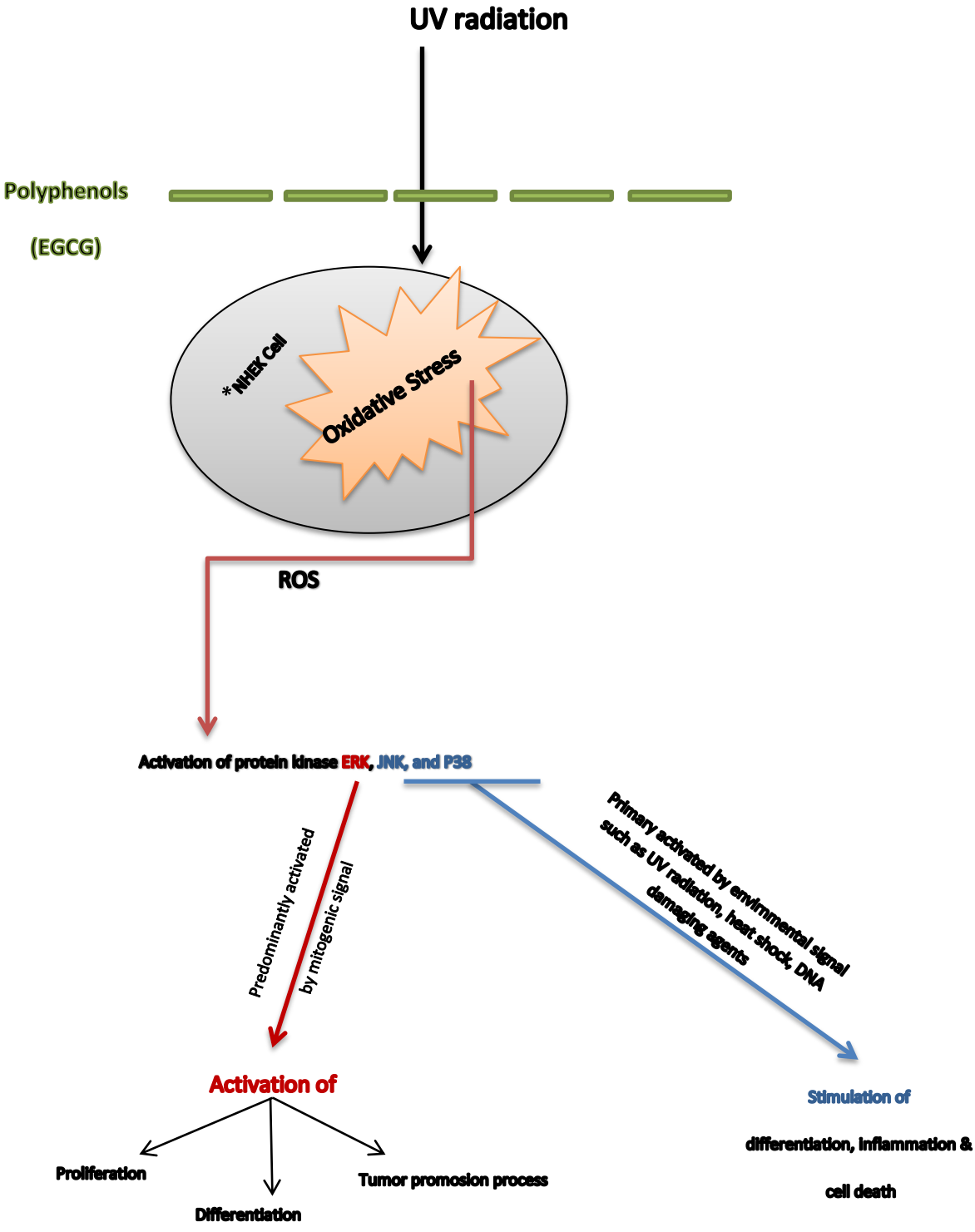

Figure 1.3 EGCG defence against UV radiation and prevent MAPK activation (adapted from (Katiyar *et al.* 2001).*Human epidermal keratinocyte

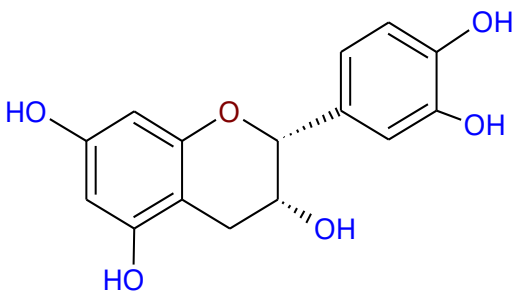

Figure 1.4 (-) Epicatechin (EC)

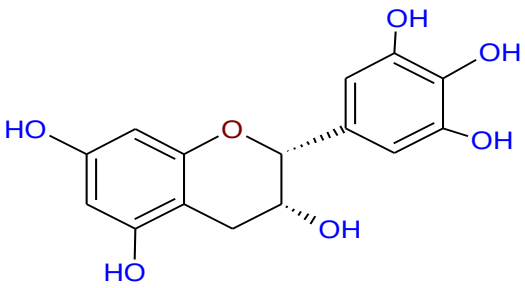

Figure 1.5 Epigallocatechin (EGC)

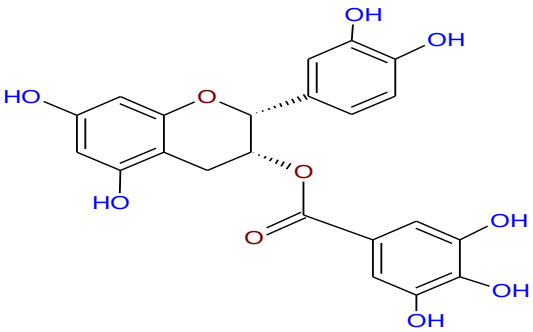

Figure 1.6 Epicatechin gallate (ECG)

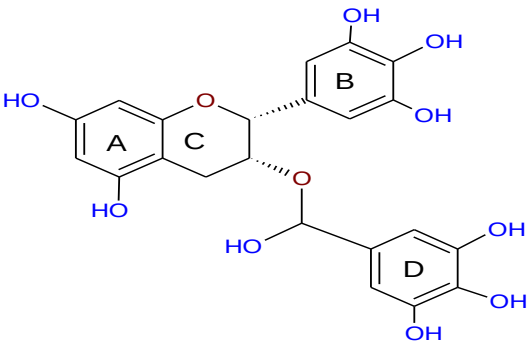

Figure 1.7 epigallocatechin-3-gallate (EGCG)

1.1.1.3 Indole-3-carbinol (I3C)

There is increasing evidence for the reverse correlation in consumption of diet rich in cruciferous vegetables such as cauliflower, broccoli and cabbage and incidence of diverse cancers such as breast, lung and colon (Bell *et al.* 2000). Decreased cancer risk during cruciferous intake is attributed in part to high content of glucosinolates which are broken down through hydrolysis catalysed by the plant enzyme myrosinase released during food preparation, producing isothiocyanates and indole products. (Verhoeven *et al.* 1997). Indole-3-carbinol (I3C) (Fig. 1.8) is a naturally occurring isothiocyanate metabolite identified in cruciferous vegetables family, in particularly *Brassica*, and released upon hydrolysis of glucobrassicin (Bradfield and Bjeldanes 1984). The first isolation from *Brassica oleracea* was in 1975 (Loub *et al.* 1975) and from that time I3C has emerged as a chemopreventive agent against a wide range of human cancers such as those of the breast, prostate, cervix and vulva (Bell *et al.* 2000).

Once I3C is exposed to acid environment of the stomach, because it is unstable in acidic medium (Kunimasa *et al.* 2010), it is converted into various derivatives of dimers, trimers, tetramers and oligomers through series of acid condensation reactions (Mcdanell *et al.* 1988) and the most famous derivatives that have been identified are 3,3'-diindolymethane (DMI) and indolo[3,2-b]carbazole (Cover *et al.* 1999). The most promising derivative that has emerged from the structural optimization of I3C recently is [1-(4-chloro-3-nitrobenzenesulfonyl)-1H-indol-3-yl]-methanol or called OSU-A9 (Weng *et al.* 2009)

OSU-A9 is considered a novel derivative because it provides considerable therapeutic advantage over the parent compound due to its greater metabolic

stability and antitumor efficacy. Although I3C is able to perturb Akt-NF-kappaB signalling mediated oncogenic signalling pathways, which is regarded as a fundamental role in pathogenesis and therapeutic resistance, its potency *in vivo* is limited and this may be attributed to different reasons, including limited bioavailability, low antitumour potency and complicated pharmacokinetic behaviours because of intrinsic metabolic instability. On the hand, it has been found that OSU-A9 is similar to I3C in blocking Akt-NF-kappaB signalling hepatocellular carcinoma (HCC) but is 100 times more potent, which is expressed as enhanced ultimately as inhibition of cell cycle progression (Omar *et al.* 2009).

Although anticancer mechanisms of I3C are unclear to some extent, recent studies have suggested indirect antiangiogenic activity of I3C to prevent angiogenesis in human umbilical vein endothelial cells (HUVEC). I3C protection activity includes prevention of tube formation and induction of caspase-dependent apoptosis in tube-forming HUVEC through inactivation of ERK1/2 (Kunimasa *et al.* 2010). Moreover, it has been found that I3C can prevent or minimize the risk of carcinogenesis initiation through its involvement in modulation of drug metabolism. This participation includes inhibition of phase 1 enzyme cytochrome P450 activity (cyp1A) which is responsible for xenobiotic activation to a DNA-reactive product. Furthermore on the other side, I3C can activate phase 2 enzymes such as NAD(P)H:quinone reductase (QR) activity, which are linked to the conjugation of activated xenobiotic with endogenous ligands such as glutathione, glucuronic- or sulphuric-, to enhance their secretion as conjugated compounds and so result in damage prevention (Gerhauser *et al.* 2003). Additionally, I3C plays a role as therapeutic agent against lung cancer and this action relies on the effect of I3C to induce caspase-8 mediated apoptosis via the Fas death receptor and induce cell cycle arrest at G0/G1 through p-p53 activation (Choi *et al.* 2010).

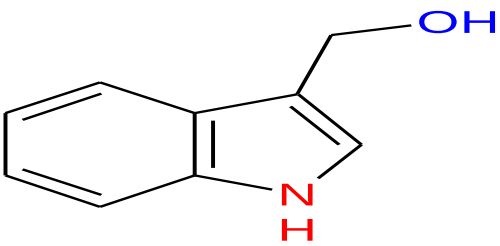

Figure 1.8 indole-3-carbinol

1.1.1.4 Sulforaphane (SFN)

Sulforaphane (1-isothiocyanato-4-(methyl-sulfinyl)butane;CH_3-SO-$(CH_2)_4$-N=C=S) (Fig.9), is a naturally occurring member of the isothiocyanate family (ITC) of chemopreventive agents, has been demonstrated to possess the ability to suppress the growth of cancer cells in different tissues and to delay the appearance of tumors (Yanaka *et al.* 2005). It is derived from the corresponding glucosinolate and is available at high concentrations in many edible cruciferous vegetables including broccoli and broccoli sprouts (Choi *et al.* 2008; Fimognari and Hrelia 2007), ingestion of which are believed to reduce the risk of different kinds of malignancies and induced apoptosis in prostate cancer cells (Singh *et al.* 2005). SFN has emerged as promising chemopreventive agent in cancer treatment at low concentration ≤ 50 μM (Choi *et al.* 2008) but it has induced apoptosis in normal cells when the concentration has been increased (Yeh and Yen 2005).

The molecular mechanism of SFN action on cancer cells has received particular attention due to the effect in prevention and/or delaying development of

preneoplastic cells. For example, SFN was capable of inducing apoptosis in human myeloid leukemia U937 cells through series of actions including effects on components of mitochondrial apoptotic pathway resulting in an increased level of ROS and this leading to loss of mitochondrial membrane potential. The apoptosis in human leukemic cells is initiated when apoptotic proteins such as cytochrome c are released into cytosol as a result of loss of mitochondrial membrane potential (MMP) and cause caspase cascades activation including caspase-9 and -3. The last activation for caspase-3 cause cleavage of specific substrates such as poly (ADP-ribose) polymerase (PARP), and eventually lead to apoptosis (Choi *et al.* 2008). A similar mitochondrial mechanism was reported for human prostate cancer cell treatment with SFN (Singh *et al.* 2005).

Physical exercise activities, particularly the moderate kind, are one of the essential components of lifestyle. The moderate exercises induce ROS generation in mild amounts which in turn cause an adaptation of antioxidant and repair systems and consequently decrease the incidence of ROS-associated diseases (Radak *et al.* 2001). On the other hand, an exhaustive and intensive exercise causes oxidative damage to muscle cells, as a sign of incomplete adaptation of antioxidant system, due to the generation of considerable amounts of ROS. Massive exercise has been demonstrated to increase thiobarbituric acid (TBARS) as oxidative stress marker, and induce oxidative protein damage, lipid peroxidation and glutathione oxidation, generation of which are attributed to high amounts of ROS (Meydani *et al.* 1993; Sastre *et al.* 1992). Acute exercise has provided an interesting oxidative stress model to study by scientists; so suggestions have been made made to prevent or minimize structural muscle damage through eliminating generation of those large amounts of ROS. It has been found that SFN plays a crucial role during exhaustive exercise protocols for rats and prevent or slow ongoing skeletal muscle damage by removing ROS amounts indirectly. The mechanism of action relies on the up-

regulation of phase II enzymes and enhancement of their activity, examples are glutathione-s-transferase (GST), glutathione reductase (GR) and NAD(P)H: quinone oxidoreductase 1(NQO1) in muscle (Malaguti *et al.* 2009).

Anti-cancer agents such as TNF-alpha provide toxicity against tumors (Tomita *et al.* 1998; Watanabe *et al.* 1986) but they have side effects simultaneously. During treatment course, transcription of anti-apoptotic and anti-inflammatory genes can be induced by these agents in tumour cells themselves which give resistance towards therapeutic treatments (Beg and Baltimore 1996; Wang *et al.* 1999). Recent studies reported that apoptosis in leukemia cells that are normally resistant to TNF-alpha alone can be triggered through combination treatments with sub-toxic doses of SFN and TNF-alpha (Moon *et al.* 2009). For example, in prostate cancer cells (THP-1, HL60, U937 and K562) (Xu *et al.* 2005) apoptosis occurs through ROS-dependent caspase-3 activation and inhibition of TNF-alpha-induced NF-kappaB activation through inhibition of IkBα degradation, IkBα phosphorylation and p65 nuclear translocation resulted in suppression of NF-kB-related genes including those linked to cell proliferation (cyclin D1, COX-2 and c-Myc), apoptosis (IAP-1, IAP-2, XIAP, Bcl-2 and Bcl-Xl) and metastasis (VEGF and MMP-9) (Moon *et al.* 2009). SFN showed a potential neuroprotective role in parkinson's disease (PD) when it caused significant increases of total GSH level, NAD(P)H:quinone oxidoreductase-1, GSH-transferase and reductase (Tarozzi *et al.* 2009).

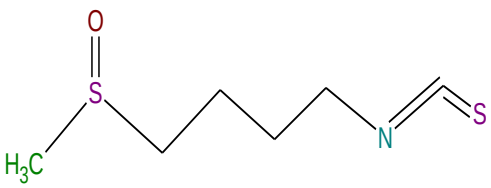

Figure 1.9 Sulforaphane

1.1.2 Assays of Antioxidant Activity

Considerable attention has been paid for antioxidants in modern society due to their relevance to human health in preventing or slowing a wide variety of diseases by scavenging the free radicals that result from cell metabolism processes (Zafra-Stone *et al.* 2007). The name of 'free radical' was given for an atom or molecule having one or more unpaired electrons which are accountable for oxidative stress including degenerations and damages that are attributed to free radicals which might be eliminated by antioxidant supplementations (Marchioli *et al.* 2001). The most reliable method to assess the antioxidant activity (AA) is 2,2-diphenyl-1-picrylhydrazyl (DPPH) spectrophotometric method (Sanna *et al.* 2012). This method is widely applied for AA assays especially for juices, vegetables, fruits and plant extracts (Magalhaes *et al.* 2008; Moon and Shibamoto 2009; Sanchez-Moreno 2002) and is based on the scavenging of the purple DPPH radical by the antioxidant, so converting the radical to a yellow species (Brandwilliams *et al.* 1995). The reaction described below involves transfer of a hydrogen (H) atom from the scavenger such as antioxidant to DPPH radical, forming DPPHH that appears as a yellow colour absorbing at 515 nm (Mishra *et al.* 2012)

$$DPPH^{\cdot} + H \longrightarrow DPPHH$$

In this work, we assessed the activity of Q, EGCG, I3C and SFN to scavenge DPPH radicals which gave further evidence to determine whether they are acting as direct antioxidants.

1.2 Cytotoxicity and cell death

In this study, one of our aims was to investigate the mechanism of cell death in HepG2 cells exposed to the oxidant tert-butyl hydroperoxide (t-BHP) under the conditions used in the cytoprotection assay, and entailed different investigations of apoptosis and its related biochemical reactions such as evaluation of glutathione content, lipid peroxidation, reactive oxygen species (ROS), and caspase-3 enzyme content and activity.

HepG2 cells are derived from a hepatocellular carcinoma, these carcinomas being the most common cancers in the world (Fukuda *et al.* 2002). HepG2 cells have been widely used as a model system of the human liver (Mateos *et al.* 2005). Although HepG2 cells are considered as cancer cells due to their ability in differentiation and dividing (Cui et al. 2007), they are valid model for application of drug testing such as the protective effects of phytochemicals against pro-oxidant effects (Jimenez-Lopez and Cederbaum 2004). This can be achieved by culturing cells in high density to allow them behave as closely as possible to those *in vivo* in regards to functionality, metabolism, and morphology (Provin et al. 2009). One of protective applications of phytochemicals in HepG2 cells can be observed by the protecting effect of quercetin where pre-treatment of HepG2 cells with 10 μM quercetin completely prevented the toxicity effect of t-BHP and prevented lactate dehydrogenase leakage (Alia et al. 2006b). Moreover, They are considered as a reliable model to study the mechanism of toxicity of the oxidant agents such as t-BHP (Piret *et al.* 2004) and proposed cell death pathways, such that HepG2 cells treated with t-BHP induced oxidative stress in HepG2 cells through induction of apoptotic cell death with reduction of glutathione content and glutathione reductase

activity (Kim et al. 1998). Moreover, the type of cell death, necrosis or apoptosis in response to hypoxia can be elucidated in HepG2 cells where calpains are involved in hypoxia-induced cell death that is likely to be necrotic (Kim *et al.* 2007).

Furthermore, HepG2 cells retain the ability to metabolise phytochemicals by similar routs as liver *in vivo* (Mateos et al. 2005; O'Leary et al. 2003; van der Woude et al. 2004), so that the exposure conditions for HepG2 cells will mimic the *in vivo* situation more closely than other cell lines which do not express xenobiotic metabolism capability. This, in turn, suggest that use of a HepG2 cell culture system for studying cytoprtection ability of phytochemicals will reflect the exposure conditions for a wide varity of cells *in vivo* which are exposed to phytochemicals in the blood following first-pass metabolism in the liver.

In our study, we cultured HepG2 cells in high density to be confluent and non-dividing to allow the identification of antioxidant behaviour and establish the possible mechanism of toxicity of t-BHP.

Chemical toxic agents that cause cell death have been reported and general features of cytotoxicity have been described (Palmieri and Sblendorio 2006; Zimmet *et al.* 2001). Such toxic agent range from glucose in high concentrations peroxides and heavy metals, and representative examples are described below.

In U-937 human promonocytic cells, treatment with low and high concentration of H_2O_2 caused cell death and displayed two different forms of oxidative stress, apoptosis and necrosis, through activation of caspase-9,and -3 and suppression the activation of caspases respectively (Troyano *et al.* 2003).

Damage of hepatic cell membranes induced by t-BHP is another picture of the deleterious oxidant effect where t-BHP decomposes and generates free radicals (Palmieri and Sblendorio 2006) (see more on free radicals in section 1.2.2).

Scientists have classified mercury as a significant environmental toxicant and warned from the deleterious effect on human health in particular neurone systems in such that it can provoke neurotoxicity in humans and animals equally. In the mid-1950s in Japan, people died when they ate fish polluted with mercury because of river pollution as a result of industrial activities (Kondo 1996; Tsuda *et al.* 1997). Moreover, 1970s poisoning with mercury has been detected in Iraq as a result of using organomercury fungicide as insecticide in agriculture (Bakir *et al.* 1973) where Poisoning had resulted from consumption of home-baked bread prepared with flour from CH_3Hg^+-treated seed grain (Sanfeliu *et al.* 2003).

A new toxicological phenomenon manifested in hyperglycemia and known as diabetes has become a major health issue around the world (Zimmet *et al.* 2001). Diabetes mellitus is classified as a chronic disease and is characterized by an increase in the level of glucose in blood higher than normal range due to impaired metabolism of glucose (Giugliano *et al.* 2008) leading to several abnormalities in the cellular pathways. The toxicity of glucose in human hepatoma HepG2 cells is represented by the high concentrations of glucose that induce apoptosis in liver cells through increased reactive oxygen species ROS generation which often plays a crucial role in cellular dysfunction and subsequently cell damage (Chandrasekaran *et al.* 2010).

Ozone depletion and global warming have played a crucial role in exposure to bombardment by irradiants of solar/cosmic origin such as nuclei of heavy elements and their decay products, neutrons, protons and ultraviolet radiation considerably and unfortunately 75% of that solar/cosmic irradiants can passes through the atmosphere without being absorbed or scattered and penetrate the ground and living organisms to be within of our environment (Chen *et al.* 1994; Chen *et al.* 2005; Chen and Mares 2008; Chen and Mares 2010; Chen *et al.* 2009; Ferrari and Szuszkiewicz 2009; Held 2009). Cosmic rays (CRs) are originally initiated in outer

space from supernova explosions and other stars, they are highly-energy charged partials and their damaging effect is greater than those of x-rays or gamma rays (Goldman 1982). The massive damaging effects of CRs are represented by the ability of CRs irradiants to penetrate the body and remove electrons from atoms or molecules leaving these molecules ionized (Muller 1955). human genes changings, mutation (Baumstark-Khan *et al.* 2003; Lau *et al.* 2010) and decline in immune system capacity (Todd *et al.* 1999) are considered to be the most important results of ionization effects. Additionally, serious diseases such as cancers can be initiated from harmful substances sent out by irradiated cells to the nearby nunirradiated cell and causes unavoidable cellular damage in phenomena called bystander effect of radiation (Brenner and Elliston 2001; Mothersill and Seymour 2006; Zhang *et al.* 2009a).

Smoking cigarettes has been reported as an implicated factor in cataracts by provoking oxidative stress and contributes in cell death through apoptosis and necrosis due to its association with cadmium accumulation. Cadmium induced toxicity in human lens epithelial cells (HLECs) and significantly generated ROS, increased lipid peroxidation and activated MAPK signalling cascade in HLECs cells which ultimately led to cell damage in smokers eyes (Kalariya *et al.* 2010).

It was found that all temperatures applied for heat shock (48-55°C) increase the releasing of cytochrome c from mitochondria to cytosol and causes a concomitant nuclear DNA fragmentation during the first six hours of heat shock at 50°C and 55°C.(Egorova *et al.* 2011).

Cell death can be caused by antioxidants that have been classified as antitumor drugs under certain conditions including concentration, time incubation, and cell type through exposure HepG2 cells high concentration of SFN (Yeh and Yen 2005).

Nearly all physiological cell death in both human health and disease proceed similarly through natural and active process called apoptosis (Green 1998; Huang 2002) or programmed autonomous cellular dismantling to avoid inflammation (Fink and Cookson 2005; Wyllie *et al.* 1980) where all dying cells are removed regularly without any trace (Green 1998) (Fink and Cookson 2005). (Fig. 1.10a) Moreover and because potentially deleterious cells are being eliminated, apoptosis can be then considered one of the most potent defences against diseases such as cancers (Martin 2006b). Inversely, necrosis is classified as the second type of cell death but it is passive, resulting from severe environmental damage to the cell and involving cell swelling, lysis, ATP depletion and with irregular release of intracellular contents resulting in inflammation of tissue (Fig.10b) (Fink and Cookson 2005).

The core effectors of apoptosis encompass proteolytic enzymes of the caspase family that exist as latent zymogens or precursors in cytoplasm of multicellular animals (Boatright and Salvesen 2003). During cell death, caspases are closly linked to be apoptosis through their two functional main groups (Los *et al.* 1999) that are called initiator and effector caspases (Boatright and Salvesen 2003). Activation of caspases is considered one of the major features of apoptosis where activation of initiator caspases leads to activation of the effector caspases which subsequently results in cellular substrate cleavage (Fink and Cookson 2005).

Oxidative stress, that induced by endogenous process as a results of imbalance between levels of oxidant and reductant in favour of the oxidants, can generate apoptosis (Bae et al. 2010). The induction of apoptosis has been associated with the depletion of endogenous antioxidant glutathione (O'Callaghan *et al.* 2002), the major modulator of oxidative stress (Reed 1990) which dictates the relative resistance or susceptibility of the cell to the apoptotic or cytotoxic activities of a toxicant (Gilmont *et al.* 1998; Mans *et al.* 1992; Reed 1990; Schneider *et al.* 1995). On the other hand, significant elevation of ROS that increase hydrogen peroxide

(Martin 2006a) have been linked with induction of apoptosis where evidence indicates that polyunsaturated fatty acid can kill cells by apoptosis (Cai et al. 1999; Rose et al. 1995). More associations between apoptosis and highest level of caspase-3 activity have been reported as well (Nicholson and Thornberry 1997).

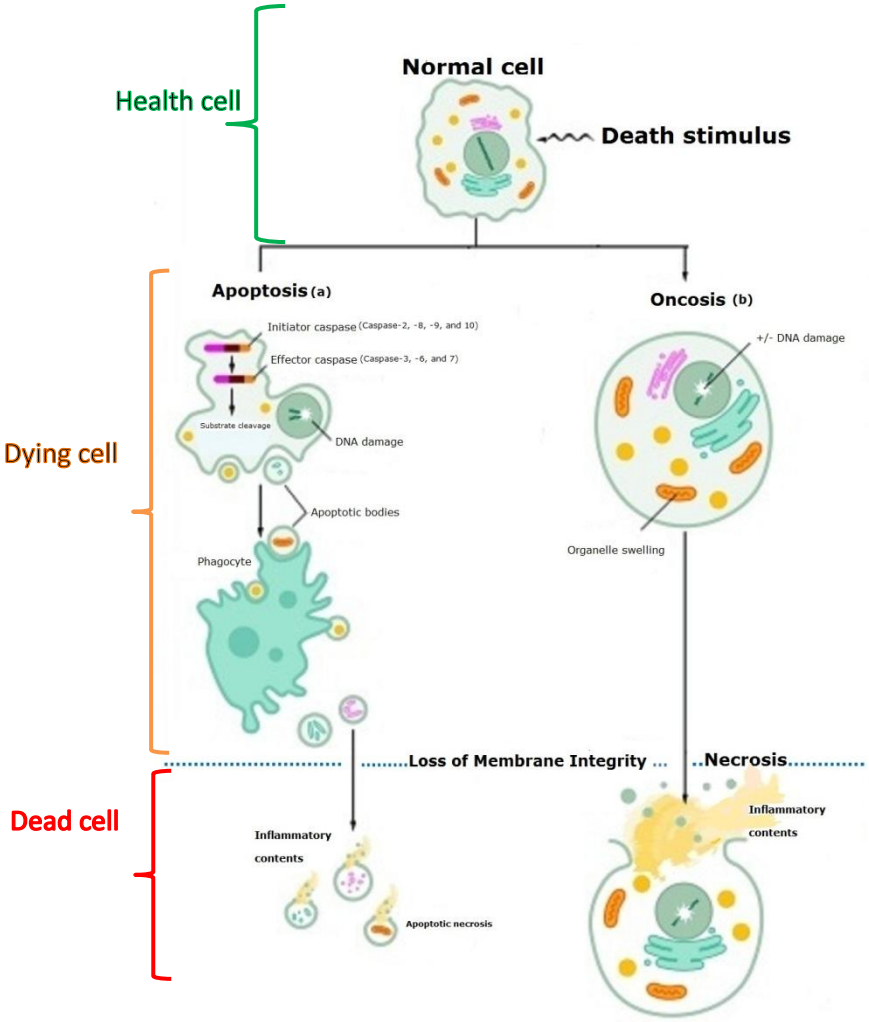

Figure 1.10 Cell death pathways. Viable cell initiates cell death pathways after responding to death-inducing stimuli. (a) Apoptosis pathway features include activation of initiator caspases first which in turn activate the effector caspases and that last activation contributes later on in cleaving cellular substrates. At this stage when apoptotic cell formed, condensation of nuclei and cytoplasm can be noticed beside DNA damage and formation of apoptotic bodies. In apoptosis pathway the intact plasma membrane can still be seen. (b) The second cell death pathway is necrosis where the cell is swells and the plasma membrane breaks down leading to release of inflammatory cellular contents (Fink and Cookson 2005).

1.2.1 tert-Butyl hydroperoxide (t-BHP)

Oxidative stress is a disturbance in the pro-oxidant antioxidant balance due to either too high level of reactive oxygen species (ROS) that could not be removed effectively by endogenous antioxidant defence system of the cell or defects in antioxidant defences or repair systems, both causes leading eventually to potential damage (Alia *et al.* 2005; Kehrer 1993). When ROS production is enhanced by oxidants, like those derived from nitrogen and oxygen, to such an extent to overwhelm the protection system this may cause oxidative modification and contribute to abnormal pathological processes and biochemical functions (Alia et al. 2005; Bae et al. 2010; Hyslop et al. 1988). *Tert*-butyl hydroperoxide (*t*-BHP) $(CH_3)_3COOH$ (Fig. 1.11) has been reported to be implicated in overproduction of intracellular ROS resulting in oxidative stress and cell injury (Lee *et al.* 2008).

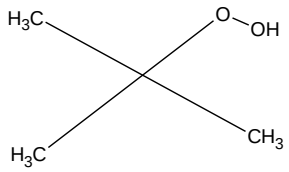

Figure 1.11 Structure of tert-butyl hydroperoxide (Sigma-Aldrich catalogue)

t-BHP is an organic short-chain analogue of hydrogen peroxide (Lee *et al.* 2008; Sohn *et al.* 2007). It has ability to produce free radicals through decomposition and participate in most oxidative stress situations such as formation of covalent bonds with cellular molecules, peroxidation of cellular lipids, and affecting cell integrity which leads to cell injury (Rush *et al.* 1985; Sohn *et al.* 2007), rapid oxidation of

reduced glutathione and loss of cell viability (Rush et al. 1985). The potential cellular toxicity mechanism of t-BHP can involve three pathways:

(A) Glutathione peroxidase (GPx) rapidly metabolizes *t*-BHP to tert-butyl alcohol and forms glutathione disulphide (GSSG) which is subsequently reoxidized by glutathione reductase (GR) resulting in regeneration of GSH and oxidation of pyridine nucleotides. High level of GPx activity can lead to oxidation of pyridine nucleotides and GSH depletion which are associated with the altered Ca^{+2} homeostasis which seems to be the critical event of plasma membrane blebbing, an early sign of toxicity induced by t-BHP (Fig.1.12) (Lotscher *et al.* 1979; Lotscher *et al.* 1980; Rush *et al.* 1985).

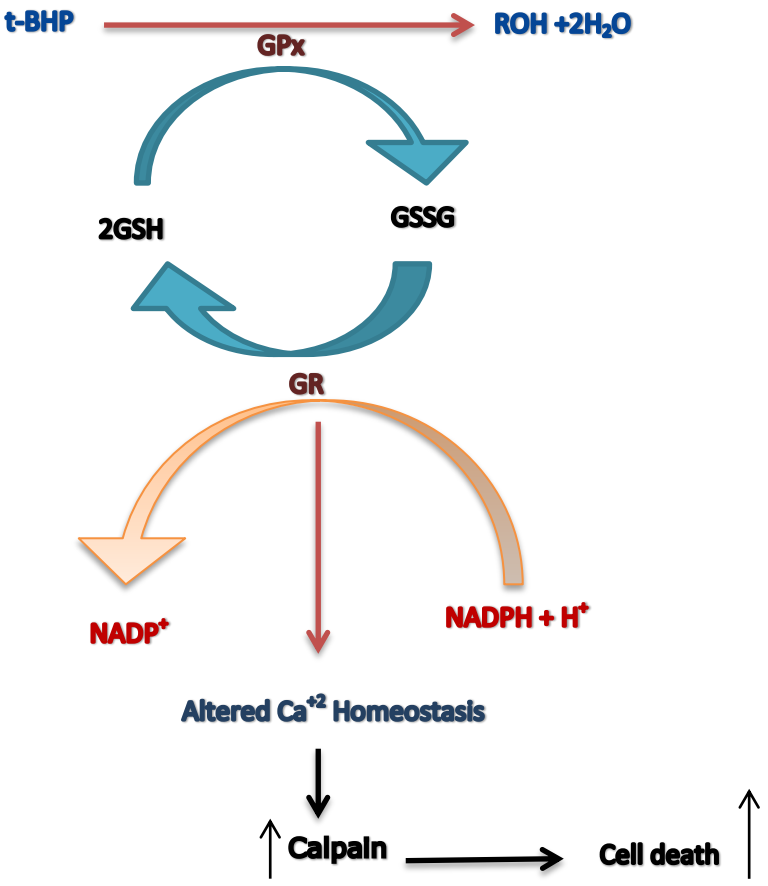

Figure 1.12 Potential mechanism of cell death induced by t-BHP (adapted from (Rush et al. 1985)

(B) Free radical intermediates can be initiated by decomposition of t-BHP, by haemoglobin (erythrocytes) or cytochrome P-450 (hepatocytes), which subsequently contributes in peroxidation of membrane lipids (fig.1.13) (Rush *et al.* 1985; Thornalley *et al.* 1983).

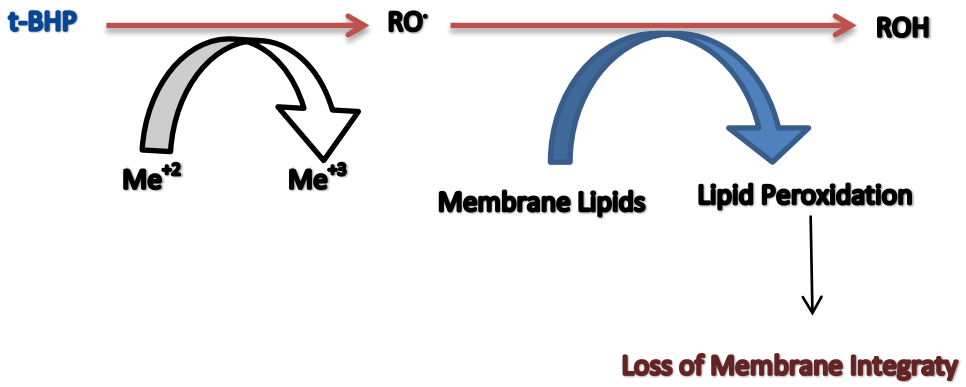

Figure 1.13 Potential mechanism of cell death induced by t-BHP (adapted from (Rush *et al.* 1985)

(C) *t*-BHP free radicals may cause cell injury through covalent bond formation with cellular molecules such as (Fig.1.14) (Orrenius *et al.* 1983; Rush *et al.* 1985).

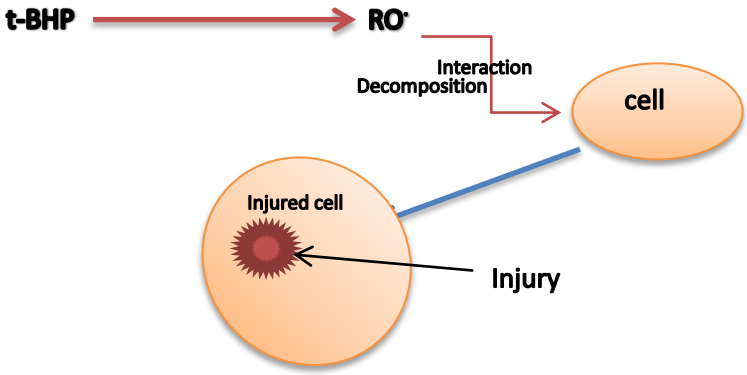

Figure 1.14. Potential mechanism of cell death induced by t-BHP (adapted from (Rush *et al.* 1985)

t-BHP is a well-known inducer of oxidative stress in biological systems and therefore it has been used as a model compound to investigate the mechanism of cell injury that induced either by apoptosis including caspase activation, DNA fragmentation, and cytochrome *c* release (Piret *et al.* 2004) or necrosis including leakage of lactate dehydrogenase (Sohn *et al.* 2007).

1.2.2 Free radical species generation

Over 2.5 billion years ago, significant mutagenic toxic amounts of oxygen appeared in Earth's atmosphere which led to formation of the ozone layer as a protector from solar ultraviolet radiation, this resulted in the survival of organisms in sea (Willcox *et al.* 2004). The primitive anaerobic organisms were unable to tolerate oxygen (O_2) due to the absence of defence mechanisms. On the other hand, the multicellular organisms including eukaryotic cells such as humans and other aerobics possess an evolving electron transport chain and other enzyme systems and therefore; O2 in mitochondria can be consumed during aerobic metabolism in parallel with the evolving antioxidant defences system to protect from toxic effect of O_2 simultaneously (Willcox *et al.* 2004; Yu 1994).

Aerobic life-style offers great advantages for organisms but is fraught with dangers because it generates free radicals which consequently result in considerable formation of reactive oxygen species ROS (Yu 1994). A free radical is any chemical species with one or more unpaired electrons (single electron being alone in an atomic or molecular orbital) and it can be initiated through three different ways:

Homolytic cleavage of covalent bond of a normal molecule to produce two fragments, each one possessing a single electron:

$$A{:}\ B \longrightarrow A^{\cdot} + B^{\cdot}$$

By losing single electron from a normal molecule:

$$A{:}\ B \longrightarrow A{:}^{-} + B^{+}$$

The type of this reaction is hetrolytic fission where one of the fragments of the parent molecule keeps the electrons of the covalent bond resulting in ions which are charged or electrically neutral.

By adding single electron to a normal molecule:

$$A + e^{-} \longrightarrow A^{-\cdot}$$

This reaction includes electron transfer and it is more common in biological system (Palmieri and Sblendorio 2006).

Free radicals are produced in animal cells either accidently or deliberately. An accidental generation can cause significant production of accumulated reactive oxygen species (Reichard and Ehrenberg 1983) which consequently result in oxidative stress (Lima *et al.* 2007), whereas the deliberate production yields profitable entities if they are targeted correctly, such as utilizing free radicals by enzymes at their active sites during catalysis process (Reichard and Ehrenberg 1983).

The deliberate generation of free radicals includes production of superoxide radical anion $O2^{\cdot -}$ and ROS at acceptable levels which are very important because they provide defence against invading microorganisms, including inactivation and destruction of viruses (Aratani *et al.* 2002; Curnutte and Babior 1987; El-Benna *et*

al. 2005; Espinosa-Garcia and Gutierrez-Merino 2003). An example for this defences action can be seen in the immune system in phagocytic cells (neutrophils, ecosinophils, macrophages, and monocytes) (Curnutte and Babior 1987), within a microbicidal mechanism which involves the initial phagocytosis of the pathogens. During action of these cells with phagocytable particle, consumption of O_2 by both of them increases considerably, leading to a situation called 'respiratory burst' resulting in activation NAD(P)H oxidase enzymes in phagocytic cells, which catalyse the reduction of molecular oxygen to produce superoxide anion $O_2^{\cdot -}$, the precursor of microbicidal oxidants, and subsequent production of important level of ROS and bactericidal protein into the phagosomes (Babior 1992; Chanock *et al.* 1994; Cross and Jones 1991; Curnutte and Babior 1987).

The importance of ROS production in immune system can be observed in granulomatous disease where patients of this disease are unable to produce $O_2^{\cdot -}$ due to a defect in NADPH oxidase enzyme which consequently leads to exacerbation of subsequent infection (Curnutte and Babior 1987; Halliwell 1994).

$$NADPH + 2O_2 \longrightarrow NADP^+ + 2\,O_2^{\cdot -} + H^+$$

The fundamental biological process that leads to $O_2^{\cdot -}$ generation is electron transport, as 'leakage' of electrons from mitochondrial electron transport chains onto molecular oxygen (O_2) produces the superoxide anion $O_2^{\cdot -}$, cytochrome *b* in mitochondria being the main site for the ($O_2^{\cdot -}$) generation. Moreover, superoxide anion can also be generated in different sites in animal cells such as in hepatocytes during detoxification process which includes electron transport systems, endoplasmic reticulum (prostaglandin synthesis, xenobiotic metabolism), nuclear membrane, and direct reaction of O_2 with autoxidisable molecules (Palmieri and

Sblendorio 2006; Yu 1994). On the other hand and in particular situations $O_2^{\cdot -}$ is considered the main source of hydrogen peroxide H_20_2 when oxygen molecule is reduced by two electrons:

$$O_2^{\cdot} + 2e^- + 2H^+ \longrightarrow H_2O_2$$

Hydrogen peroxide is not a free radical but is classed within the ROS category because the latter includes oxygen free radicals as well as non-radical oxygen derivatives which are contributed in oxygen radical production. Hydrogen peroxide can also be produced through reaction between two superoxide molecules (Palmieri and Sblendorio 2006):

$$2O_2^{\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$$

This reaction is called dismutation reaction because the free radical reactants generate non-radical products and can be catalysed by superoxide dismutase enzyme (SDO) or take place spontaneously (Mccord and Fridovic.I 1969; Palmieri and Sblendorio 2006). Moreover, H_2O_2 is an oxidizing agent but not reactive. It's main importance comes from it being the main source for hydroxyl radicals in the presence of reactive transition metal ions such as iron(Fe^{+2}):

$$H_2O_2 + Fe^{+2} \longrightarrow OH + OH^- + Fe^{+3}$$

Hydroxyl radical is an extremely reactive oxidizing radical and it is able to cause large amounts of damage when it can react with biomolecules before it diffuses any significant distance within the cell (Palmieri and Sblendorio 2006).

1.2.3 Reactive Oxygen Species (ROS)

Among the metabolic processes occurring within an organism, oxidation reactions are regarded an essential part of normal metabolism. These reactions include superoxide radical production in electron flow system that produces ATP due to the transfer of an electron to the electron acceptor, oxygen molecule (Davies 1995). The energy production, ATP, and electron flow should be coupled to prevent free radical generation of reactive oxygen species (ROS) which emerged to cause serious health issues (Nohl *et al.* 2005). ROS are continually generated within a cell as a result of mitochondrial electron transfer (as highlighted in section 1.2.2) The amounts of ROS are checked permanently by the endogenous antioxidant defence system to prevent their accumulation in the body (Tiwari and Kakkar 2009). If this balance is disturbed because of overproduction of ROS quantities or weakness of the endogenous antioxidant system defences (Henricks and Nijkamp 2001), diseases such as malignant neoplasm (Valko *et al.* 2006), cardiac dysfunction (Keith *et al.* 1998), and Alzheimer disease (Christen 2000) may ensue.

Moreover, the most important biological processes, in particular ATP synthesis and oxidative phosphorylation, are continually achieved by mitochondria which used 90% of the consumed O_2 for this purpose. In these processes, the mitochondrial proteins are probably implicated in superoxide $O_2^{\cdot -}$ production which consequently lead to ROS generation (fig.1.14) (Kakkar and Singh 2007). Therefore, the mitochondria are considered the intracellular sources for normal and abnormal ROS quantities generation during normal and impaired mitochondrial oxygen reduction respectively.

Beside their harmful effects, ROS are important in many physiological processes as they are involved in the removal of dead cells (Henricks and Nijkamp 2001). They are now being recognized as regulators of cell function and modulators of cell signalling pathways, as they are considered as a part of the signalling process responsible for apoptosis induction and elimination of cancer cells (Lopaczynski and Zeisel 2001). Different mechanisms and pathways have been suggested for ROS generation and their participation in dead cell elimination, in such that mitochondria pathway via Fas activation where rapid and transient ROS are generated and apoptosis is induced by the potent carcinogen such as cadmium Cd as an example (Oh and Lim 2006). Moreover, ROS achieve certain functions during apoptosis in such that, they are signal transducers of cell death as the following: first and at early stages of apoptosis, they are just signals, and, secondly, when there are impaired mitochondrial oxygen reduction this causes alterations in mitochondrial permeability leading to production of ROS. Finally, the produced ROS induce the depolarization of the mitochondrial membrane and lead to further mitochondrial dysfunction (Oh and Lim 2006; Segovia and Berges 2009).

1.2.4 Lipid peroxidation

Lipid peroxidations (LPO) are generated in mitochondria when the free radicals attack the double bonds of carbon-carbon of unsaturated fatty acid (Devasagayam et al. 2003; Raha and Robinson 2000). During the process of lipid peroxidation, toxic by-products which are known as 'second messengers' are formed and cause damage to the functions of the cell (Raha and Robinson 2000).

Polyunsaturated fatty acids (PUFAs) are considered as the basis on which the fluidity of phospholipids of the bilayer of biological membranes is maintained. When preoxidative attacks by free radicals on PUFAs of a biological membrane they cause extensive damages lead to loss its important function as a barrier. Therefore, large molecules such as cytosolic enzymes are able to leak out considerably (Riceevans and Burdon 1993). Moreover, LPO can cause further disorders to the membrane by cross-linking of membrane components which lead to restriction of the mobility of membrane proteins, decreasing the fluidity and electrical resistance of the membrane as well as changing the phase properties of the membranes (Devasagayam *et al.* 2003; Riceevans and Burdon 1993). So, LPO has been implicated in decrease of enzyme activities associated with membrane, such as cytochrome P450 and glucose-6-phosphatase. LPO also contributes to inactivation of membrane pumps that are responsible for cellular ion homeostasis maintenance (Raha and Robinson 2000).

LPO has been implicated in a large varty of diseases, including Alzheimer disease and type 1 diabetes, diabetes, Parkinson disease, premature birth disorders, atherosclerosis, chronic inflammatory conditions, preeclamsia and eclamsia, fibrosis and cancer (Castranova and Vallyathan 2000; Devasagayam *et al.* 2003; Yagi 1987). Oxidation of lipids has been noticed since ancient times and has been determined as a major issue in the deterioration of exposed to air. Metals such as iron participate in LPO and their products such as aldehydes initiate where this process, which can be terminated by antioxidants such as vitamin E and alpha-totopherol (reaction 6 and 7, Fig. 1.15).

It has been found that iron plays an important role in LPO synthesis where it attacks LH, the unsaturated fatty acid, and generates L. radical (reaction 1, Fig. 1.15). This reaction includes generation of methylene group (-CH2) with weak double bond of unpaired electron on carbon atom which is very susceptible to any

attack. On the other hand, L. radical can be generated from another source which is lipid peroxyl radical (LOO.) (reaction 3, Fig.1.15) that formed in (reaction 2, Fig. 1.15) when oxygen molecule react with the resultant .CH radical from (reaction 1, Fig.1.15) and LP propagation. LOOH is formed during reaction 3 where hydrogen atom can be abstracted from LH by lipid peroxyl radical LOO. . In addition, LOO. radicals are unstable and can degrade into various products encompassing aldehydes such as malondialdehyde (MDA) (reaction 4, Fig. 1.15). Furthermore, through the Fenton reaction that includes oxidized ferrous Fe+2 to ferric Fe+3 also participate in LOP radicals formation by breaking down of LOOH to LO. (reaction 5, Fig. 1.15) (Devasagayam *et al.* 2003).

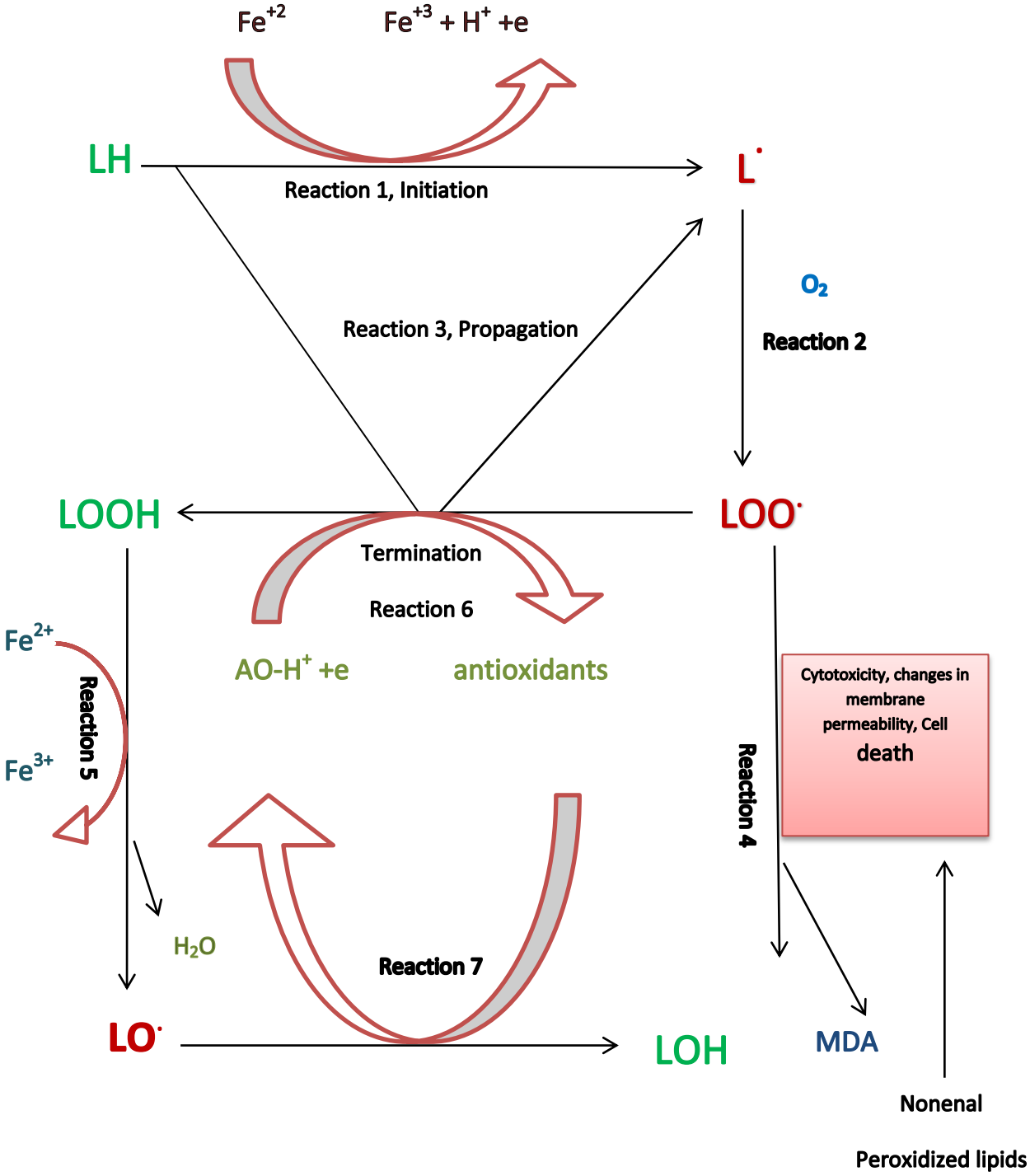

Figure 1.15 Role of iron and antioxidant in Lipid peroxidation initiation and termination respectively.

1.2.5 Oxidative Stress

The importance of oxygen molecule has appeared since fifteenth century due to its bilateral role in life and the famous words ‘‘the same thing that makes you live can kill you in the end’’ by Neil Young was a really perfect description for the multifaceted property of oxygen as a life donor and killer at the same time (Davies and Pryor 2005). It has been found recently that numerous medical illnesses are attributed primarily to the damaging effect of the generated oxygen free radicals through electron transport in mitochondria result in ROS accumulation (Tiwari and Kakkar 2009). To overcome this puzzling issue, body are regularly checked on the excess amounts of ROS and eliminate them through particular defence system called endogenous antioxidant system (Rahman *et al.* 2006). To be aware about the crucial role of the intracellular defence system in the maintenance of cellular redox balance and defence against free radical attack we should first know its mechanism of action.

1.2.6 Apoptosis: programmed cell death

Apoptosis or programmed cell death is a normal physiological mechanism used by metazoans during development, morphogenesis and homeostasis of organs and tissues; since it carves the body, shapes the organs, and sculpt the fingers and toes (Meier *et al.* 2000). Moreover, this process is essential for controlling the number of the cells (Vaux and Korsmeyer 1999), balancing between cell division and cell death

(Pedrera *et al.* 2012), regulation the immune system and elimination of pathogen-infected cells (Mccarthy *et al.* 1992; Vaux and Korsmeyer 1999).

The term "apoptosis" is Greek and means "falling of" like leaves of trees. It was firstly given in 1972 by Kerr and his colleagues to describe the distinctive phenotypic phenomenon of cellular shrinkage of cells that were eliminated during embryonic development, atrophy upon hormone withdrawal, and normal cell turnover in healthy adult tissue (Kerr *et al.* 1972). Moreover, another definition has been given for apoptosis relying on the occurrence of the events consecutively depending on the cellular metabolism that causes the specific morphology of the cell damage (Martin 2006a). By the second definition, apoptosis can be distinguished easily from the other types of cell death, necrosis, autophagy and oncosis (Lopaczynski and Zeisel 2001; Martin 2006a).

The main morphological features of apoptosis are characterized by nuclear and cytoplasm condensation, nuclear fragmentation into mono-and oligonucleosomal units, plasma membrane blebbing, and cell shrinkage before the cell breaks into small membrane-surrounded fragments called apoptotic bodies. These fragmented bodies are taken up by phagocyte cells and degraded before deletion within phagosomes without inciting inflammation (Kerr *et al.* 1972; Lauber *et al.* 2004; Rodriguez and Schaper 2005). Whereas Necrosis, the passive and accidental cell death, can be distinct from apoptosis by uncontrolled release of inflammatory cellular contents due to cellular and organelle swelling and membrane breakdown (Fink and Cookson 2005) (Fig. 1.16).

Regulation of cell death is indispensable for normal development and maintenance of tissue homeostasis in multicellular organisms (Schafer and Kornbluth 2006). Metazoans cells die through different stereotypical modes including apoptosis, necrosis, autophagy, and oncosis but apoptosis is the most notable among all. Misregulation or failure of apoptosis may result in development of cancer (Green *et*

al. 1994) when apoptotic cells loses responsiveness to death inducing and becomes aberrant viable cell which can then switched to tumour cell characteristics (Malaguarnera 2004).

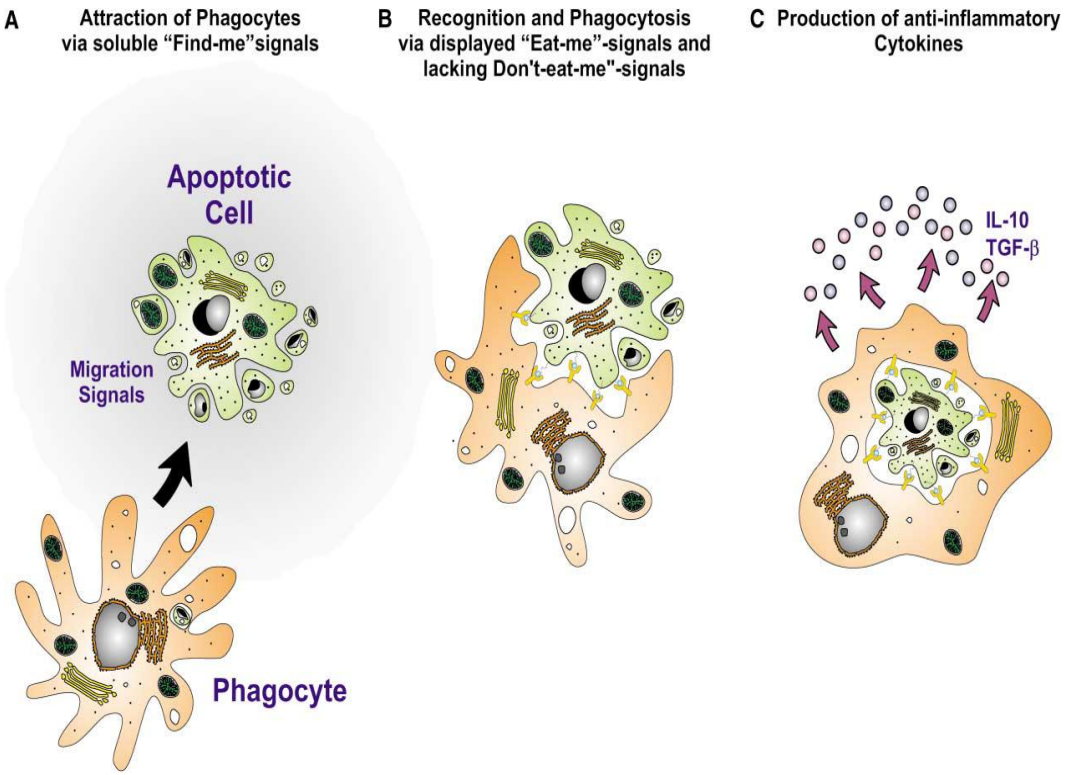

Figure 1.16 The Three Steps of Apoptotic Cell Removal (a) soluble find-me signals are released by dying cells to attract professional phagocytes. (b) During this action of encounter of phagocyte cells with its prey, phagocytable particles which display eats-me and the lack of don't-eat-me signals on its surface, phagocyte cell are able to internalize of the apoptotic cell corpse. (c) To prevent inflammation after ingestion of apoptotic remains, the phagocyte starts producing anti-inflammatory cytokines, like IL-10 and TGF- around the site of the apoptotic cell death (Lauber *et al.* 2004).

1.2.6.1 Apoptosis pathways

Apoptosis is characterized by an executioner program including a family of highly conserved proteases known as caspases which occupy a critical position in signal transduction cascades associated with immune reactions (Riedl and Shi 2004). Furthermore, they are in particular responsible for dismantling cells by cleaving cellular substrates. Aberrant caspase regulation seems unequivocally to be implicated considerably in pathogenesis of variety of diseases such as cancers and neurological disorders (Riedl and Shi 2004). All caspases exist as inactive precursor enzyme (proenzyme) (Creagh *et al.* 2003; Fink and Cookson 2005) and exist as latent zymogenes (procaspases); they are expressed as a single-chain composed of three domains: an N-terminal propeptide (or prodomain) followed by region of two-subunit effector domain, one small and one large subunit (Creagh *et al.* 2003; Walker *et al.* 1994; Wilson *et al.* 1994). Members of caspases family can be classified based on their physiological roles which are significantly different although all family members are similar in amino acid sequences, structure and substrate specificity (Creagh *et al.* 2003). Two main groups can be distinguished: caspases related to caspase 1 (caspase-1, -4, -5, -13, and 14 as well as caspase -11, and -12) and caspases that are implicated in apoptosis (caspase-2, -3, -6, -7, -8, -9, -10) which can be further divided into initiator and effector subgroups. The initiator caspases (caspase-2, -8, -9, and -10) are responsible for activating the effector caspases cascades (caspase-3, -6, and -7), which are involved in cellular substrate cleavages (Los *et al.* 1999) (Fig.1.17).

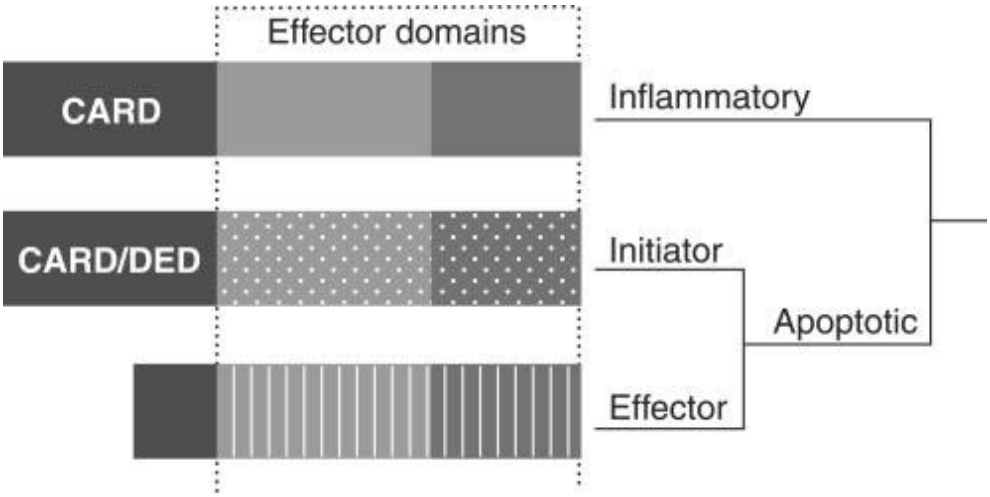

Figure 1.17 Caspases are divided into two main groups: those implicated in apoptosis (caspase-2, -3, -6, -7, -8, -9, and -10) and those related to caspase-1 (caspase-1, -4, -5, -13, and -14, as well as murine caspase-11 and -12) which their role appears to be proinflammatory and cytokine processing cell death. The caspases involved in apoptosis can be further divided into initiator and effector subgroups: Initiator caspases (caspase-2, -8, -9, and -10) which are used to activate the effector caspases (caspase-3, -6, and -7) to cleave cellular substrates DED= death effector domain, CARD= caspase recruitment domain (Fink and Cookson 2005).

Caspases are intimately involved in both the intrinsic or extrinsic pathways of apoptosis (Fig.1.18) (Schafer and Kornbluth 2006). The extrinsic pathway involves activation of initiator caspases such as pro-caspase-8 through binding of extracellular ligands such as FasL to their cognate receptors such as FAS to form cytosolic adaptor protein called FADD which then can be recruited to activate pro-caspase-8 and subsequently activate the effector caspases for dismantling cellular substrates (Nagata 1999). Activation of effector caspases can also be achieved during transmission of the emanating pro-apoptotic signals from internal stressors such as DNA damage via intrinsic pathway. The extrinsic and intrinsic pathways converge on mitochondria. The result of this convergence is permeablization of the

outer membrane of mitochondria which subsequently leads to release of cytochrome c and other pro-apoptotic molecules into the cytosol (Green and Kroemer 2004; Olson and Kornbluth 2001). Caspase-8 can cleave and activate the proapoptotic Bcl-2 family member Bid (into Bax and Bak) to promote mitochondrial cytochrome c release (Danial and Korsmeyer 2004) or impede the release by Bcl-2 and Bcl-XL (Cory and Adams 2002). The released cytosolic cytochrome c is essential for caspases effectors activation because it binds with Apaf-1 protein and forms a large complex called apoptosome which activates the initiator caspases such as caspase-9 to induce apoptosis (Liu *et al.* 1996; Srinivasula *et al.* 1998; Zou *et al.* 1997).

Many disorders and diseases such as AIDS, Alzheimer's, Cancer, Parkinsons disease, infectious diseases (Thompson 1995), and autoimmunity such as Addison's disease which is endocrine disorder (Zhang *et al.* 2009b) have risen due to the physiological defects in apoptosis pathways. Some of these apoptotic defects that contribute in medical illnesses can be attributed to the deregulation in cell death processes meaning that either too much or too little cell death occurs. Such examples includes self-renewing tissues of skin where cell death occurs physiologically but when normal cells fail to undergo apoptosis for some reasons related to defect in apoptosis mechanisms results in cell accumulation and cancers can arise (Reed 2001). The key for therapeutic strategies in future includes using the components of cellular regulation of apoptosis such as Bcl-2 proteins as integral regulators of the mitochondria apoptotic pathway, death receptors that trigger apoptosis from cell surface, endogenous caspases inhibitors, and caspases as the executioner enzymes as targets to maintain the regulated apoptosis pathways which eventually prevent disease (Fischer and Schulze-Osthoff 2005).

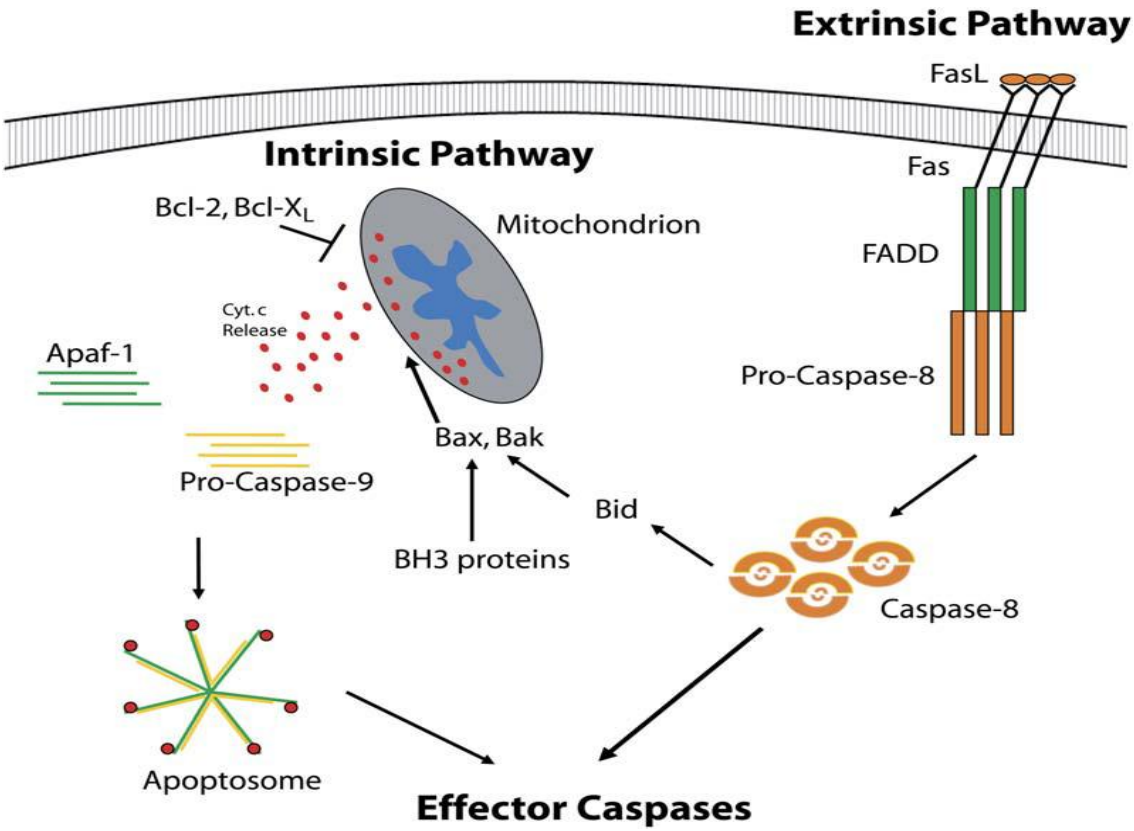

Figure 1.18 Intrinsic and Extrinsic Pathways of Apoptosis. Extrinsic Pathway encompasses caspase activation which includes binding of extracellular ligands such as FasL to their receptors Fas to form FADD, the intracellular adaptor proteins, which its primary role is caspase- 8 activation and subsequent downstream effector caspases. Whereas intrinsic pathway involves the cytosolic cytochrome c release from mitochondria into the cytosol the subsequent steps include formation of the apoptosome, activation of caspase- 9 and then cleavage of effector caspases (Schafer and Kornbluth 2006)

1.2.7 Endogenous antioxidant system

The antioxidant defence system has evolved to protect cellular homeostasis from damaging effects of free radicals that are generated through the reduction of oxygen molecules, and result in a balance of the equation of aerobic life (Cotgreave et al. 1988; Palmieri and Sblendorio 2006). The system defence is regarded as an integrated "network" (Cotgreave *et al.* 1988) and includes two main categories: members of one group that are used to prevent production of free radicals and members of another group which intercept any attack from radicals (Mittal *et al.* 2001; Mourao *et al.* 2009). The members in each group are enzymes such as catalase (CAT), superoxide dismutases (SOD), GSH redox system encompassing glutathione peroxidase (GPx), glutathione reductase (GR), and in addition glucose-6-phosphate dehydrogenase, glutaredoxins, heme oxygenase-1, peroxiredoxins, and thioredoxins (Rahman *et al.* 2006) and also non enzymatic entities such as ascorbic acid, glutathione, and uric acid (Mittal *et al.* 2001; Palmieri and Sblendorio 2006), are localized in membrane and aqueous segments of the cell (Palmieri and Sblendorio 2006). Moreover, those enzymes that are involved in ROS metabolism and detoxification of oxidative products should complement each other in the prooxidant cell states to maintain the redox balance as well as the efficient mechanism of cellular defence which eventually results in protection of the cell integrity (Halliwell *et al.* 1992; Sies 1993). By looking at those enzymes, a really complete coordination can be recognized to save the defence system but they should be in steady level state. Enzyme activity, in particular SOD, can be affected slowly by the accumulated amounts of hydrogen peroxide which leads to inactivate this enzyme gradually but the presence of GPx and catalase conserve SOD by reducing H_2O_2 water (Rahman *et al.* 2006) (Fig.1.19). Moreover, a crosstalk is

possible between the protective enzymes such as glutathione reductase GR and glutathione peroxidase GPx, such that GR reduces the oxidized form of glutathione GSSG to glutathione GSH, which is a specific substrate to GPx that is used to catalyse reduction H_2O_2 to H_2O (Alia *et al.* 2005). Thus all reactants and products preserve their steady state amounts which result in protect mechanism of defence perfectly (Rahman *et al.* 2006).

Superoxide dismutase enzymes are classified within metalloenzymes family and are localized in all cells in the body (Mourao *et al.* 2009; Simurda *et al.* 1988). They play an important role in defence against oxygen toxicity through catalysis disproportionation or dismutaion of superoxide free radical anions to a less potent hydrogen peroxide H_2O_2 (Cerutti 1985; Mccord and Fridovic.I 1969; Rahman *et al.* 2006). Moreover, they can be found in three major forms like superoxide dismutase SOD, manganese superoxide dismutase (MnSOD), and extracellular superoxide dismutase (ECSOD) based on their inducibility, requirements for metal ions, localization in cell and structure (Rahman *et al.* 2006). Recently, changes in antioxidant enzyme activity has been regarded as a sensitive biomarker to measure the cellular response to oxidative stress (Alia *et al.* 2005). In this study we measured glutathione (GSH) depletion and considered it as a biomarker for oxidative stress induced in HepG2 cells by *tert*-butyl hydro peroxide (*t*-BHP).

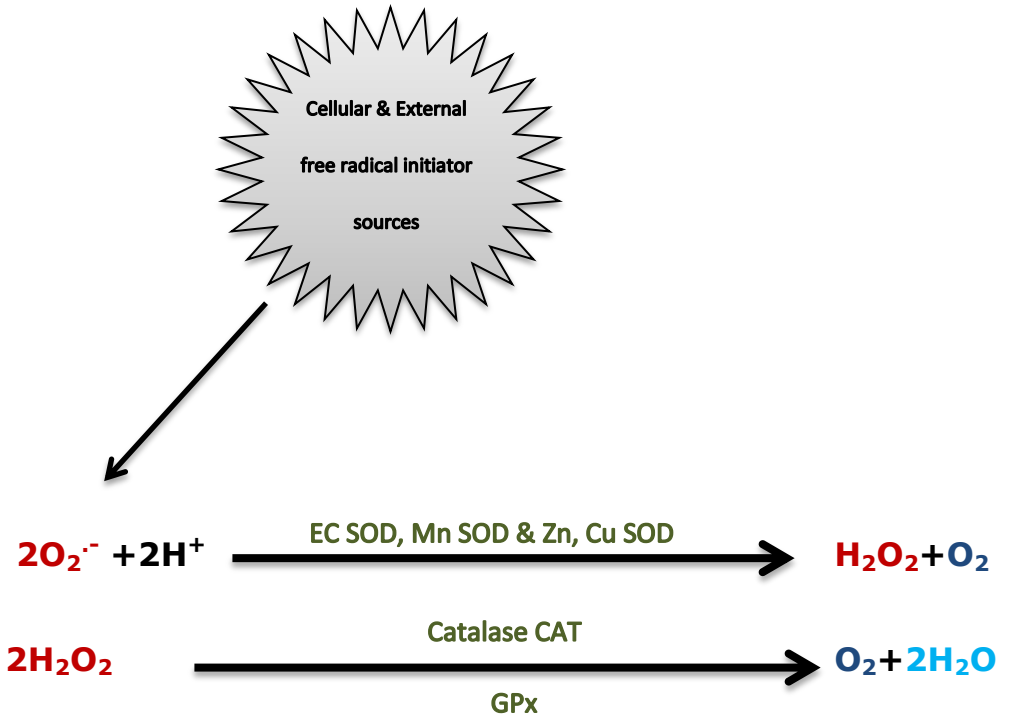

Figure 1.19 Antioxidant defence system including superoxide dismutase SOD, catalase, and glutathione peroxidase GPx which are very important enzymes in scavenging the extra- and intracellular generated superoxide anion $O_2^{\bullet -}$ and convert it to hydrogen peroxide H_2O_2 and then to H_2O. Therefore, hydroxyl radical $^{\cdot}OH$ is intercepted from being generated, resulting in prevention of cell damage (adapted from (Rahman *et al.* 2006).

1.2.7.1 NAD(P)H:Quinone reductase 1 (NQO1)

The protection provided by phase 2 enzymes has become an effective strategy against the carcinogenicity and cytotoxicity of many compounds. NAD(P)H:Quinone oxidoreductase 1 (NQO1; EC 1.6.99.2) is considered one of the major phase 2 hepatic detoxification enzymes (Talalay 2000).

NQO1 or DT-diaphorase is a cytosolic flavoprotein that exist as a homodimer and ubiquitous (Ross and Siegel 2004) but its main localization is in gastrointestinal tract, liver and kidney (Riley and Workman 1992). It is characterised by its ability to detoxify reactive environmental xenobiotics that possess quinone structure, catalyzing the two-electron reduction of substrate which is responsible for providing the detoxifying properties of NQO1 (Lee *et al.* 2005) to produce less reactive hydroquinone derivatives. Thus, NQO1 participates effectively in preventing the generation of ROS and toxic semiquinone radicals (Ross and Siegel 2004). Moreover, other main characteristics of NQO1 are its ability to utilize either or NADPH or NADH as reducing cofactors (Siegel *et al.* 1997), and its inhibition by the anticoagulant dicumarol which is a specific characteristic that can be used as an indicator for NQO1-mediated metabolism in biological system (SantaCruz *et al.* 2004) for example in quinone detoxification (Joseph et al. 2000; Moran *et al.* 1999).

Beside the major function of NQO1 as cancer preventive enzyme (Benson *et al.* 1980; Talalay and Benson 1982; Talalay *et al.* 1988; Talalay and Prochaska 1987), different biological functions of NQO1 have reported such as catalyzing the generation of antioxidant alpha- tocopherohydroquinone (TQH_2) from reduction of alpha-tocopherolquinone (TQ), a product of alpha-tocopherol oxidation (Siegel *et al.* 1997).

Different studies have reported the potential effects of many dietary constituent compounds such as flavonols, and quercetin (Q) in particular, on activity level of phase 2 enzyme, NQO1 (Lee *et al.* 2005). These effects included either activation (Valerio *et al.* 2001) or inhibition of enzyme activity (Wiegand *et al.* 2009).

1.2.7.2 Glutathione

Compounds that are generated by the interaction of ROS with nucleic acids, proteins, carbohydrates, and lipids are highly reactive, and antioxidant enzymes such as SOD, GPx, catalase, and thioredoxin reductase, the first line of defence, are not totally effective in counteracting oxidative attack and providing protection to these biological macromolecules (Masella *et al.* 2005). Therefore, the second line of defence against ROS-mediated damages is provided by enzymes such as glutathione *S*-transferase (GST), glutathione peroxidase (GPx), aldehyde dehydrogenase, and aldo-keto reductase (Armstrong 1997; Brigelius-Flohe 1999; Kuhn and Borchert 2002). Detoxification products that are produced by reaction of those enzymes with oxidative products are removed from cell by GSH (Akerboom and Sies 1989) which has emerged as a major modulator of oxidative stress because its content dictates the relative resistance susceptibility of a cell to toxicants (Gilmont *et al.* 1998; Mans *et al.* 1992; Reed 1990). Thus, GSH play a central role in all the lines of protection against ROS (Sies 1999). Additional functions of GSH include modulation of immune cell functions (Jeannin *et al*. 1995; Staal *et al.* 1994) , and maintenance of the reduced form of protein thiols (Reed 1990).

GSH, *gamma*-glutamylcysteinylglycine, is a cysteine-containing tripeptide (Meister and Anderson 1983), synthesized in various organisms from glycine and y-glutamylcysteine by GSH synthetase enzyme, the active sulfur having been provided by methionine through the cystathionine pathway (Beatty and Reed 1980). Moreover, it is present in all living cell types and exists as oxidized forms (GSSG), known as glutathione disulphide which are converted to the reduced form by glutathione reductase enzyme (GR) (Briviba *et al.* 1999; Kalyanaraman *et al.*

1996). Under normal cellular redox conditions, the bulk of cellular GSH in the endoplasmic reticulum, nucleus, and mitohondria (Pompella *et al.* 2003). GSH has numerous cellular functions as nucleophile and reductant and is involved in many biological reactions such as redox reactions to detoxify ROS and electrophilic xenobiotics, due to its functional thiol (Reiners *et al.* 2000).

1.2.8 Aims of objective

There is abundant evidence that consumption of fruits and vegetables is protective against a wide variety of chronic diseases as indication in earlier sections of this chapter. for this reason, there is intense interest in identifying and developing further the active constituents of these fruits and vegetables. The antioxidant capability of these active constituents appears to be a major contributor to their beneficial effect, and numerous researchers have applied a variety of screening approaches to identify antioxidant capacity of phytochemicals or plant extracts.

This present work aimed to gain further insight into developing a sensible strategy to identify antioxidant capacity of selected phytochemicals. The objectives of the work are set out below.

To compare the antioxidant behaviour of Q, EGCG, I3C, and SFN measured in a chemical assay (DPPH screening) and in a cell-based assay (protection of HepG2 cells from oxidative stress initiated by t-BHP). This work is presented in chapter 2 and 3.

To compare two different treatment regimens to test the antioxidant capacity in the cell-based assay, one based on co-exposure with oxidative stressor, and one based

on exposure before the oxidative stress, this latter to allow for identification of 'indirect' antioxidant behaviour. This work is presented in chapter 3.

To determine the influence of presence of serum in the culture medium on the cytoprotective effects of these phytochemicals. This work is presented in chapter 3.

To establish the mechanism of toxicity of t-BHP to HepG2 cells under the conditions of cytoprotection assay, this to provide indication of the possible mechanism of antioxidant activity of the phytochemicals. This work is presented in chapter 4.

To assess the possible utility of a recent published cell-based assay for induction of NQO1, this to provide further information on the ability of phytochemicals to act as 'indirect ' antioxidants by virtue of induction of cytoprotective proteins. This work is presented in chapter 5.

By obtaining information from these objectives, it was hoped to shed new light on development of a cellular protection assay to identify novel plant extracts and phytochemicals which may be developed for therapeutic reasons.

Chapter 2

Phytochemical properties: Direct and indirect antioxidants

Chapter 2

Phytochemical properties: Direct and indirect antioxidants

2.1 Introduction

Increased consumption of dietary citrus fruits, dark-green vegetables and cruciferous vegetables has been inversely associated with wide a range of diseases such as cancers (Barrera *et al.* 2012; Riboli and Norat 2003), which may be attributed to the ability of antioxidants in those of foods to prevent oxidative stress (Agudo *et al.* 2007). The main characteristic of antioxidants is the capacity to scavenge free radicals produced during cell metabolism and thus they contribute to lowering the risks of many diseases such as neurodegenerative and cardiovascular diseases (Zafra-Stone *et al.* 2007).

Several methods have been used to assess the antioxidants behavior but the most trusted, reliable and common methods are based on the scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and ferric reduction activity potential (FRAP) assays which are often being used in parallel whilst measure the total phenolics content (TPC) is usually considered as marker for antioxidant capacity (Clarke *et al.* 2013).

The DPPH· radical is stable in solution and appears as a violet colour in methanol solution absorbing at 515 nm. The first conception of this method was illustrated by Blois in 1958 when DPPH· free radicals accepted H atom from cysteine molecule:

$$DPPH^{\cdot} + H \longrightarrow DPPHH$$

The principle of assay is based on the fact that DPPH radical accepts hydrogen atom from the scavenger such as antioxidant to produce DPPHH that appears yellow colour absorbing at 515 nm. This assay has been adopted in different laboratories with some modification (Mishra *et al.* 2012).

The aim of this study was to evaluate the antioxidant properties of selected four compounds Quercetin (Q), Epigallocatechin-3-gallate (EGCG), Indole-3-carbinol (I3C), and Sulforaphane (SFN) as determined by the DPPH assay. These data would then form the basis for subsequent cellular antioxidant assays, with a view to establishing the distinction between direct and indirect antioxidants.

2.2 Materials and methods

2.2.1 DPPH free radical scavenging assay

The power of phytochemicals to scavenge free radicals was achieved by using 1,1 diphenyl-2-pycrylhydrazl (DPPH) radical from Sigma, UK as a model of free radical, the method was based on that of (Nara *et al.* 2006) with some modifications into 96-well plate in triplicate and for the blank assay, 20 µl of DMSO (from Sigma) is

added to 180 µl of 0.004% DPPH in methanol working solution. For sample assay 20 µl of antioxidant solution Q, EGCG, I3C, and SFN (320 µg/ml, 160 µg/ml, 80 µg/ml, 40 µg/ml, 20 µg/ml and 10 µg/ml) was added to 180 µl of DPPH solution. Leaving the plate for 15 min in dark place to avoid dissociation, the absorbance was measured spectrophotometrically (MULTISKAN ASCENT, Made by Thermo ELECTRON CORPORATION) at 540 nm after shaking for one minute.

By using equation below, scavenging of DPPH radical percentage was calculated from the difference between the control run with no antioxidant addition and the absorbance in the presence of antioxidant (Nara *et al.* 2006).

$$\% \text{ Scavenging} = 100 \times [A_0 - (A_{+DPPH} - A_{-DPPH})]/A_0$$

Where A_0 is an absorbance of sample solvent (DMSO) plus DPPH, A_{-DPPH} is an absorbance of DMSO in methanol, and A_{+DPPH} is an absorbance of sample (i.e. phytochemicals) with DPPH.

2.3 Results

2.3.1 Scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals

Radical-scavenging activity of EGCG, Q, I3C and SFN were investigated. The results from this assay demonstrated that EGCG and Q possessed radical-scavenging activity and act as direct antioxidants, with 100% scavenging being achieved at a concentration of 160 µg/ml. While SFN and I3C did not display any antioxidant activity in that both failed to scavenge DPPH radicals and remained inactive in the

concentrations range 0-320 µg/ml; therefore the intensity of the DPPH colour did not reduce (Fig 2.1).

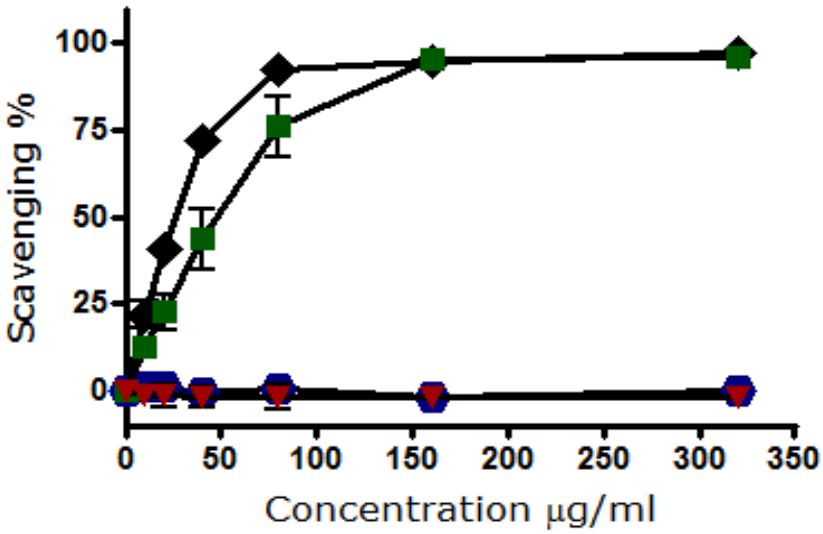

Figure 2.1 Activities of Q ( ), EGCG ( ), I3C ( ), SFN ( ) in scavenging free radicals. (n=3).

2.4 Discussion

Our results indicated that Q and EGCG are direct antioxidant while I3C and SFN are not.

The mechanism of direct trapping action of free radical is based on the structure of the antioxidant and hydroxyl groups in particular.

Q and EGCG exhibited a notable antiradical activity and this may be attributed to the hydroxyl groups present in those compounds, where Q has 5 and EGCG has 9 groups on their structure. These groups represent the possible attack sites for the free radicals resulting in radicalization of all hydroxyl groups (Chiodo *et al.* 2010; RiceEvans and Miller 1997). This reaction includes transfer of hydrogen atoms from antioxidant to the active radicals to produce oxidized antioxidant radicals which are less reactive than the active free radical attacker.

Q has showed potential antioxidant activity during inactivation of lipid peroxide radicals; (Chiodo *et al.* 2010) and (Fiorucci *et al.* 2004) have described the oxygenolysis process by Q. Moreover, (Trouillas *et al.* 2006) have confirmed that hydroxyl groups on ring B of Q are responsible for antioxidant properties of Q and when 3-OH group on ring B is blocked for example by adding sugar such as in rutin, the antioxidant activity is decreased significantly (RiceEvans *et al.* 1996).

Our results about EGCG was compatible with Salah et al. (1995) who have described the superior capacity of EGCG to scavenge free radicals and attributed this active action to the contribution of multiple number of hydroxyl groups of EGCG in this process, in particular the *ortho*-dihydroxyl groups on ring B which confers a high stability for oxidized EGCG.

Although I3C has one hydroxyl group on its structure, it did not show any action and this may because the insufficient hydroxyl groups which means decrease the number of attacking sites by free radicals.

As the structure of SFN has no any hydroxyl group, then any donation for hydrogen atom is missing resulting in that SFN is inactive completely and DPPH radicals are accumulated without any trapping.

2.5 Conclusion

In conclusion, this investigation indicates that Q and EGCG possess direct antioxidant properties which can be used in further cellular studies. I3C and SFN did not appear to possess any direct antioxidant behaviours during DPPH radical scavenging. Thus, any cytoprotection exerted by either I3C or SFN would be due to mechanisms other than a direct antioxidant mechanisms.

Chapter 3

Cytoprotection activity of phytochemicals

Chapter 3

Cytoprotection activity of phytochemicals

3. Introduction

There is considerable interest in the cytoprotective effect of phytochemicals including compounds such as antioxidants, as they possess biological cytoprotection properties, and defence against toxic agents (Son *et al.* 2008). These compounds contribute to minimising the risks of tissue damage caused by ROS (Kang *et al.* 2006) where free radicals either inactivates by transfer single electron or hydrogen atom from antioxidant (Chiodo *et al.* 2010) as highlighted in section 1.2.2.

Quercetin (Chiodo *et al.* 2010) and epigallocatechin-3-gallate (Hu and Kitts 2001) are classified as direct antioxidants (chapter 2); they contribute in radical scavenging by donating hydrogen atoms to the active radical and deactivate it. Indole-3-carbinol (Gerhauser *et al.* 2003) and sulforaphane (Malaguti *et al.* 2009) are classified as indirect antioxidant. The mechanism of action is based on the inactivation of phase 1 enzymes such as cytochromes P450, which activate xenobiotics, and induce phase 2 enzymes, which deactivate the active compounds, such as NQO1 (Gerhauser *et al.* 2003).

Serum albumin is the most abundant protein in the circulatory system (Trnkova *et al.* 2011); it forms approximately 50% or more of protein interstitial fluid in human aortas (Smith and Staples 1982). It binds to a large variety of exogenous and endogenous ligands such as drugs, fatty acid, nutrients, and hormones, and transports them to their sites of action. On the other hand, the binding of xenobiotics to albumin can affect their metabolism and biological activity (Trnkova *et al.* 2011). Therefore, presence of albumin within experimental constituents such as culture medium may impact on the results obtained by assays (Alia *et al.* 2006b).

The present study aimed to establish the use of a cellular protection assay in the screening of antioxidant behaviour as a model of phytochemicals which are known to act as direct and/or indirect antioxidants. The cellular protection assay is based on the protection of HepG2 cells against oxidative stress induced by tert-butyl hydroperoxide t-BHP: these assays involved two cytoprotection patterns: (a) 5 hour exposure to phytochemicals and t-BHP simultaneously where direct cytoprotection is provided by antioxidant against oxidative stress; (b) 20 hour exposure to phytochemical followed by 5-hour exposure to t-BHP, the long incubation with antioxidant allows defence against oxidative stress induced by t-BHP indirectly by up-regulation of phase 2 enzymes. Moreover, we focused on the duration of treatment and the effect of presence or absence of serum on the antioxidant activity.

3.1 Materials and methods

3.1.1 Chemicals

Human hepatoma HepG2 cells were purchased from ECACC, Salisbury, UK. Quercetin (Q), epigallocatechin-3-gallate (EGCG), sulforaphane (SFN), indole-3-carbinol (I3C), Hanks' balanced salt solution [H9394 ,without Ca+2 and Mg+2. with phenol red, sterile filtered] Trypsin-EDTA , MEM Eagle [M2279, with Earle's salts and sodium bicarbonate, without glutamine, sterile filtered], gentamicin [G1397, 50 mg/ml], glutamine [G7513, 200mM], Foetal Bovine Serum [F7524], MEM Non-essential Amino Acid solution [M7145], neutral red, ethanol solution, tert-butyl hydroperoxide (t-BHP), and glacial acetic acid were purchased from Sigma Chemical Co. Ltd., Poole, Dorset. UK.

3.1.2 HepG2 cells culture

HepG2 cells were cultured in 175 cm^2 Nunclon culture flasks in 5% CO_2-in-air atmosphere using 50 ml of minimum Essential Eagles MEM medium supplemented with 10% (v/v) foetal calf serum, 2 µg/ml fungizone, 0.05 mg/ml gentamicin, 1% (v/v) non-essential amino acid solution, 2 mM L-glutamine at 37°C. Culture medium was changed every 72 hours, and cells were confluent after 7 days of sub-culturing. For experiments including treatments, cells were sub-cultured at high density in wells of a 24-well plate in 1 ml medium; under these conditions confluence was achieved within 24 hours.

3.1.3 Treatments

Stocks solutions of Q, EGCG, I3C, and SFN were prepared in DMSO and stored at 4 ºC until use.

3.1.3.1 Direct cytoprotection

Different series of concentrations (0, 25, 50, 60, 75, and 100) µg/ml of Q, EGCG, I3C and SFN and 0.8 mM tert-butyl hydroperoxide (t-BHP) in complete culture medium with or without FBS were added simultaneously to the cells for 5 hours (i.e., no pre-exposure to the antioxidants).

3.1.3.2 Indirect cytoprotection

HepG2 cell cultures were treated with different series of concentrations (0, 25, 50, 60, 75, and 100) µg/ml of Q, EGCG, I3C and SFN in complete culture medium with or without FBS for 20 hours prior to exposure to 0.8 mM t-BHP for 5 hours. A solvent control (culture medium containing 0.5% DMSO) was run concurrently.

3.1.3.3 Effect of serum

The influence of serum on cell survival was assessed by culturing HepG2 cells in culture medium lacking foetal bovine serum or containing FBS in the concentration range 0.1 to 10% (v/v) for short (5 hours) and long exposure (20 hours).

3.1.3.4 Neutral red assay

Neutral red uptake assay was used to determine the viable cells after treatment and is based on the incorporation and binding of neutral red into the lysosomes of viable cells (Repetto *et al.* 2008). The antioxidant actvity of compounds can be measured using this method; cells are seeded in 24-well plate and are treated with chemicals and then incubated for 1 hour with medium containing neutral red (contains 3.3 g neutral red/L of DPBS) after cells are washed subsequently by warm PBS. Cold fixative (is prepared by mixing 250 ml ethanol, 250 ml distilled water, and 5 ml glacial acetic acid) is used to display the stain and the absorbance is read using a spectrophotometer at 540 nm.

3.2. Statistical analysis

Results were entered into Graphpad Prism 4, from which mean ± S.D. and TC_{50} values were determined for SFN and I3C whilst EC_{50} values were determined for Q, EGCG, I3C, and SFN. Values were derived from at least 3 independent experiments. One-way ANOVA followed by selected comparisons by the Bonferroni method was used to compare the EC_{50} values obtained under different culture conditions.

3.3 results

3.3.1 Phytochemical mediated protection against *tert*-butyl hydroperoxide t-BHP induced cytotoxicity in HepG2 cells

Cytoprotection assay is based on the protection of human hepatoblastoma HepG2 cells under oxidative stress induced by t-BHP. The prolonged exposure to the phytochemical would allow any up-regulation of cytoprotective proteins to have occurred (Alia *et al.* 2006b). For the present work, the four selected phytochemicals Q, EGCG, I3C, SFN were incubated with HepG2 cells for (a) 5 hour with t-BHP simultaneously using serum free medium and medium containing 2% or 10% serum (b) 20 hours to prior exposure to t-BHP for 5 hours using serum free medium and medium containing 10% serum.

3.3.2 Direct cytoprotection

Q and EGCG were selected as active direct cytoprotectants. Under conditions of direct cytoprotection scenario and using 10% serum, cell viability was not affected by the incubation with Q for 5 hours and 100% cells survived. Moreover, Q was able to protect HepG2 cells against oxidative stress induced by t-BHP directly and cell viability was reached to about 85-90% at concentration 100 µg/ml of Q and EC_{50} was 39±4.5 (µg/ml) (Fig 3.1a). A notable positive relationship was reported for EGCG and cell viability for 5 hours, in that direct cytoprotection was increased when the concentration of EGCG was increased and EC_{50} was 84.7±7.4 (µg/ml). Furthermore, EGCG was not toxic to HepG2 cell during the period of incubation and cell viability was 100% (Fig.3.1b). In addition, Q was significantly more active than

EGCG (lower EC_{50}, $p \leq 0.001$) (table 3.1). Both indirect antioxidants SFN (Malaguti *et al.* 2009) and I3C (Krajka-Kuzniak *et al.* 2011) were not tested as they do not exhibit direct antioxidant behaviour (Fig. 2.1).

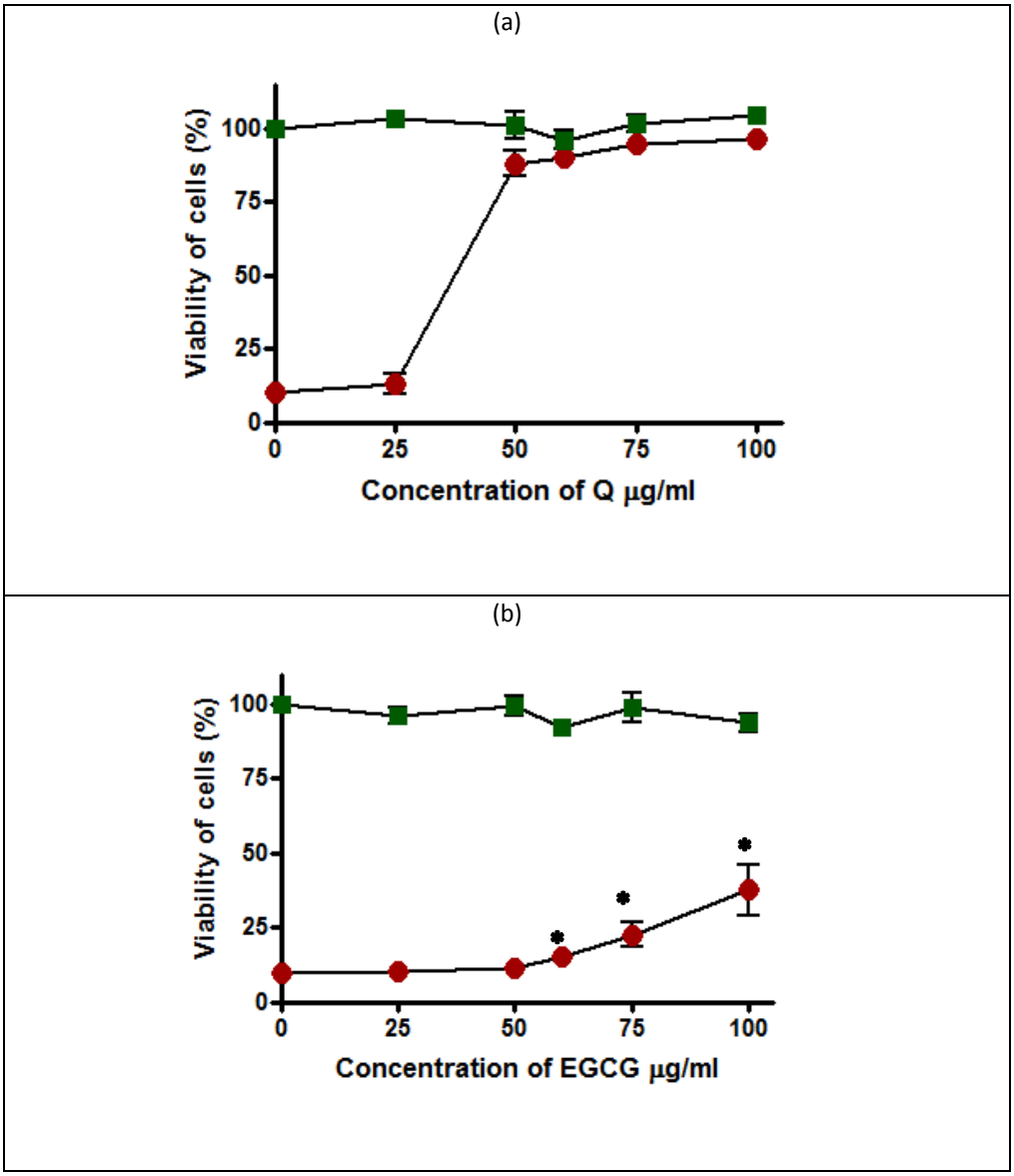

Figure 3.1 Effect of 5-hour exposure to (a) Q (0-100 µg/ml) and (b) EGCG (0-100 µg/ml) on cell viability in the absence (■) or presence (●) of t-BHP, conducted in the presence of 10% serum. EC_{50} (µg/ml) for Q and EGCG was 39±4.5 and 84.7±7.4 respectively and not determined for I3C and SFN. * Means values of phytochemicals with t-BHP were significantly different to values without t-BHP ($p \leq 0.05$). Statistical significance was only tested at those concentrations of polyphenol at which there was some evidence of protection emerging. Values are Mean±SEM of 3 independent experiments.

3.3.3 Indirect cytoprotection

Results showed that Q was not toxic to the cells in the concentration range tested, in that viability was maintained when cells were incubated with Q for 20 hours. Although Q failed to provide full cytoprotection at low concentrations such as 25 µg/ml where viability was 25%, it provided high protection and viability increased to reach 80% at 100 µg/ml and EC_{50} was 34±5.3 µg/ml (Fig.3.2a).

EGCG showed no cytotoxicity at any concentration in the range tested and maintained cell viability of the cells at 100% to each concentration in the range. On the other hand, cells incubated with EGCG overnight were partially protected from t-BHP toxicity at 50 µg/ml of EGCG to obtain approximately 50% of viability and after that viability of cells declined slowly to reach about 25% at 100 µg/ml and EC_{50} was more than 100 µg/ml (Fig 3.2b).

Incubation of HepG2 cells with I3C for 20 hours to detect the toxicity effect of this compound showed an inverse relationship between cell viability and concentration of I3C. At 25µg/ml, cell viability was recorded 100% and it gradually declined to about 60% at 100 µg/ml of I3C and TC_{50} was 98.1±0.0. Moreover, when the cells were maintained with I3C for 20 hours and then exposed to t-BHP for 5 hours, there was evidence of cytoprotection against t-BHP and EC_{50} could not be determined. I3C provided cytoprotection at 60 and 75 µg/ml and the highest cell viability was about 50% at 60µg/ml but when the concentration of I3C was increased, viability dropped to approximately 0% at 100µg/ml (Fig.3.2c).

SFN was toxic to the cells at concentrations higher than 1µg/ml, with a marked decline in cell viability from 100% to about 0% when concentration of SFN increased from 5 to 20µg/ml where TC_{50} was 3.9±0.91 µg/ml. Moreover, SFN was

inactive and failed to give any small protection to the HepG2 cells at any concentration in the range tested and EC_{50} could not be determined (Fig.3.2d).

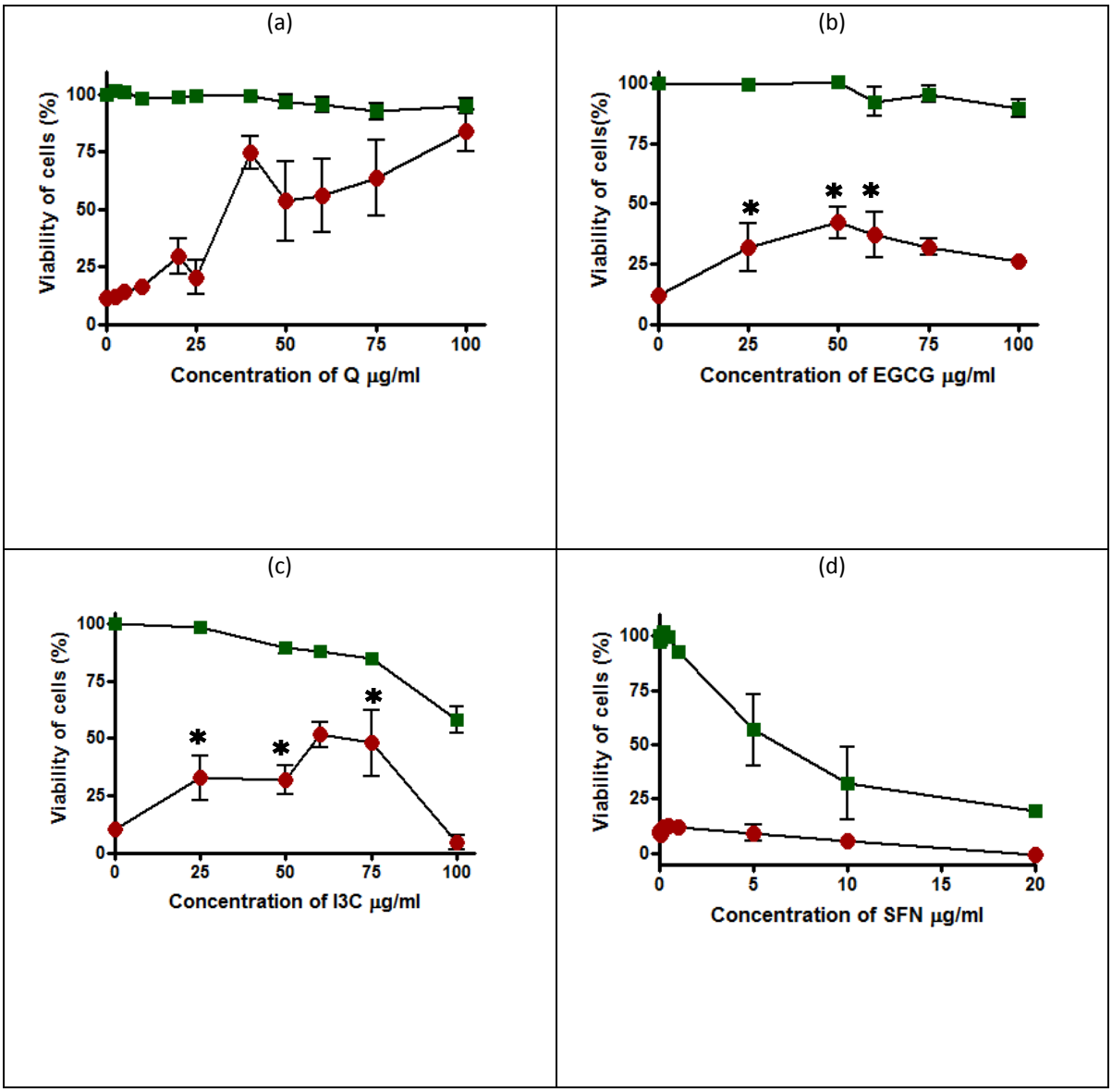

Figure 3.2 Effect of 20-hour exposure to (a) Q (0-100 µg/ml), (b) EGCG (0-100 µg/ml), (c) I3C (0-100 µg/ml), and (d) SFN (0-20 µg/ml) on cell viability in the absence (■) or presence (●) of t-BHP, conducted in the presence of 10% serum. EC_{50} (µg/ml) for Q was 34±5.3, EGCG ≥100, and not determined for I3C and SFN. TC_{50} ((µg/ml) for SFN was 3.9 ±0.91 and for I3C was 98.1±0.0. * Means values of phytochemicals with t-BHP were significantly different to values without t-BHP ($p \leq 0.05$). Statistical significance was only tested at those concentrations of polyphenol at which there was some evidence of protection emerging. Values are Mean±SEM of 3 independent experiments.

3.3.4 Effect of serum

Serum had no effect on the viability of the cells when different serum concentrations were used for short (5 hours) and long (20 hours) exposure periods (Fig.3.3a and b). This allowed a further study of the cytoprotection provided by the chemicals, this time under both reduced serum (2% as opposed to 10%) and serum-free conditions.

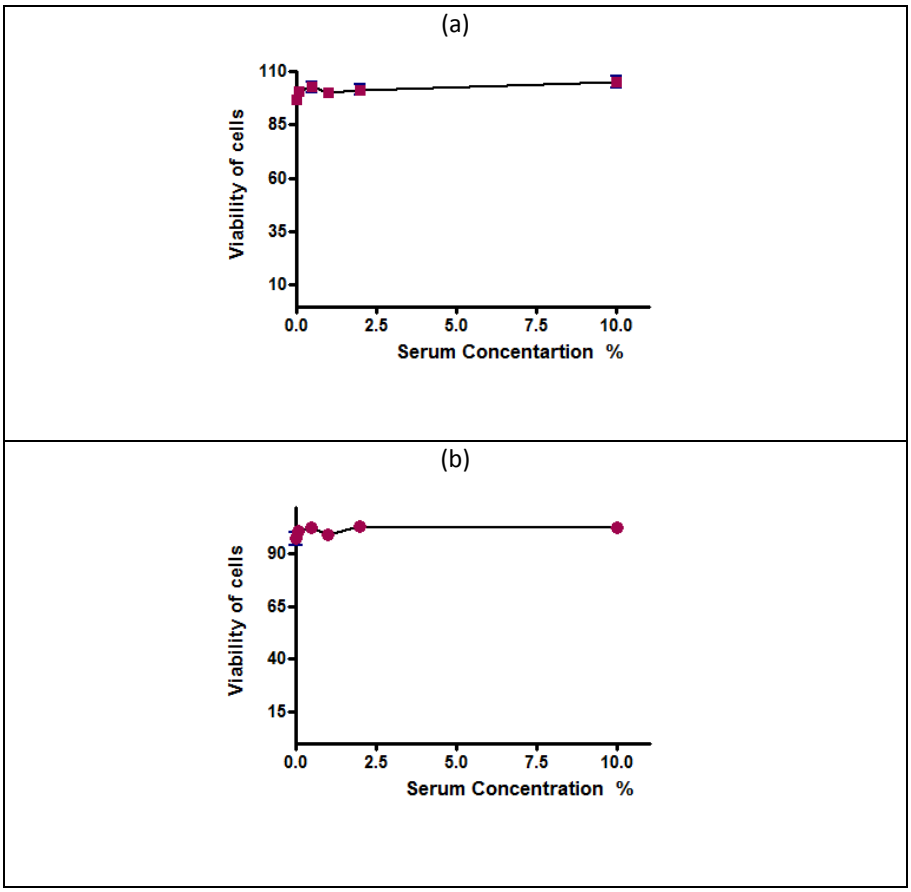

Figure 3.3 Effect of (a) 5-hours and (b) 20 hours exposure to culture medium containing different serum concentration on HepG2 cell viability. Values are Mean±SEM of 3 independent experiments.

3.3.5 Direct cytoprotection: effect of serum

(a) Medium 2% serum

As the previous results showed that serum has no effect on the viability of HepG2 cells, experiments were repeated involving 5-hour exposure to Q, EGCG, I3C and SFN with t-BHP simultaneously, this time in medium containing 2% serum.

Results displayed no loss in cell viability when HepG2 cells were incubated with different concentrations of Q (0-100 µg/ml). On the other hand when cells were exposed to t-BHP for 5 hours in the presence of Q, cells were protected against toxicity by Q and protection was very notable when cell viability increased as concentration of Q increased to reach to about 90-95% at 100 µg/ml of Q and EC_{50} was recorded 16.1±2.4 µg/ml (Fig.3.4a).

Under similar condition of incubation, viability of cells was maintained 100% during incubation with EGCG and EGCG was not toxic at any concentration used. An enhancement in protection has shown this time when HepG2 cells exposed to t-BHP in the presence of EGCG; cell viability was gradually increased to 75% at 100 µg/ml of EGCG and EC_{50} was 51.2±8.3 µg/ml (Fig.3.4b).

I3C and SFN were inactive due to their inability to protect cells from oxidative stress induced by t-BHP, the viability of the cells near from 0% where EC_{50} for both was unable to determine (Fig 3.4c and 3.4d). Moreover, a decline in the viability of cells recorded when cells were exposed to SFN at concentration higher than 1µg/ml and reached 10-15% at 10 µg/ml with a TC_{50} of 4.6±0.78 while I3C was not toxic to the cells by itself at any concentration in the range this time and cell viability remained unaffected.

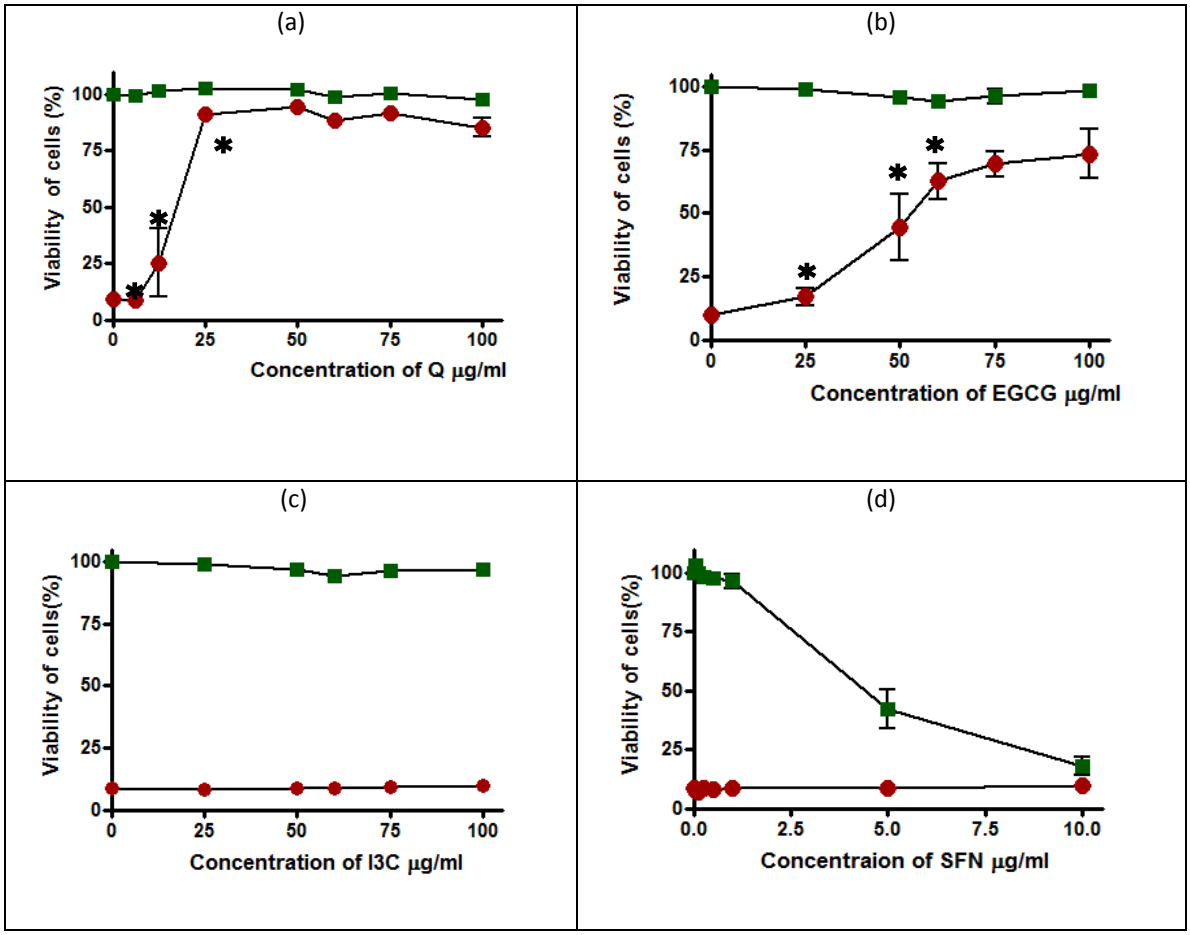

Figure 3.4 Effect of 5-hour exposure to (a) Q (0-100 µg/ml), (b) EGCG (0-100 µg/ml), (c) I3C (0-100 µg/ml), and (d) SFN (0-10 µg/ml) on cell viability in the absence (■) or presence (●) of t-BHP, conducted in the presence of 2% serum. EC_{50} (µg/ml) for Q was 16.1±2.4, EGCG 51.2±8.3, I3C and SFN ≥100. TC_{50} for SFN was 4.6±0.78. * Means values of phytochemicals with t-BHP were significantly different to values without t-BHP ($p \leq 0.05$). Statistical significance was only tested at those concentrations of polyphenol at which there was some evidence of protection emerging. Values are Mean±SEM of 3 independent experiments.

(b) Serum-free medium

Under certain conditions of serum-free medium, Q and EGCG were not toxic to the cells when both of them are incubated individually with HepG2 cells (Fig.3.5a and b). When cells incubated with Q and t-BHP simultaneously for 5 hours, protection increased and cell viability reaching 80-100% at concentrations of 25-100 µg/ml and EC_{50} was 17.9±18 µg/ml (Fig.3.5a). Similarly, EGCG increased the protection of the cells against oxidative stress induced by t-BHP and increased the viability of the cells to about 55-80% at concentrations of 25-100 µg/ml and EC_{50} was determined as 23.4 ±7.2 µg/ml (Fig.3.5b).

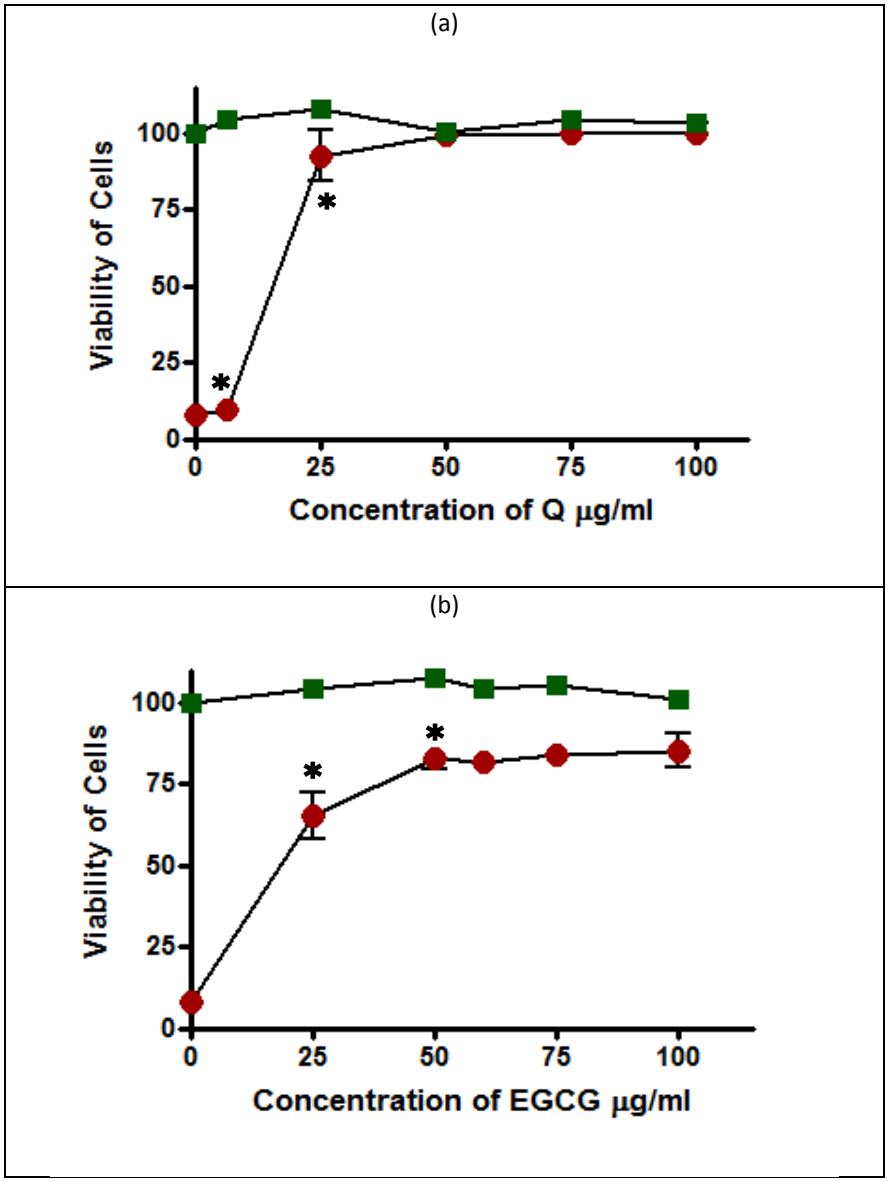

Figure 3.5 Effect of 5-hour exposure to (a) Q (0-100 µg/ml) and (b) EGCG (0-100 µg/ml) on cell viability in the absence (■) or presence (●) of, using serum-free medium. EC_{50} (µg/ml) was for Q 17.9±18, EGCG 23.4 ±7.2. ¤ Means values of Q with t-BHP were significantly different to values without t-BHP ($p \leq 0.05$). * Means values of phytochemicals with t-BHP were significantly different to values without t-BHP ($p \leq 0.05$). Statistical significance was only tested at those concentrations of polyphenol at which there was some evidence of protection emerging. Values are Mean±SEM of 3 independent experiments

3.3.6 Indirect cytoprotection: effect of serum

Under serum-free conditions, Q was not toxic to the cells at any concentration in the range and cells were still a live by 100%. Furthermore, Q produced a concentration-dependent protection against t-BHP with total protection above 50 µg/ml and EC_{50} was 33.8±4.0 µg/ml (Fig.3.6a).

Similarly EGCG was not toxic to the cells at 50 µg/ml and viability was reached about 100%, before it declined to reach about 80% at 100 µg/ml where EC_{50} was 27.8±9.9 µg/ml (Fig.3.6b).

Viability of cells was markedly affected by I3C which was obviously toxic and caused a decline in viability; it decreasing from about 80 % at concentrations of 50 µg/ml no viable cells at 100 µg/ml of I3C and TC_{50} was 72.13±5.9 µg/ml. Moreover, I3C under these conditions failed to protect HepG2 cells against t-BHP toxicity where EC_{50} was more than 100 µg/ml (fig.3.6c). Results obtained by SFN were identical to the results from cellular assay when HepG2 cells were exposed to SFN for 20 hours using medium containing 10% serum prior to exposure to t-BHP (Fig.3.6d) and EC_{50} was more than 100 while TC_{50} was 5.81±0.27 µg/ml.

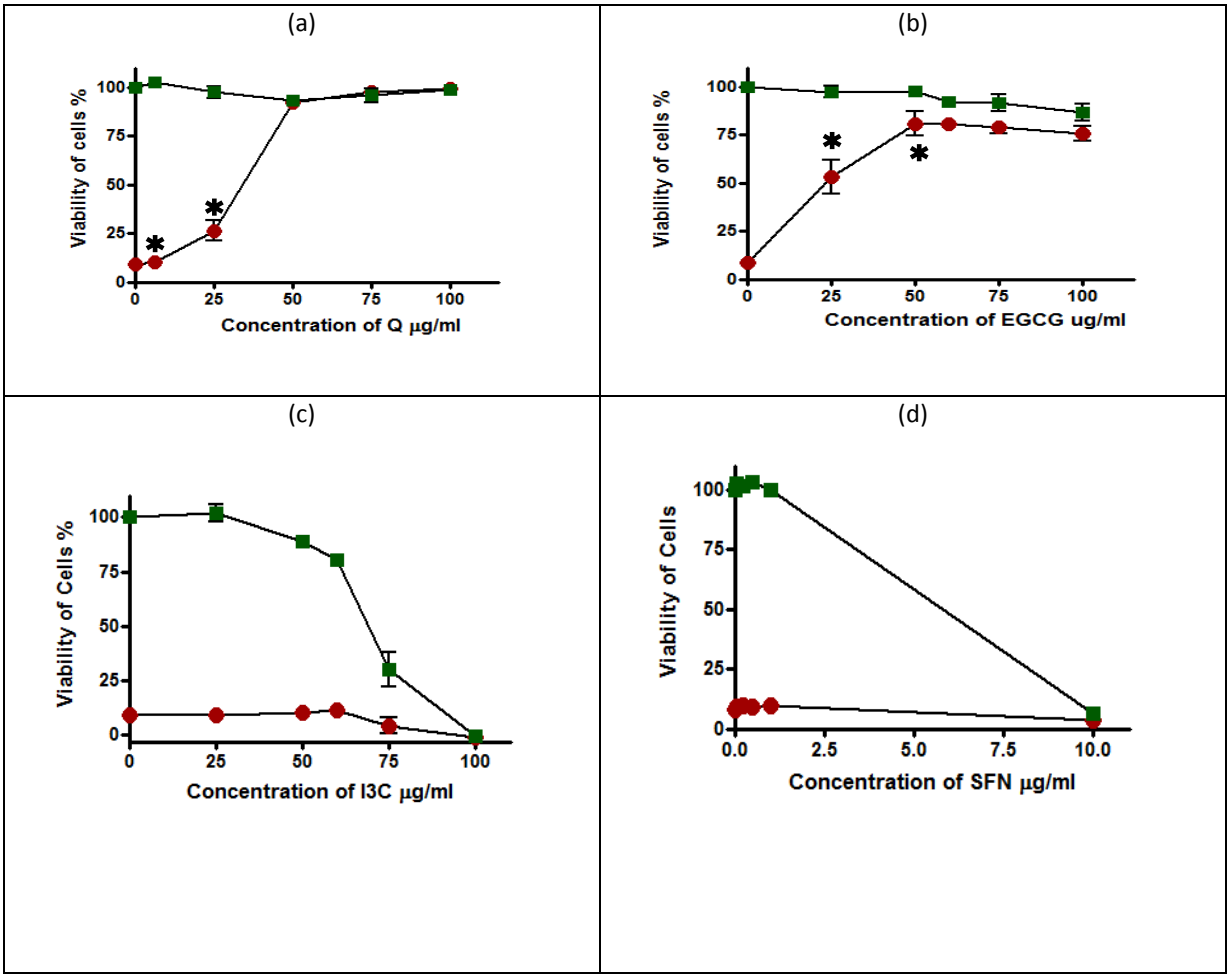

Figure 3.6 Effect of prior 20-hour exposure to (a) Q (0-100 µg/ml), (b) EGCG (0-100 µg/ml), (c) I3C (0-100 µg/ml), and (d) SFN (0-10 µg/ml) on cell viability in the absence (■) or presence (●) of t-BHP, using serum-free medium. . EC_{50} (µg/ml) was for Q 33.8±4.0, EGCG 27.8±9.9, I3C and SFN ≥100. TC_{50} (µg/ml) for I3C was 72.13±5.9 and for SFN was 5.81±0.27. * Means values of phytochemicals with t-BHP were significantly different to values without t-BHP ($p \leq 0.05$). Statistical significance was only tested at those concentrations of polyphenol at which there was some evidence of protection emerging. Values are Mean±SEM of 3 independent experiments.

Antioxidant	EC_{50} (µg/ml)				
	20-hour Exposure		5-hour Exposure		
	Medium Serum 10%	Medium Serum-free	Medium Serum 10%	Medium Serum 2%	Medium Serum-free
Q	34.1±5.3 n =8	33.8±4.0 n=3	39.9±4.5 n=5	16.1±2.4* n =7	17.9±1.8* n=3
EGCG	NE	27.8±9.9 n=3	84.7±7.4 n=4	51.2±8.3* n= 3	23.4±7.2*, º n=3
I3C	~60' n=2	NE	ND	NE	ND
SFN	NE	NE	ND	NE	ND

Table (3.1).EC_{50} (the effective concentration of phytochemical providing 50% protection).Values are reported as mean ±S.D; n=number of experiments, *significantly different to 5-hour (10% and 2% serum) (P<0.001). *Significantly different to 5-hour 10% serum (P<0.001), º significantly different to 5-hour 2% serum (P<0.001). NE= no effect, ND= not determined, ' = Response varied between experiments. One-way ANOVA followed by selected comparisons by the Bonferroni method was used to compare the EC_{50} values obtained under different culture conditions.

Antioxidant	TC_{50} (µg/ml)		
	20-hour Exposure		5-hour Exposure
	Medium Serum 10%	Medium Serum-free	Medium Serum 2%
I3C	98.1±0.0	72.13±5.9	ND
SFN	3.90 ±0.91	5.81±0.27	4.60±0.78.

Table (3.2).TC_{50} (the effective concentration of oxidant providing 50% toxicity).Values are reported as mean ±S.D; n=number of experiments.

3.4 Discussion

There is considerable current interest about the involvement of natural antioxidants in cytoprotection against oxidative stress (Alia *et al.* 2006b).

Our main goal was to detect the influence of the four phytochemicals Q, EGCG, I3C and SFN on cell viability and to what extent can they provide protection against cell death stimuli such as t-BHP in the presence and/or absence of serum in culture medium. According to this, different patterns of cellular assay including direct and indirect cytoprotection were used for this purpose.

Two aspects can help to explain the findings, role of albumin and structure of the antioxidant compounds.

Although albumin has antioxidant properties where it can define against toxicity effect of DHA, cis-4, 7, 10, 13, 16, 19-docosahexaenoic acid which can be found in fish-oil (Kanno *et al.* 2011), it interferes in the running of the assay and effect on results (Alia *et al.* 2006a; Gulden *et al.* 2006) through binding to antioxidant resulting in decrease antioxidant activity (Gulden *et al.* 2006).

Albumin is a highly soluble protein contains 585 amino acid and has a molecular weight of 66 kDa (Rothschild *et al.* 1988). It represents approximately 50% of total plasma protein, thus the amount of albumin is 3.5 g/100ml, and for 10% serum the albumin amount equals 0.35 g/100ml which is the amount of albumin in medium contain 10% serum which was used. This amount is not much different from the amount of albumin in interstitial fluid (IST). Thus, albumin content in (IST) from human aortas has been estimated to be 0.2 g/100ml (Smith 1982), interstitial fluid/plasma ratio for albumin 0.42 (Rutile 1977), and interstitial fluid from rat skin 2.1 g/100ml which is represent 63% of plasma albumin concentration, in that the amount of albumin in 10% is 0.21 g/100ml (Patel *et al.* 2010).

Accordingly, the use of medium containing 10% serum approximated exposure of cells to (IST) in most tissues (Rutili and Arfors 1977; Smith and Staples 1982)

We found that serum has no effect on cell viability during incubation for 5 and 20 hours (Fig 3.3a and b). This indicates that any variations in cytoprotection later on may be attributed to the presence of foreign substances such as oxidants and antioxidants.

With regards to direct cytoprotection, the mechanism is based on the principle of scavenging free radicals directly through donation of hydrogen atom from scavenger, an antioxidant, to the radical and so inactivating it (Chiodo *et al.* 2010).

Therefore, compounds that possess hydroxyl groups in their structure such as Q and EGCG are considered as main source for hydrogen atoms donation during hydroxyl sites attack by free radicals (Chiodo *et al.* 2010; Xiao and Kai 2012). Decrease the potency of Q and EGCG to scavenge free radicals during cytoprotection may be attributed to the presence of albumin where it binds to hydroxyl groups and prevent them from being free for radicals attack and hydrogen donation (Xiao and Kai 2012). This is clear when the activity of Q and EGCG was increased as the amounts of albumin decreased from 10% to 2% and serum-free medium and the hydroxyl groups were free to some extent from binding to that little amount of albumin resulting in increased the capability to scavenge radicals considerably (Gulden *et al.* 2006). On the other hand, I3C and SFN failed to scavenge free radicals, although I3C has one hydroxyl group in its structure, and this is attributed to the lack of any direct antioxidant properties.

With respect to indirect cytoprotection, the four compounds displayed different cytoprotective activities. Antioxidant activity of Q was increased and EGCG was enhanced when albumin was omitted. It is obvious that the absence of albumin was responsible for increase of antioxidant activity considerably, EGCG in particularly where Q and EGCG were being free from binding to albumin. On the other hand, I3C contributed partially in cytoprotection and this incomplete cytoprotection is mainly attributed to cytotoxicity effect of I3C at higher concentrations where it caused cell death during cellular incubation. Moreover, absence of albumin increased the toxicity effect of I3C because it holds antioxidant activity which participates in providing cellular protection (Kanno *et al.* 2011) from I3C and this was clear from decline TC_{50} value from 98 t0 72 µg/ml.

SFN failed to provide any cytoprotection at any concentrations due to the toxicity effect of SFN which kills cell even at low concentrations. Under this condition, toxicity of SFN was proportional with albumin and TC_{50} was increased when amount

of albumin was decreased. This means that albumin may be participated in transporting and distributing SFN in its site of action in cell and when it is reduced or omitted from culture medium resulting in minimizing the toxicity effect of SFN.

3.5 Conclusion

The antioxidant activity of Q, EGCG, I3C, and SFN was influenced by both duration of treatment and presence of serum proteins.

It is likely that the effect of duration of treatment is linked to the different actions of direct versus indirect antioxidants where the short treatment including free radical scavenging, while the long one is based on up-regulation of proteins. On the other hand, the effect of serum is likely linked to the binding of the phytochemicals to the plasma proteins.

Phytochemical treatments by using medium 10%, 2% serum, and serum-free medium were approximately expressed their effect in vivo where 10% serum represents the amount of albumin in interstitial fluid whilst serum-free medium represents the entities of proteins in brain.

Q and EGCG were not toxic in any pattern of cellular assay while toxicity effect of I3C and SFN were influenced by time of incubation and presence of albumin.

Chapter 4

Mechanism of toxicity of t-BHP

Chapter 4

Mechanism of toxicity of t-BHP

4. Introduction

Programmed cell death or apoptosis is a second type of cell death beside necrosis. It is described as an active programmed process of autonomous cellular dismantling to remove unwanted cells (Fink and Cookson 2005) that enter the program either as part of physiological cell death because of age or in response to cellular stress or infection (Green 1998) where there is no inflammation and cellular contents are not released (Fink and Cookson 2005; Green 1998). Therefore, apoptosis is important for elimination of pathogen-infected cells in human disease and for regulation of immune system in human health (Mccarthy *et al.* 1992).

Moreover, apoptosis is used to maintain a constant number of cells through balance between the rapid proliferation of cells that are produced normally in humans every day and cell death (Huang 2002). If this balance is changed in different direction then it causes pathological consequences such as in cancers where lossing is increased (Thompson 1995).

Apoptosis occurs via two pathways, the intrinsic and the extrinsic. The extrinsic includes caspases activation after receptor involvement, while intrinsic pathway

involves release of cytochrome c from mitochondria into the cytosol. All events in both pathways subsequently lead to effector caspases cleavage and induction of apotosis (Schafer and Kornbluth 2006). Apoptosis can be induced by internal and external stimuli such as DNA damage, presence of cytokines (Fas ligand, TNF-alpha), oxidative stress, viruses, etc. (Piret *et al.* 2004).

tert-butyl hydroperoxide is well known as a source of external peroxy radicals through interaction with ferrous iron in reaction similar to Fenton reaction in cytosol (Kim *et al.* 1988; Slater *et al.* 1995; Williams and Jeffrey 2000). Therefore, t-BHP contributes in intracellular ROS production and consequently is implicated in oxidative stress and cell injury (Williams and Jeffrey 2000). Moreover, t-BHP has been applied to investigate the mechanism of cell damage initiated by oxidative stress (Rush *et al.* 1985) such as in hepatocytes where the intrinsic pathway has been proposed for t-BHP to cause cell death and induce changes in mitochondrial permeability and depolarization of the mitochondrial potential (Nieminen *et al.* 1997; Nieminen et al. 1995).

The endogenous antioxidant system is responsible for eliminating ROS. However, since these defences are not totally perfect, oxidative stress can be induced and impaired normal biological function occurs when endogenous antioxidant system are overwhelmed by ROS and become imbalanced (Gulati *et al.* 2007; Sohn *et al.* 2007).

ROS can serve as a signal during early stages of apoptosis while their production at later stages because of alteration in mitochondrial permeability is associated with the final destruction of the cell (Segovia and Berges 2009). Therefore, ROS may contribute directly in mitochondrial dysfunction but their function during apoptosis remains controversial. Later investigations have demonstrated that ROS are derived from mitochondria as a result of Fas activation (Bauer *et al.* 1998; Castaneda and Kinne 2001; Denning *et al.* 2002; Jayanthi *et al.* 1999; Um *et al.* 1996). Moreover

it has been found that during apoptosis in intestinal epithelial cells, ROS was capable of triggering Fas- and Bax-mediated death pathway (Oh and Lim 2006).

Intracellular enzymes such as glutathione peroxidase contribute to removal of ROS by using glutathione (GSH), an antioxidant, to catalyze the elimination reaction of H_2O_2.

The aim of this study was to evaluate the effect of different concentrations of t-BHP on HepG2 cells in the presence and/or absence of phytochemicals. Moreover, two selected concentrations of t-BHP that cause cell death were used to determine GSH content, lipid peroxidation, ROS generation, caspase-3 activity, and caspase-3 activation which are indicated for apoptosis induction for up to 2.5 hours.

4.1 Materials and methods

4.1.2 Treatment of cells

HepG2 cells were cultured and seeding as shown in section 3.1.2 and then treated with different phytochemicals (Q, EGCG, I3C and SFN) for 20 hours prior to incubation with different concentrations (0.05, 0.1, 0.2, 0.4 and 0.8 mM) of t-BHP for 5 hours in serum-free medium.

In related experiments, the effect of t-BHP on the viability of HepG2 cells was studied to determine whether t-BHP can induce apoptosis in these cells or not. HepG2 cells were distributed into 24-well plate and exposed to t-BHP (0.4 or 0.8 mM) for 5 hours in serum-free medium and medium containing 2% serum.

4.1.3 Effect of phytochemicals on caspase-3 activation by t-BHP: western blot

The effect of t-BHP on treated HepG2 cells with I3C and Q was studied using western blotting and viability test.

HepG2 cells were incubated with I3C (25 µg/ml) using medium 10% serum for 20 hours prior exposure to 0.4 and 0.8 mM of t-BHP for different periods of time (0, 15, 30, 60, 90, 120, 150 minutes) for viability test while cells were exposed for 60 and 120 minutes for western blot using medium containing 2% serum. In addition, HepG2 cells were incubated with Q (100 µg/ml) and 0.4 and 0.8 mM of t-BHP simultaneously for different periods of time (0, 15, 30, 60, 90, 120, 150 minutes) in medium containing 2% serum. For western blot, HepG2 cells were incubated with Q (100µg/ml) and 0.4 and 0.8 mM of t-BHP simultaneously for 60 and 120 minutes using medium containing 2% serum.

4.1.4 Apoptosis: programmed cell death detection

4.1.4.1 Preparation of Cell lysate samples

After exposure of the cells to the various test incubations, the plate was placed on ice, medium removed and 150 µl of lysis buffer pH 7.6 (was prepared from dissolving 12.1g of Tris (20 mM), 1.9g of EGTA (1 mM), 500 µl of Triton X-100

(0.1%), 0.021g of NaF (1mM), 1.08g of *b*-glycerophosphate (10 mM) in 500 ml distilled water) was added. The pellet was re-suspended and homogenised using a pestle to make sure that all protein was released from the cell. The lysate (20 µl) was placed into one Eppendorf tube for estimation of protein by Lowry assay (see section 4.1.3.2), whilst another 100 µl was placed into another tube for western gel.

4.1.4.2. Determination of protein level

The total level of protein was determined by using Lowry method which is based on the conversion of Cu^{+2} to Cu^{+} under alkaline conditions (Lowry *et al.* 1951). A stock solution of 1mg/ml of bovine serum albumin (BSA) was prepared in water. From that solution, a series of dilutions were made to produce standard curve (see table below).

Tube number	D.H_2O (µl)	BSA (µl)	Protein concentration (mg/ml)
0	200	0	0
1	190	10	0.05
2	180	20	0.10
3	170	30	0.15
4	160	40	0.20
5	150	50	0.25
6	140	60	0.30
7	130	70	0.35
8	120	80	0.40
9	110	90	0.45

Lowry solution AB was prepared by mixing 20 ml of Lowry solution A (a solution prepared by dissolving 2 gm of sodium hydroxide NaOH, 1 gm of sodium dodecyl sulfate SDS, and 10 gm of sodium carbonate Na_2CO_3 in 500 ml of distilled water) with solution B (100 µl of 2% of sodium-potassium tartrate and 100 µl of 1% of copper sulphate $CuSO_4$), and then 1 ml of Lowry solution was added to all samples and BSA standards. All samples and BSA standard solutions were incubated for 10 minutes at room temperature and then, 100 µl of Folin reagent dilution 1:1 (1 ml of Folin reagent added to 1 ml of distilled water) was added to all samples and standards solutions. Solutions were transferred after mixing on the vortex to 96-well plate and incubated at room temperature for at least 45 min, and no more than 3 hours. The resulting absorbance was determined on spectra MAX 340pc plate reader at 750 nm.

4.1.5 in-Cell Western for Determination of Caspase-3 Activity

Following exposure of HepG2 cells to t-BHP for up to 120 minutes, the cells were washed with PBS, and then fixed by adding 150 µl of (4% w/v) paraformaldehyde (20g paraformaldehyde solid dissolved in 500ml PBS containing 7 drops 10M NaOH) to each well and incubating for 30 minutes at room temperature. Fixative was aspirated. Cells were re-washed with PBS before 150 µl of (0.1% v/v) Triton solution (0.1 ml Triton X-100 dissolved in 100ml PBS solution) was added to each well, and then incubated for 15 minutes at room temperature then aspirate. Cells were re-washed with PBS, and then 150 µl of (5% w/v) milk, the blocking agent, (5g milk powder dissolved in 100ml PBS) was added to each well then left on plate shaker for an hour at room temperature, and then aspirate. Following this, 50 µl of

primary antibodies, (2:1000) caspase-3 (2 µl of caspase-3 antibody diluted in 1 ml of 5% milk) and (0.25:1000) GAPDH (0.25 µl of GAPDH antibody diluted in 1 ml of 5% of milk), were added to each test well. The plate was incubated overnight on a plate shaker in cold room (4 - 6°C). Next day, primary antibodies solution was aspirated before each well was washed three times with PBS each 5 minutes, and then aspirated. Following aspiration of the plate, 100 µl of secondary antibodies solution (8 µl of goat anti rabbit IR800 and 6 µl of goat anti mouse IR680 were diluted in 30 ml of 5% of milk) was added to each well and incubated on closed plate shaker for 60 minutes at 37 ºC. The secondary antibody was light sensitive so addition to the assay plate was carried out quickly and the assay plate was covered during incubation. When incubation was finished, solutions were aspirated, and each test well was washed three times with PBS, aspirating after each wash. After aspirating the third wash, the assay plate was scanned by an Odyssey infrared fluorescent imaging system (LI-COR) and analysed using Odyssey V3 software.

4.1.6 Immunocytochemistry

HepG2 cells were cultured on coverslips present in 24-well plate for 24 hours, next day cells were treated with 0.4 and 08 mM t-BHP using medium 2% serum for 0, 60, and 120 minutes. After treatment, HepG2 cells were fixed by paraformaldehyde (4%) in phosphate buffered saline (PBS) for 30 min at room temperature, after then cells were washed by PBS and permeabilized using 0.1% Triton-X100 in PBS for 30 minutes. Non-specific biding sites were blocked by incubating cells with PBS containing 1% bovine serum albumin (BSA) for 60 minutes at room temperature. Following this, 50 µl of primary antibodies, (1:200) caspase-3 (from Cell Signaling Technology) (2 µl of caspase-3 antibody was diluted in 200 ml of 5% milk) were

added to each well and incubated overnight at 4 °C. Next day, primary antibody was aspirated and the wells washed with PBS. After PBS washing, FITC-conjugated goat anti-rabbit IgG (1:800) (from Jackson Immuno Research) was added and the plate incubated for 1 hour at 37 °C. Cell nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI, Molecular Probes) dye (1µl:1000 µl PBS) for 5 minutes at room temperature to visualize all cells on coverslips. After extensive washing with PBS, coverslips were mounted with mounting medium without DAPI (Vectashield) and viewed through 63 x glycrine immersion lens objective with a Leica DMRA2 fluorescent microscope. Images were cropped and brightness and contrast were adjusted equally using the ImageJ Software.

Negative controls without primary antibody were included to ensure mininmal non-specific staining.

4.1.7 Determination of caspase activity

Activation of specific proteases called caspases is considered to be one of the initial biochemical events of apoptosis (Alnemri *et al*. 1996). A Homogenous Caspases Assay kit from Roche was used for determination of caspase-3 activity.

After treatment, medium was aspirated, and then to each well of treated cells 175 µl of working substrate was added; working substrate was immediately prepared by mixing 200 µl of 500 µM of DEVD-R110 stock solution in DMSO with 2.8 ml of incubation buffer (already prepared in bottle). While 87.5 µl of working substrate was added to 87.5 µl of working positive control which was prepared by mixing 10 µl of stock positive control (lysate from apoptotic U937 cells treated with camptothecin) with 90 µl of incubation buffer. For standard curve 87.5 µl of working

substrate was added to 87.5 µl of series of dilution 5, 2, 1, 0.5, 0.25, and 0.125 µM that were prepared from 10 µM of working standard solution; it was prepared by mixing 10 µl of 1 mM of stock R110 standard with 990 µl of media. For blank, 175 µl of medium containing 2% serum was added.

After substrate addition, the plate was covered with a lid and incubated not less than an hour at 37 ºC. Fluorescent plate reader was used to measure caspase-3 activity at excitation of 470-500 nm and emission of 499 nm.

4.1.8 Determination of intracellular ROS production

The level of intracellular ROS was measured by using the oxidation-sensitive fluorescent dye 2′, 7′-dichlorodihydrofluorescein diacetate DCFH-DA (Sakuma *et al.* 2009). This dye is a stable nonpolar compound and cell-permeable indicator of ROS because it can get readily into the cell as non-fluorescent compound DCFH which is oxidized by ROS to the highly fluorescent DCF (Oh and Lim 2006; Segovia and Berges 2009). DCFH-DA was dissolved in DMSO to a final concentration of 5 mM and then diluted to 10 µM in complete PBS containing Ca^{+2} and Mg^{+2} before use. For ROS measurement, HepG2 cells were washed with complete PBS after pre-incubation with the probe DCFH-DA for 30 minutes. The washed cells including DCFH-DA as DCFH were then treated with t-BHP for different periods of time. Following the treatment, ROS was measured immediately in fluorescence spectrophotometry by using 485 nm and 520 nm wavelengths for excitation and emission respectively. The amount of ROS that are produced by the cells is proportional to the intensity of fluorescence generated (Oh and Lim 2006).

4.1.9 Determination of cellular glutathione (GSH) content

GSH in its reduced form (gamma-glutamylcysteinylglycine) is a major intracellular antioxidant in most eukaryotic and prokaryotic cells. It is considered as an important monitor for human diseases that are related to detoxification processes and oxidative stress (Lewicki *et al.* 2006). GSH was determined by the method of (Hissin and Hilf 1976).

Following treatment, cells were washed with PBS, and then 1 ml of 10% trichloroacetic acid (TCA) (10g of TCA was dissolved in 100 ml distilled water) was added to each well. The plate was covered and placed on a shaker for 30 minutes to mix and extract glutathione into TCA. The supernatant was collected in plastic tubes. Into 96 well-plates, 50 µl of cell/TCA supernatant, glutathione standard, and TCA blank was distributed before adding 200 µl of K_2HPO_4/OPT mixture; this mixture was prepared before use shortly by adding 2.5 ml of *o*-phthalaldehyde (OPT) (10 mg of OPT dissolved in 10 ml of methanol and stored in dark in fridge, this reagent was prepared weekly) to 7.5 ml of 0.4 M of K_2HPO_4 buffer (pH 8.2) (35g of K_2HPO_4 dissolved in 500 ml distilled water). The plate was left for 15 minutes to stand, and then the fluorescence was read on a fluorescent plate reader by using 360 nm and 460 nm wavelengths for excitation and emission respectively. The concentration of glutathione in samples was determined by reference to the standard solutions of GSH.

For glutathione standard curve, a series dilution of 100 µM, 50 µM, 25 µM, 12.5 µM and 6.25 µM were freshly prepared from 1 mM of glutathione (3.07 mg of reduced form of glutathione was dissolved in 10 ml of 10% TCA) by using TCA. TCA was used as blank.

4.1.10 Determination of lipid Peroxidation (LPO)

After treatment, cells were collected in plastic tubes by adding 150 µl of 20% TCA (20g of TCA dissolved in 100 ml distilled water) to each well and mixing. The tubes were left to stand for 10 minutes, and then centrifuged, the precipitate being discarded. To 100 µl of supernatant (or standard), 100 µl of 0.67% TBA (2-thiobarbituric acid) (0.67g of TBA was dissolved in 100 ml distilled water) were added, and then tubes were heated at 95 ºC for 20 minutes. The tubes were placed on ice to cool for 10 minutes; the pink colour appeared gradually. Finally, 1.5 ml of butanol was added to each tube and mixed before left to stand for 5 minutes. The fluorescence of the butanol layer was read on a fluorescent plate reader by using 530 nm and 590 nm wavelengths for excitation and emission respectively.

For standard curve, from 100 mM of 1,1,3,3-Tetramethoxypropane (178.7 µl was added to 10 ml of 20% TCA) a series dilution of 10, 5, 2.5, 0.625, 0.1, 0.05, 0.025, and 0.0125 mM was prepared.

4.2 Results

4.2.1 Toxicity of tert-butyl hydroperoxide (t-BHP)

4.2.1.1 Incubation of HepG2 cells with phytochemicals, Q, EGCG, I3C, SFN using different concentration of *tert-butyl hydroperoxide* (t-BHP) in serum-free medium

The viability of HepG2 cells treated with or without (100 µg/ml of Q, EGCG, and I3C and 10 µg/ml of SFN) for 20 hours, using serum-free medium prior exposure to different concentrations (0.05, 0.1, 0.2, 0.4 and 0.8 mM) of t-BHP for 5 hours was shown in (Fig. 4.1). This experiment was done to establish concentrations of t-BHP to use in subsequent studies.

Similar results were obtained from different sets of incubation of HepG2 cells with t-BHP in the absence of the four phytochemicals (table 4.1). Moreover, cell viability was decreased when the concentration of t-BHP was increased and reached to about 5% at 0.4 mM above in the absence of phytochemicals (Fig.4.1).

On the other hand, when HepG2 cells were incubated with phytochemicals for 20 hours prior to incubation with different concentration of t-BHP for 5 hours, different results were obtained. *T*-BHP had no effect of viability of the cells treated with Q and cells remained alive 100% during 5 hours of incubation (Fig. 4.1a) and TC50 was ≥ 0.8 mM (table 4.2). Identical results were obtained with EGCG where TC50 was ≥ 0.8 mM (table 4.2) but cell viability was slightly declined from 100% to about 90% and 85% at 0.4 and 0.8 mM respectively (Fig.4.1b). On the other hand, I3C protected HepG2 cells partially from toxicity of specific concentrations of t-BHP

where viability of cells was dropped from 100% by using 0.2 mM of t-BHP to about 60% and 10% by using 0.4 and 0.8 mM of t-BHP respectively (Fig.4.1c). Moreover, it was toxic to the cells and cause cell death at concentration lower than 0.6 mM t-BHP where TC50 was 0.54 (table 4.2). Similarly to I3C, partial protection was provided by SFN against the effect of t-BHP where viability of the treated cells with SFN was decreased to 80% by using 0.4 mM of t-BHP while it declined to about 25% by using 0.8 mM of t-BHP (Fig.4.1d), additionally, SFN cause cell death at concentration lower than 0.6 mM t-BHP where TC50 was 0.62 mM (table 4.2).

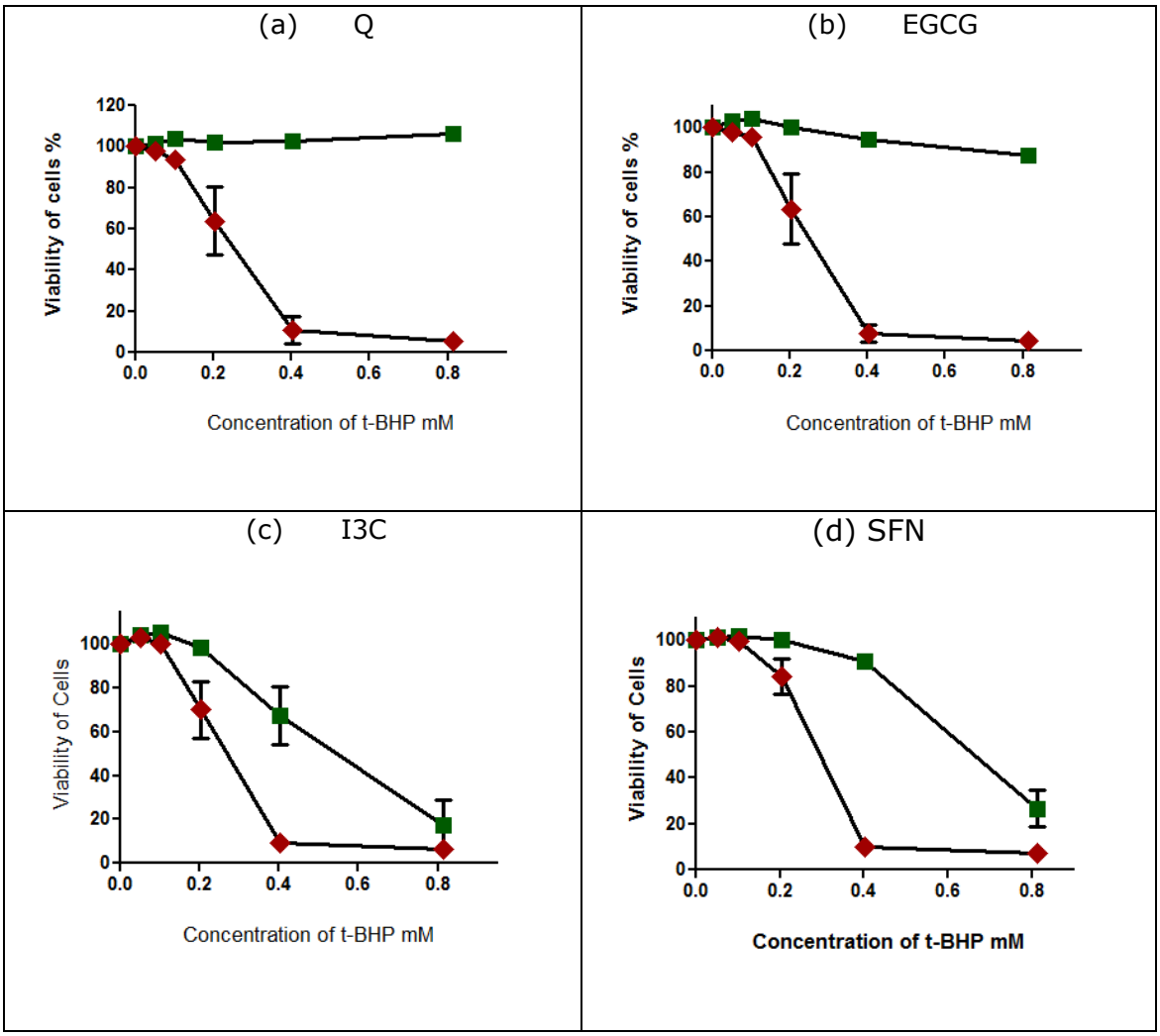

Figure 4.1 Effect of different concentrations of t-BHP (0.05, 0.1, 0.2, 0.4 and 0.8 mM) on Viability of HepG2 cells in the presence (■) and absence (◆) of phytochemicals for up to 5 hours using serum-free medium values are Mean±SEM of 3 independent experiments.

Phytochemical µg/ml	Toxicity of t-BHP (expressed as TC_{50}, mM)	
	Absence of Phytochemical	Presence of phytochemical
Q	0.29 ± 0.05	≥ 0.8
EGCG	0.27 ± 0.07	≥ 0.8
I3C	0.21 ± 0.08	0.54 ± 0.098
SFN	0.17 ± 0.07	0.62 ± 0.063

Table 4.1 TC_{50} values for untreated and treated HepG2 cells with four chemicals Q, EGCG, I3C, and SFN for 20 hours prior exposure to different concentration of t-BHP (0, 0.05, 0.1, 0.2, 0.4, and 0.8 mM) for 5 hours using serum-free medium. Values are Mean±SEM of 3 independent experiments.

4.2.1.2 Time dependence of t-BHP toxicity on HepG2 cells

Although 0.4 and 0.8 mM t-BHP elicited similar level of toxicity (see Fig.4.1), it was apparent that I3C and SFN provided good cytoprotection only at the 0.4 mM concentration of *t*-BHP. Accordingly, 0.4 and 0.8 concentration of *t*-BHP were employed in further studies into the possible mechanism (s) underlying the toxicity of *t*-BHP to HepG2 cells and protection gained by the phytochemicals.

(a) In presence of 2% serum-containing medium

When serum concentration in culture medium was set at 2%, results showed that HepG2 cells retained their viability during an incubation time of 5 hours in the absence of t-BHP. On the other hand, viability of cells was affected by the presence of 0.4 mM of t-BHP when they were incubated for similar periods of time. It is

notable that with this concentration of t-BHP, cell viability dropped gradually from 100% at zero time to reach about 10% after 5 hour of incubation. It is seems that about 20% of cells dead after one hour of incubation. Similarly HepG2 cells recorded decline in their viability when 0.8 mM of t-BHP was used. A cell death was notable with higher concentration of t-BHP and about 90 % of cells were dead after two hours (cell viability reached till about 10% after two hours incubation) (Fig.4.2)

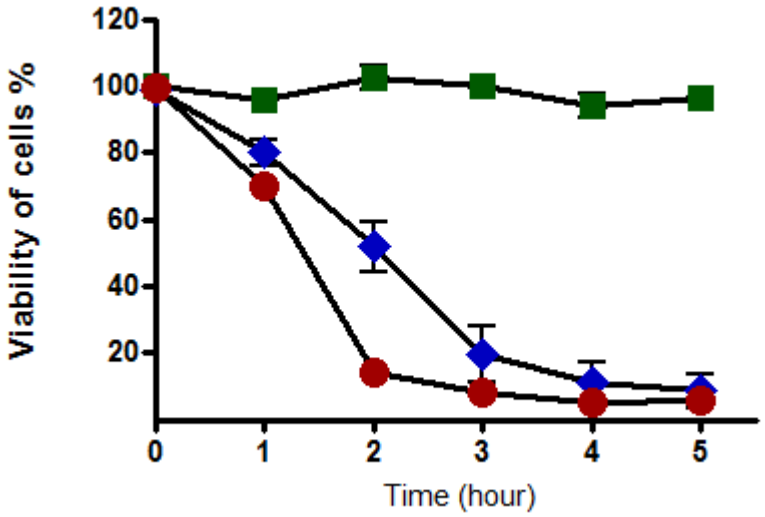

Figure 4.2 Viability of HepG2 cells incubated in the absence (■) and presence of t-BHP at 0.4 mM (◆) or 0.8 mM (●) for up to 5 hours in medium containing 2% serum. Values are Mean±SEM of 3 independent experiments.

(b) In presence of serum-free medium

Results displayed that HepG2 cells were still alive during incubation with serum free medium for different periods of time (0-5 hours) and the omission of serum from culture medium for this had no effect on the viability of cells. Number of dead cells started to increase when cells incubated with 0.4 mM *t*-BHP after one hour and complete cell death occurred after four hours of incubation where non-viable cells was 100%. Similarly, extreme cell death was notable with 0.8 mM *t*-BHP during incubation time course where more than 50% of cells were dead after two hours of incubation and all cells were dead after three hours when cell viability recorded to be 0% (Fig.4.3).

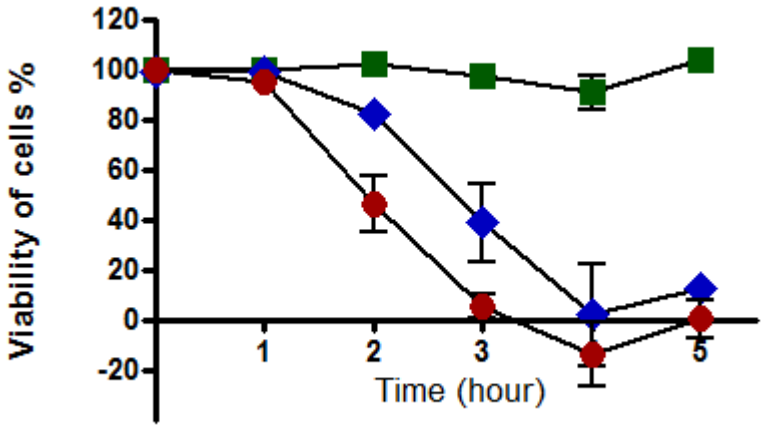

Figure 4.3 Viability of HepG2 cells incubated in the absence ( ) and presence of *t*-BHP at 0.4 mM ( ) or 0.8 mM ( ) for different periods of time in serum-free medium. Values are Mean±SEM of 3 independent experiments.

4.2.2 Detection of caspase-3 activation

(a) With western blotting

Viability of untreated HepG2 cells was unaffected and cells retained 100% viability during incubation up to 150 minutes. A notable decline in viability of HepG2 cells was recorded after 60 minutes of incubation with 0.4 and 0.8 mM and cell death was increased as the incubation time increased to reach the maximum at 150 minutes of incubation where viability of treated HepG2 cells with 0.4 mM was dropped to about 60% whilst it dropped to about 25% with 0.8 mM (Fig 4.4). Therefore, HepG2 cells were incubated with t-BHP using 0.4 and 0.8 mM for specific time of incubation (60, 90, and 120 minutes) in 2% serum medium to detect caspase-3 activation under these conditions.

Equivalent amounts of protein from all HepG2 cell studies were loaded onto the electrophoresis gel after determination of protein content by Lowry assay by interpretation from a linear standard curve (Fig 4.5).

The positive control of Jurkat cells and the positive control of HepG2 cells treated with 50 mM of glucose for 72 hours were showed strong bands of procaspase-3 and active caspase-3 at 35 and 17 KDa respectively. Moreover, the procaspase-3 band of negative control of Jurkat cells at 35 KDa was also marked while no activation for caspase-3 could be seen at 17 KDa (Fig 4.6). These evidences show that caspase cleavage can be detected in HepG2 cells undergoing apoptosis. Procaspase-3 for untreated and treated HepG2 cells with t-BHP at 60, 90 and 120 min has been shown with strong bands at 35 KDa except samples treated with 0.8 mM of t-BHP at 90 and 120 minutes where bands were lighter than others. On the other hand, no strong evidence for caspase activation could be reported where all samples

displayed faint bands of active caspase-3 at 17 Kda but the less faint bands from all of them were the untreated HepG2 cells at 60 and the treated cells with 0.8 mM of t-BHP at 90 and 120 minutes (Fig 4.6) and (Fig 4.7 a and b).

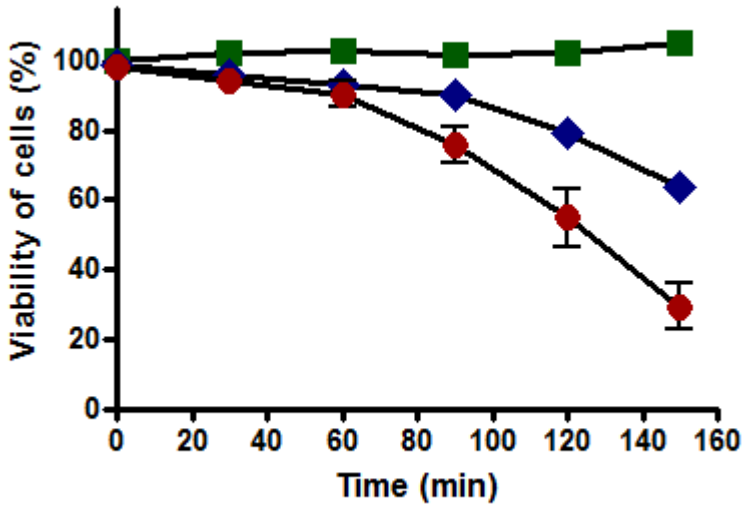

Figure 4.4 Viability of HepG2 cells incubated in the absence (■) and presence of t-BHP at 0.4 mM (◆) or 0.8 mM (●) for different periods of time (0, 30, 60, 90, 120, and 150 minutes) in medium containing 2% serum. Values are Mean±SEM of 3 independent experiments.

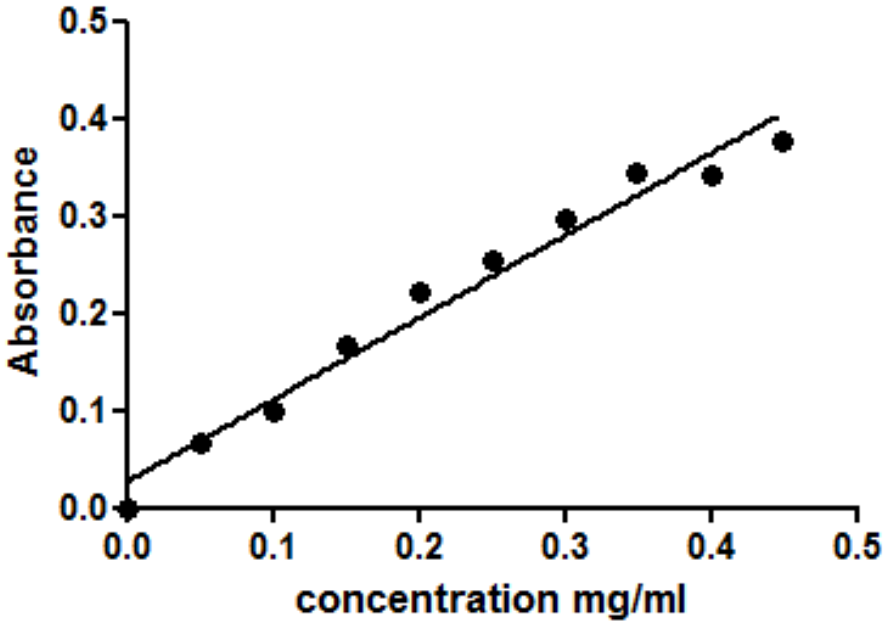

Figure 4.5 Standard curve for protein.

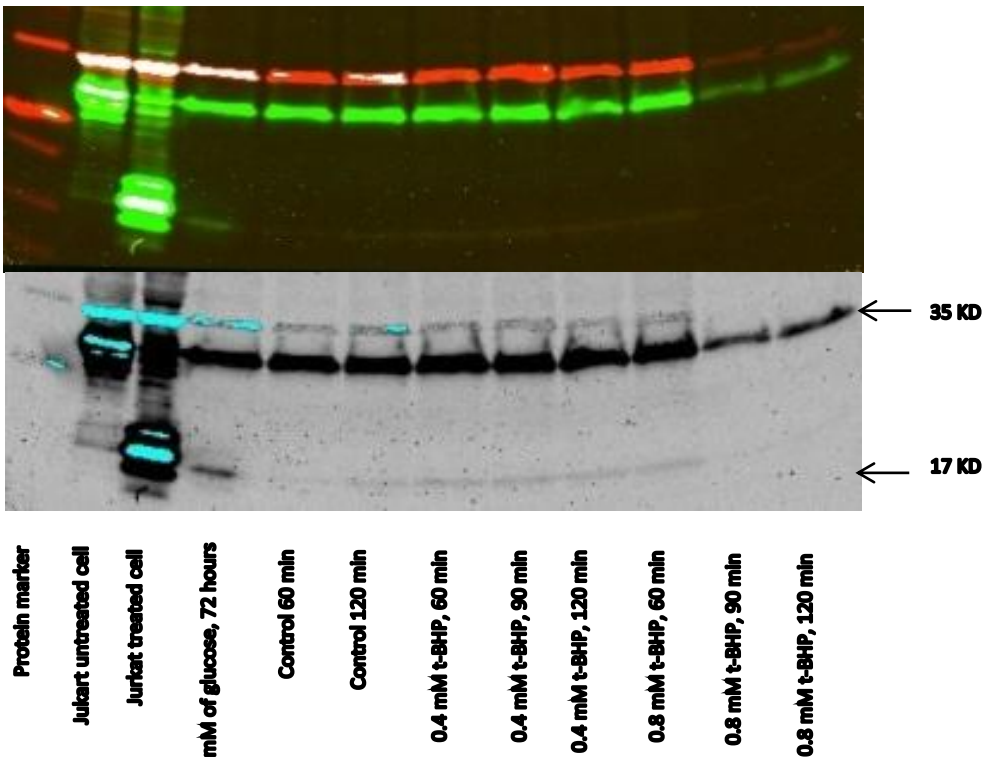

Figure 4.6 Western blot of caspase-3 activation in HepG2 cells. blot showed the bands of procaspase-3 and its cleavage product at 35 and 17 KDa respectively for untreated and treated Jurkat cells and HepG2 cells treated with 50 mM of glucose for 72 hours and untreated and treated HepG2 cells with 0.4, 0.8 mM of t-BHP for 60, 90, 120 minutes using medium 2% serum.

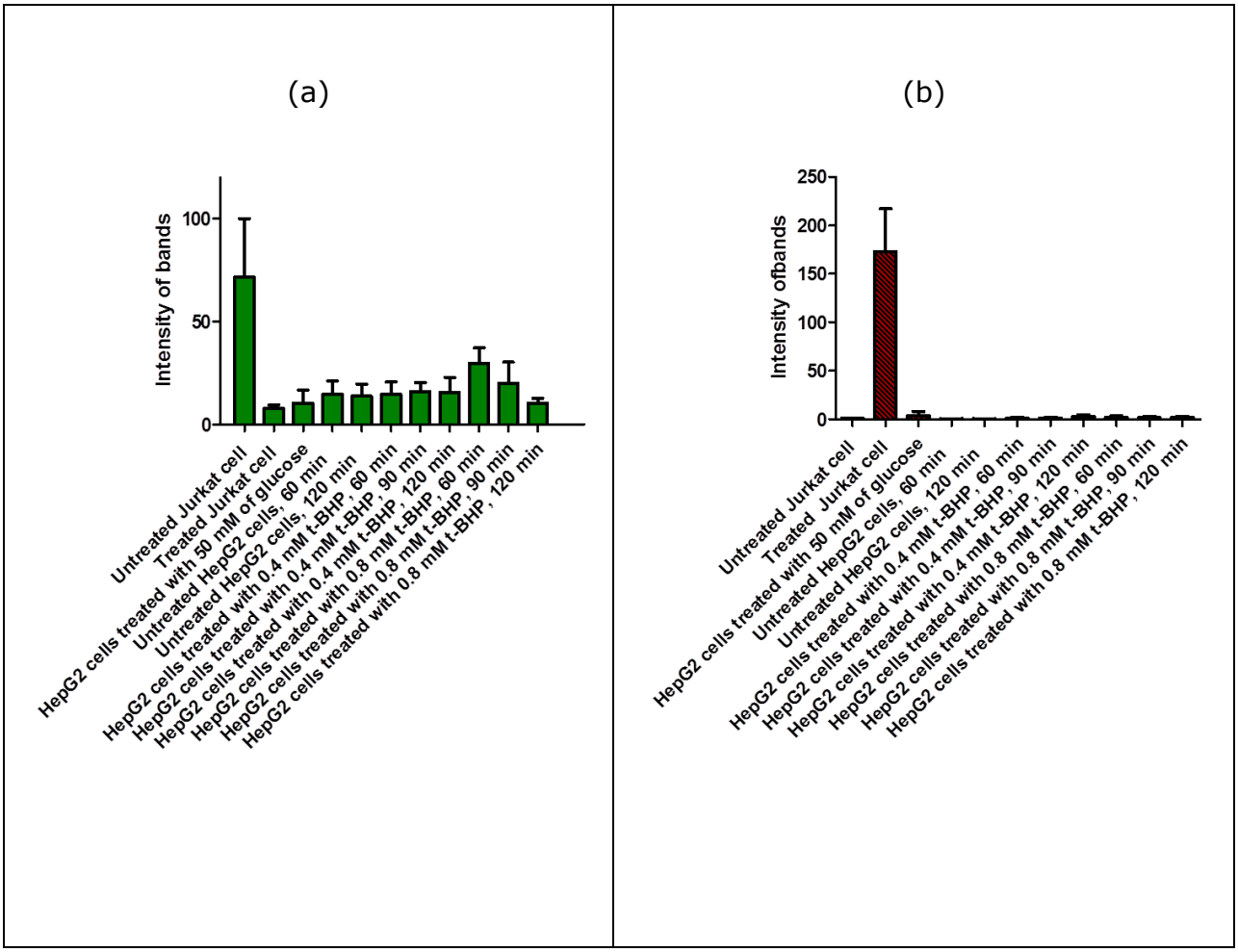

Figure 4.7 Quantitative procaspase-3 (a) and active caspase-3 (b) of untreated and treated Jurkat cells and untreated and treated HepG2 cells with 50 Mm of glucose for 72 hours and with 0.4 and 0.8 mM of t-BHP for 60, 90, and 120 minutes using medium 2% serum. Values are Mean±SEM of 3 independent experiments.

(b) In-cell western

Results showed that there was no noticeable variation between untreated and treated HepG2 with 0.4 and 0.8 mM t-BHP for 60 and 120 minutes (Fig 4.8). For 60 minutes incubation, no differences between incubated cells with 0.4 and 0.8 mM t-BHP and control. Similarly for 120 minutes incubation, no variations between untreated HepG2 cells treated with 0.4 and 0.8 mM. Results showed that there was no evidence for activation of caspase-3 (Fig 4.9a and b).

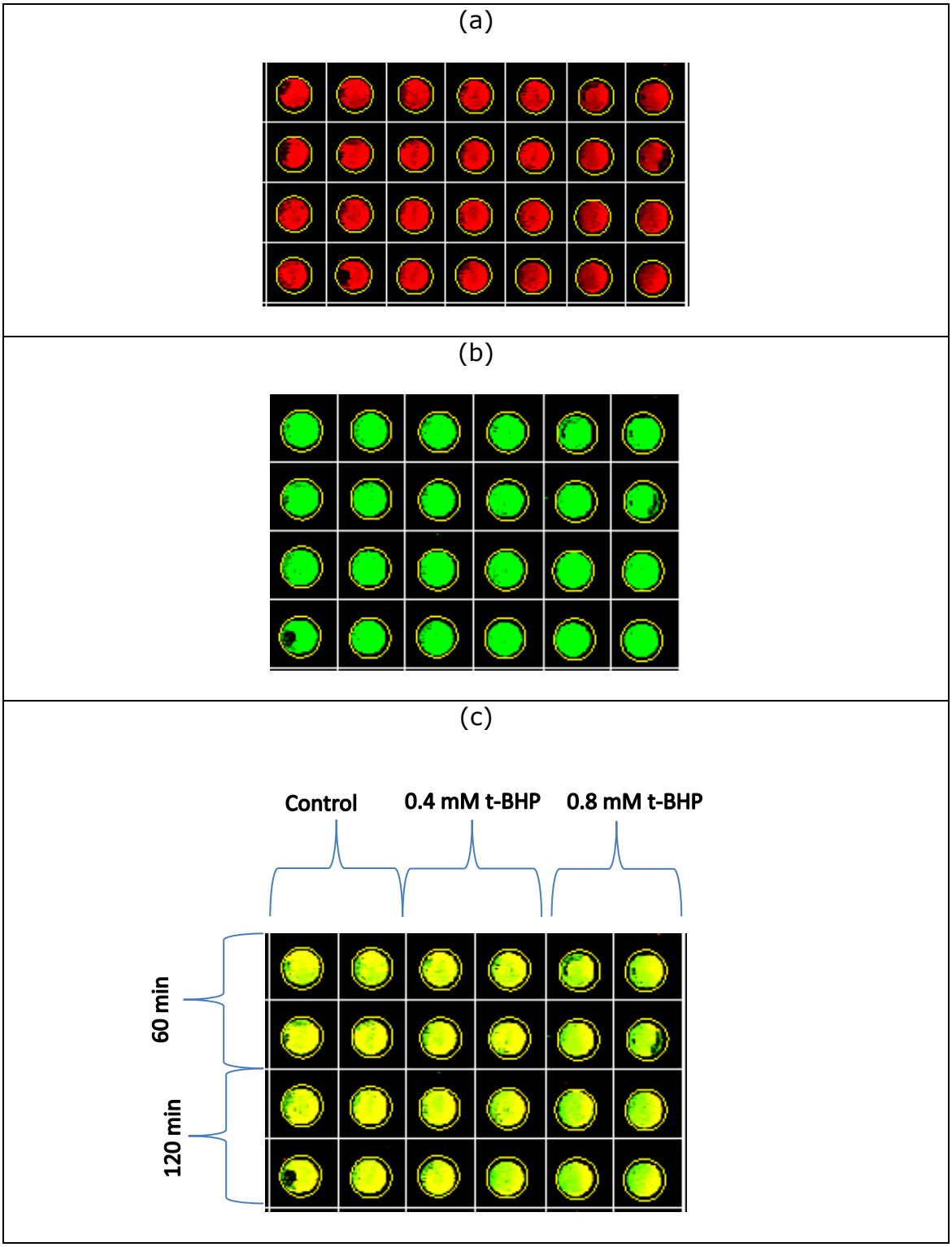

Figure 4.8 in cell-western for untreated and treated HepG2 with 0.4, 0.8 mM of t-BHP for 60 and 120 minutes. (a) GAPDH mg/ml (B) caspase-3 (c) caspase-3/GAPDH). Values are Mean±SEM of 3 independent experiments

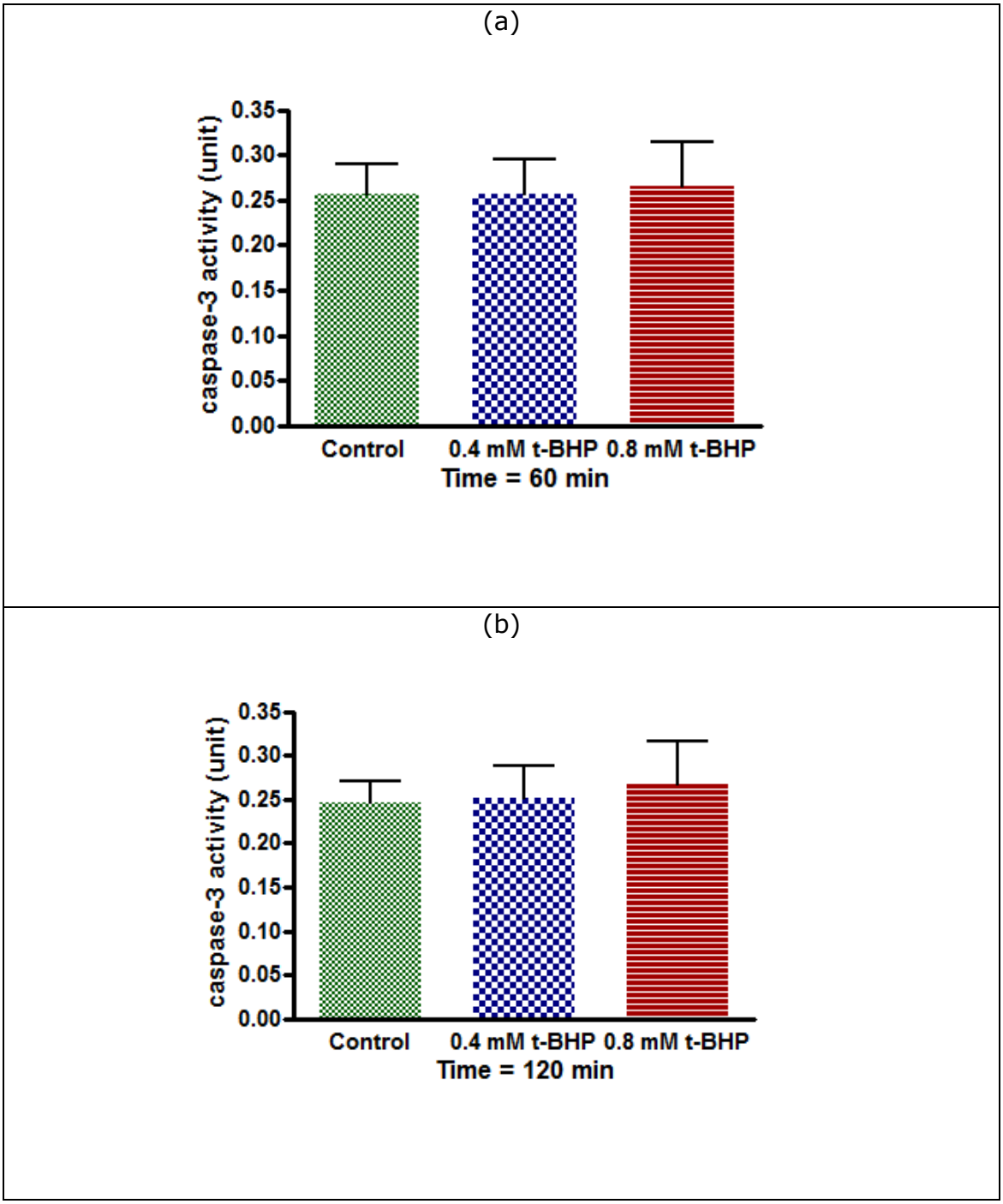

Figure 4.9 caspase-3 activity production (a) untreated and treated HepG2 with 0.4 and 0.8 mM of t-BHP for 60 (b) untreated and treated HepG2 with 0.4 and 0.8 mM of t-BHP for 120 minutes. Values are Mean±SEM of 3 independent experiments.

(c) Immunocytochemistry

Although the number of dead cells increased with the increase of incubation time of HepG2 cells with 0.4 and 0.8 mM of t-BHP using medium 2% serum (Fig 4.4), the immunocytochemistry results showed that incubation of HepG2 cells in the absence or presence of 0.4 and 0.8 mM t-BHP at zero, 60, and 120 minutes had small effect on the activation of caspase-3 where a little variation can be seen during incubation with 0.4 and 0.8 mM t-BHP at 60 and 120 minutes (Fig 4.10).

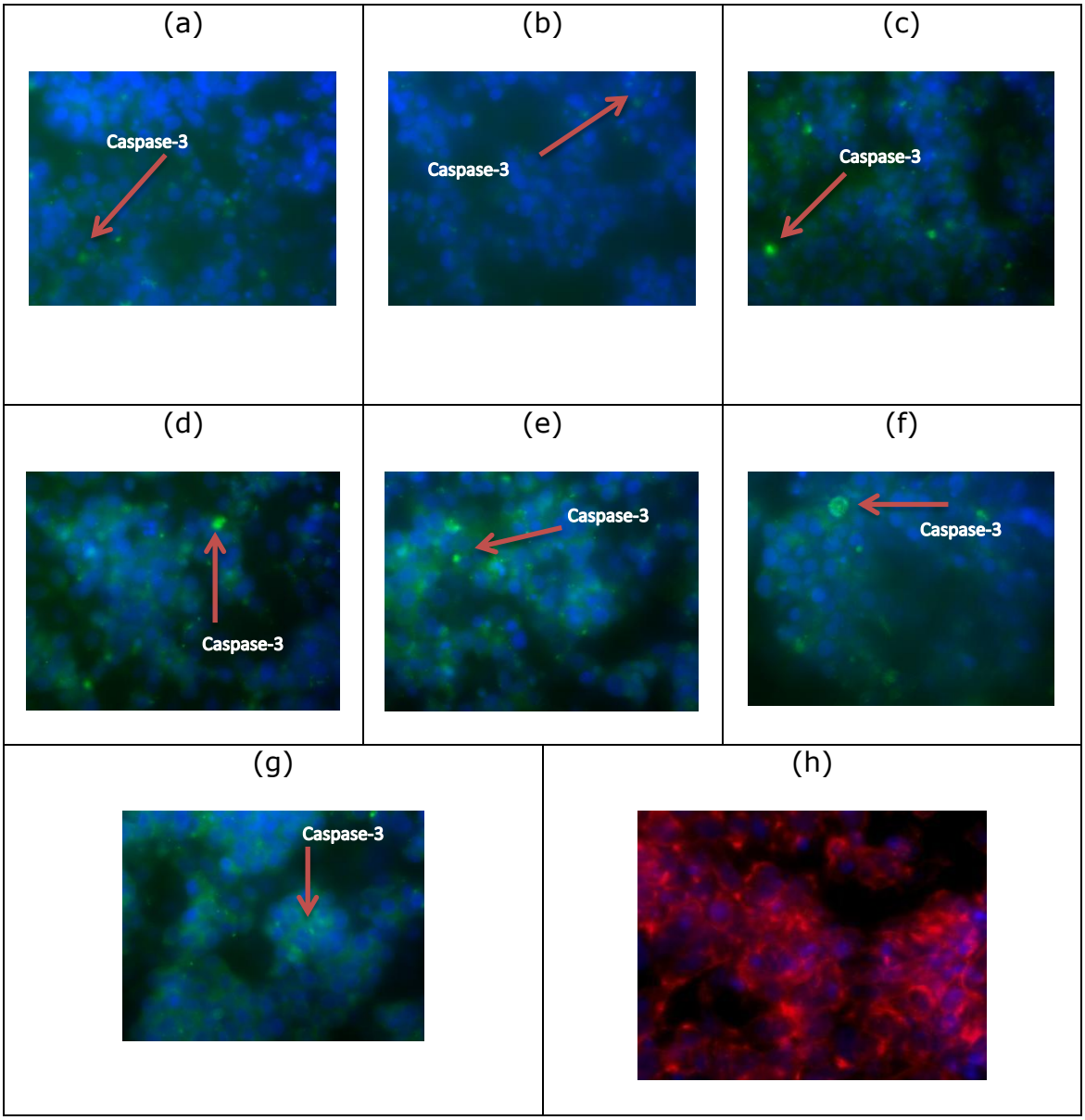

Fig.4.10 immunocytochemistry for HepG2 cells (a) Incubation of untreated HepG2 cells for zero minutes (b) Incubation of untreated HepG2 cells for 60 minutes (c) Incubation of untreated HepG2 cells for 120 minutes (d) Incubation of treated HepG2 cells with 0.4 mM of t-BHP for 60 minutes (e) Incubation of treated HepG2 cells with 0.4 mM t-BHP for 120 (f) Incubation of treated HepG2 cells with 0.8 mM t-BHP for 60 minutes (g) Incubation of treated HepG2 cells with 0.8 mM t-BHP for 120 minutes (h) Incubation of HepG2 cells with medium containing 2% for 120 minutes(negative control). The red arrow points to the green spots of caspase-3. This is single experiment.

4.2.2.1 Caspase activity

The standard curve for caspase-3 activity was displayed a linear response (Fig 4.11). Cell viability was recorded that untreated HepG2 cells remained alive through incubation time for up to 150 minutes but it was not the same for treated cells with 0.4 and 0.8 mM t-BHP where it was declined from 90% to about 60% and 30% respectively (Fig 4.12a).

Results of caspase enzyme activity showed that the activity of enzyme in untreated HepG2 cells was unchanged during the incubation time up to 150 minutes. Cells treated with 0.4 and 0.8 mM t-BHP displayed similar behaviours as their caspase enzyme activity declined after 30 minutes of incubation from 400 to reach about 300 and 200 for cells treated with 0.4 and 0.8 Mm t-BHP respectively (Fig 4.12b).

When caspase activity was adjusted for viability there was some variations observed at 150 minutes for HepG2 cells treated with 0.8 mM t-BHP. Results obtained from Friedman test for untreated and treated HepG2 cells with 0.4 and 0.8 mM t-BHP at 150 minutes showed significance and p value was 0.0278 ($P \leq 0.05$) and results obtained by Dunn's Multiple Comparison Test indicated a significant increase in caspase activity at 0.8 mM t-BHP but not at 0.4 mM t-BHP (Fig 4.12c and d).

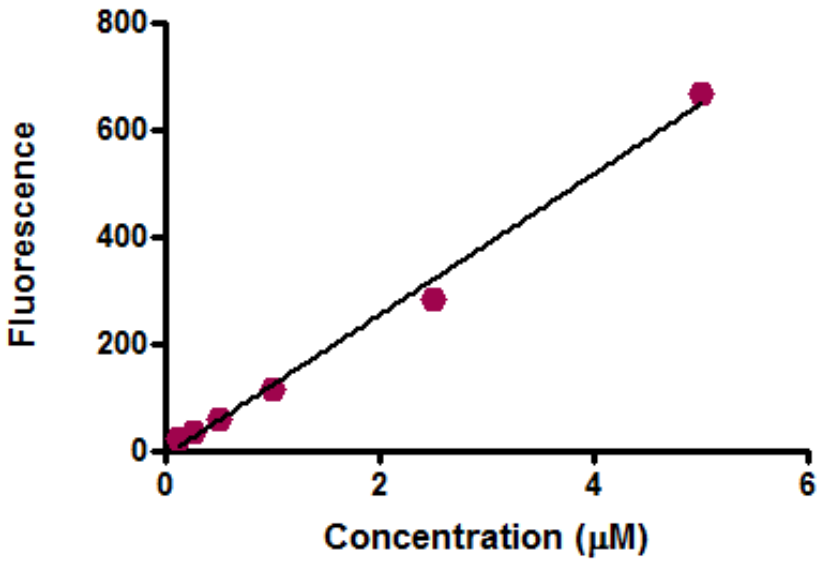

Figure 4.11 Standard curve of caspase activity

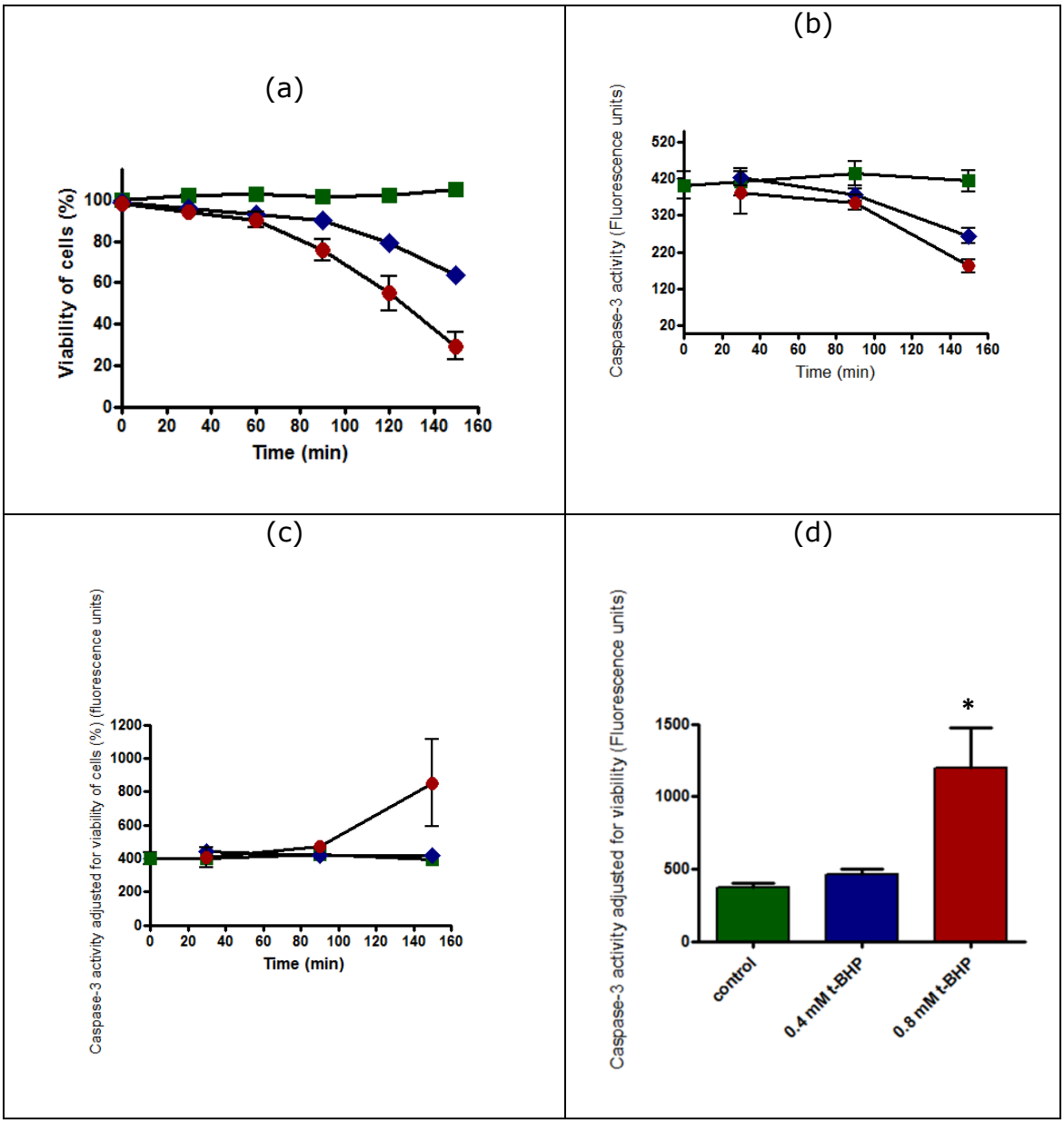

Figure 4.12 Caspase activity of HepG2 cells (a) Viability of untreated ( ) and treated HepG2 cells with 0.4 ( ) and 0.8 mM of t-BHP 0.8 mM ( ) for different periods of time (b) caspase-3 activity production by untreated ( ) and treated HepG2 cells with 0.4 ( ) and 0.8 mM t-BHP ( ) for 30, 90, and 150 minutes (C) caspase-3 activity production by untreated ( ) and treated HepG2 cells with 0.4 ( ) and 0.8 mM t-BHP ( )and adjusted for viability (d) caspase-3 activity production by untreated (green bar) and treated HepG2 cells with 0.4 (blue bar) and 0.8 (red bar) mM t-BHP at 150 minutes. * Significant where P≤ 0.05 and equal 0.0278 according to Friedman test and Dunn's Multiple Comparison Test. Values are Mean±SEM of 3 independent experiments.

In summary, results of western bolt, in-cell western, and immunocytochemistry, indicate that there was no much evidences for apoptosis when HepG2 cells incubated with 0.4 and 0.8 mM for 60, 90, and 120 minutes using medium containing 2% serum but there was significance increased in caspase-3 activity at 0.8 mM t-BHP. On the other hand, cell death was increased under the same applied conditions.

4.2.2.2 Effect of phytochemicals on t-BHP: western blot data

Although there was only modest indication for apoptosis in western blot, in-cell western and immunocytochemistry during incubation of HepG2 cells with t-BHP for up to 2 hours, there was obvious evidence for cell death and decline in viability of cells under these conditions. Therefore, the purpose of this experiment was to show the cytoprotective effect of direct and indirect antioxidants against the toxicity effect of t-BHP to induce apoptosis even the induction was slightly.

We chose 60 and 120 minutes specifically because at these times caspase-3 was activated slightly and faint bands appeared at 17 KDa (Fig 4.6). Moreover, Q was selected as example of direct antioxidant and I3C as example of indirect antioxidant to attain the cytoprotection effect against t-BHP.

To achieve the best cytoprotection by antioxidant, we relied on specific conditions from previous cytoprotection experiments including nontoxic concentration of antioxidant, cytoprotection pattern, and serum concentration.

(a) Direct cytoprotection

The best cytoprotection by Q against t-BHP effect was displayed by using direct cytoprotection pattern where HepG2 cells were incubated with Q (100 µg/ml) and 0.8 mM of t-BHP simultaneously for 5 hours using medium containing 2% serum (Fig 3.4a) in chapter 3.

Therefore, incubation of treated HepG2 cells with Q (100 µg/ml) and 0.4 and 0.8 mM t-BHP simultaneously for up to 150 minutes using medium containing 2% serum was studied. Under these conditions, cell viability was unaffected throughout the incubation with Q which means that Q was not toxic to the cells at that selected concentration and cell viability was retained 100%. Moreover, Q protected cells from the toxic effect of t-BHP and no decline in viability of cells was recorded at any time of incubation (Fig 4.13). Furthermore, caspase-3 for all the treated samples at 60, 90 and 120 min has been shown at 37 KDa with no evidence for caspase-3 cleavage at 17 KDa being identified at any time during the incubation (fig.4.14 and 4.15).

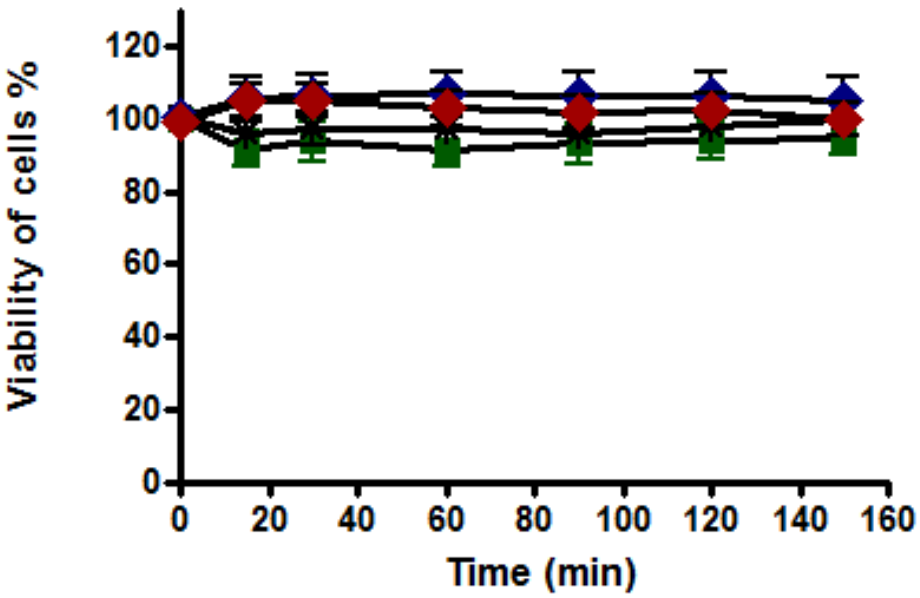

Figure 4.13 Viability of HepG2 cells incubated in the absence ( ) and presence of Q (*) and (Q +0.4) ( ) or (Q + 0.8) mM of t-BHP ( ) simultaneously for up to 150 minutes in medium containing 2% serum. Values are Mean±SEM of 3 independent experiments.

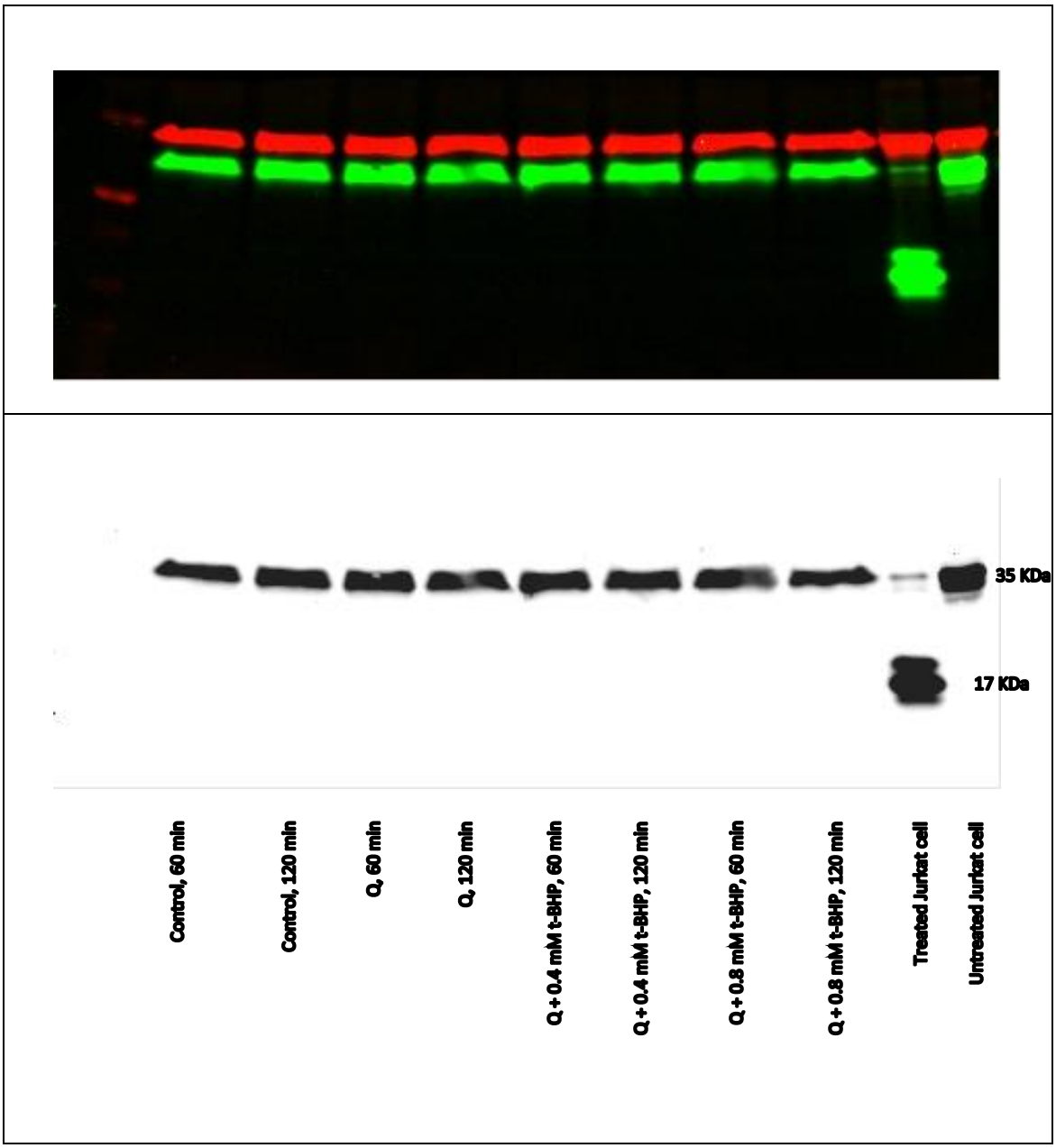

Figure 4.14 Western blot of caspase-3 in HepG2 cells. Blot showed the bands of procaspase-3 at 35 KDa for untreated and treated Jurkat cells and untreated and treated HepG2 cells with Q, and (Q+0.4 and Q+ 0.8) mM t-BHP for 60, 120 minutes using medium 2% serum. This blot was also showing caspase-3 activation of treated Jurkat cells and its cleavage at 17 KDa.

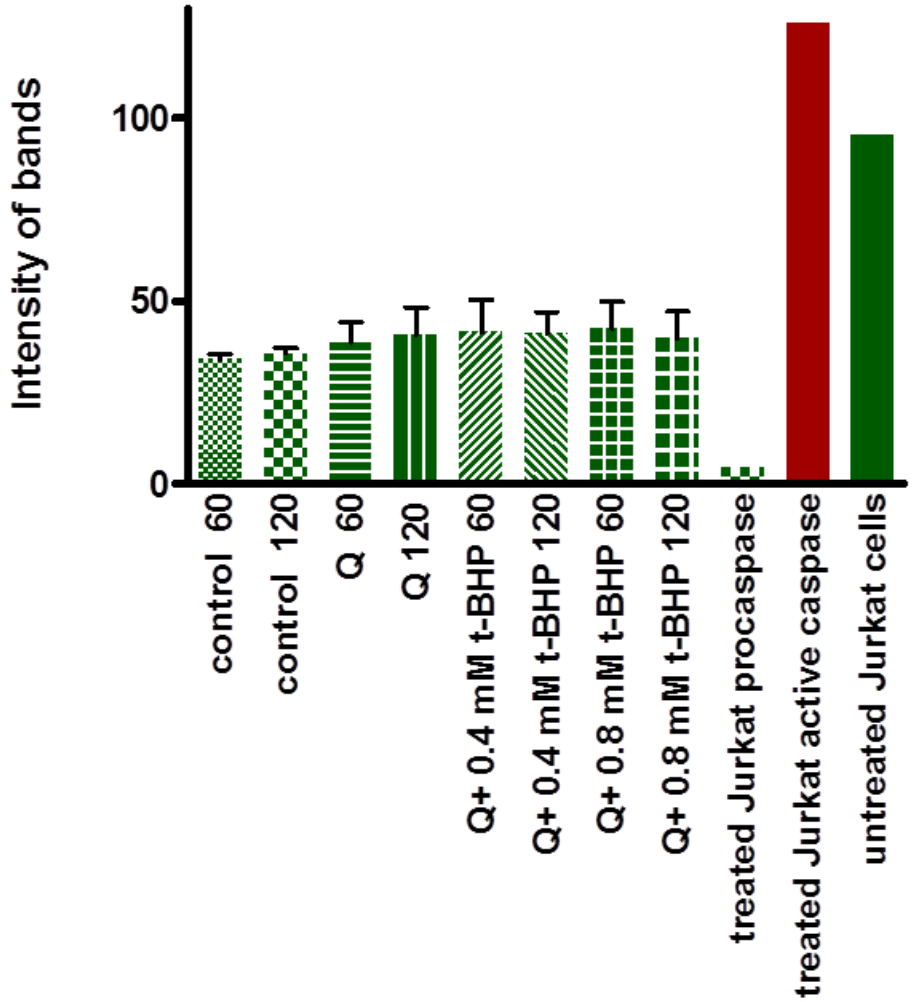

Figure 4.15 Quantitative analysis of active caspase-3 of treated Jurkat cells and the procaspase-3 of untreated and treated Jurkat cells and untreated and treated HepG2 cells with Q , (Q+ 0.4) and) Q+0.8) mM t-BHP for 60, and 120 minutes using medium 2% serum. Values are Mean±SEM of 3 independent experiments.

(b) Indirect cytoprotection

The best cytoprotection by I3C against the toxicity effect of t-BHP was shown by using indirect cytoprotection pattern where HepG2 cells were incubated with I3C (25µg/ml) for 20 hours in medium containing 10% serum prior exposed to 0.8 mM t-BHP for 5 hours using medium containing 2% serum (Fig 3.2c) in chapter 3.

Incubation of treated HepG2 cell with I3C (25 µg/ml) using 10% serum for 20 hours prior exposure to 0.4 and 0.8 mM t-BHP for up to 150 minutes displayed good results. Under these conditions, results of cell viability indicated that I3C was not toxic to the cells at that selected concentration and cells remained alive by 100% during all the time. Moreover, it was capable of protecting cells from the toxic effect of t-BHP till 90 min and recorded 100% cell viability but after that time a decline in viability was recorded at 120 and 150 min which was decreased to 75 and 50 % respectively (Fig 4.16). Moreover, caspase-3 for all the treated samples at 60, 90 and 120 min was visible at 37 KDa whereas the cleaved caspase-3 was not apparent at 17 KDa min comparing with the positive control (Fig.4.17 and 4.18).

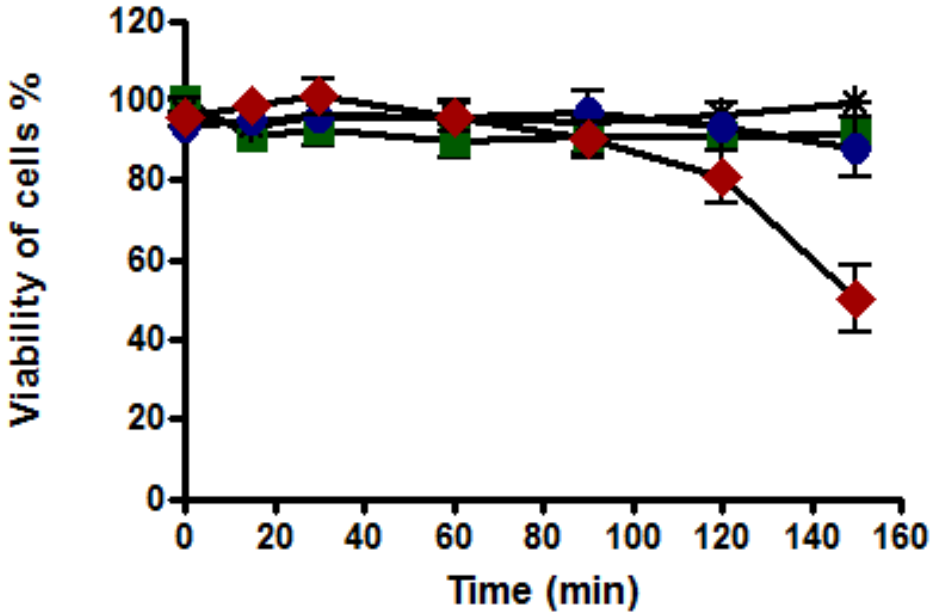

Figure 4.16 Viability of untreated HepG2 cells ( ) and incubated cells with I3C for 20 hours (*) using medium containing 10% serum prior exposure to 0.4 mM ( ) or 0.8 mM ( ) t-BHP for up to 150 mintes in medium containing 2% serum. Values are Mean±SEM of 3 independent experiments.

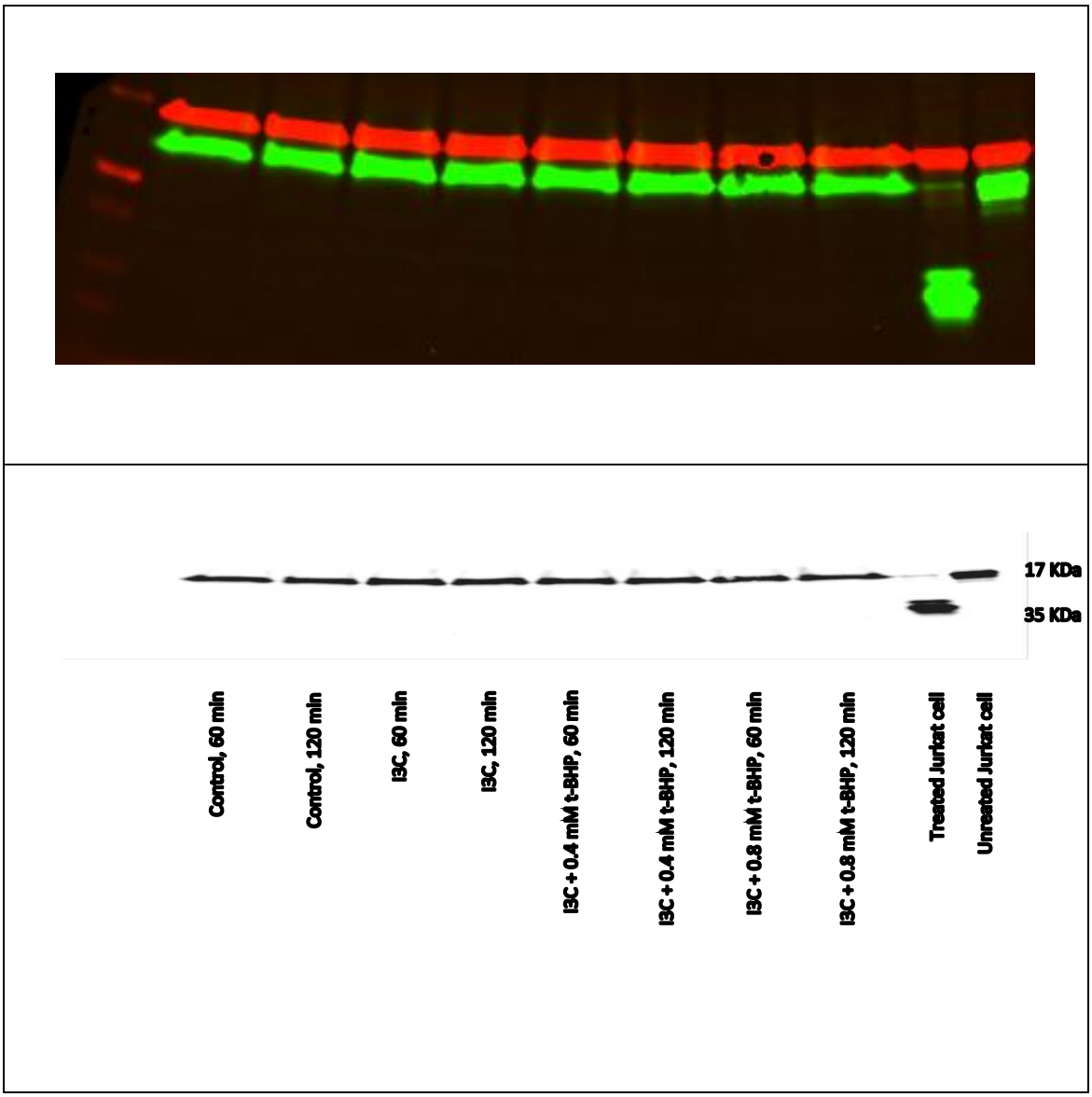

Figure 4.17 Western blot of caspase-3 in HepG2 cells. Blot showed the bands of procaspase-3 at 35 KDa for untreated and treated Jurkat cells and untreated and treated HepG2 cells with I3C for 20 hours using medium containing 10% serum before exposure to medium containing 2% serum for 60 and 120 minutes, and with I3C for 20 hours using medium containing 10% serum before exposure to 0.4 and 0.8 mM t-BHP for 60, 120 minutes using medium 2% serum. This blot was also showing caspase-3 activation of treated Jurkat cells and its cleavage at 17 KDa.

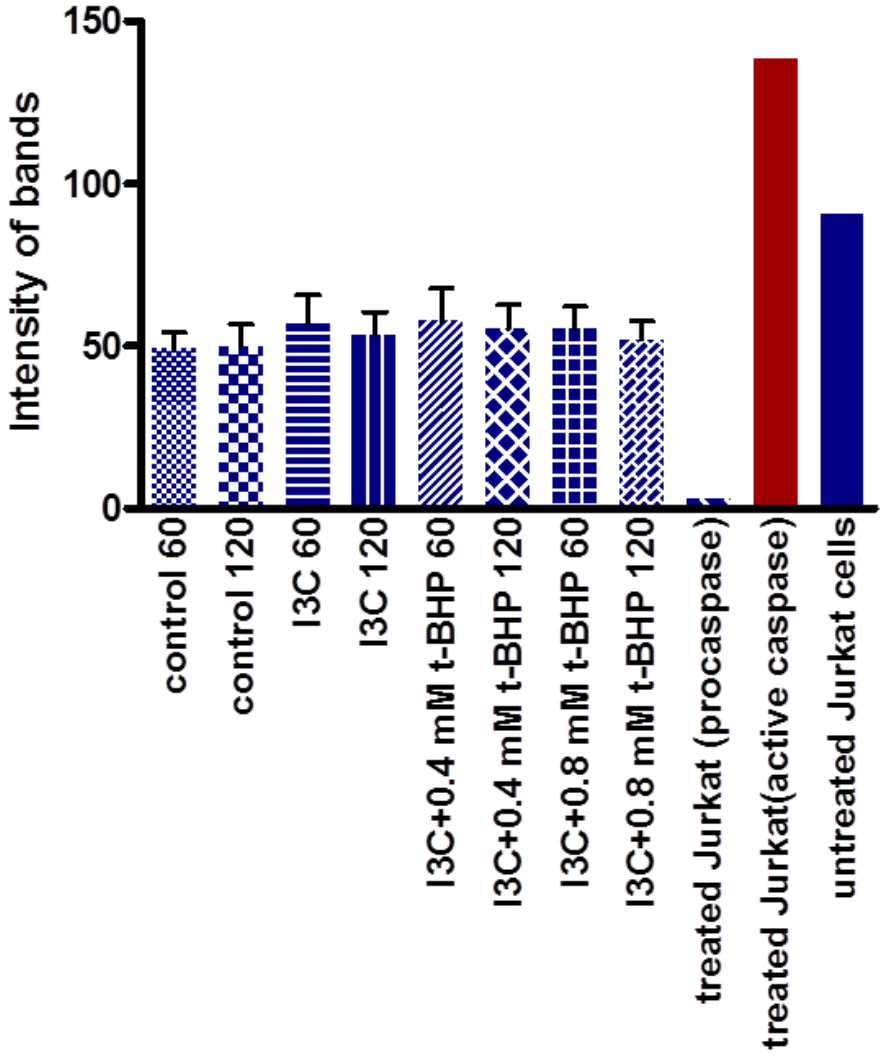

Figure 4.18 Quantitative analysis of active caspase-3 of treated Jurkat cells and the procaspase-3 of untreated and treated Jurkat cells and untreated and treated HepG2 cells with I3C for 20 hours using medium containing 10% serum before exposure to medium containing 2% serum for 60 and 120 minutes, and with I3C for 20 hours using medium containing 10% serum before exposure to 0.4 and 0.8 mM of t-BHP for 60, 120 minutes using medium 2% serum. Values are Mean±SEM of 3 independent experiments.

4.2.3 Reactive Oxygen Species (ROS)

Results showed that cell viability of untreated HepG2 cells were not affected during all incubation time and viability of cells was 100% during the incubation while viability of the treated cells with 0.4 and 0.8 mM t-BHP decreased from 80% to about 60% and from % 75 to 25% after 2.5 hour of incubation with 0.4 and 0.8 mM t-BHP respectively (Fig 4.19a).

ROS production increased gradually with incubation time of HepG2 cells with or without t-BHP, using medium 2% serum. HepG2 produced ROS during incubation with 0.4 and 0.8 mM t-BHP for up to 150 minutes (Fig 4.19b). No significant variation could be detected between untreated HepG2 and HepG2 cells treated with 0.4 mM of t-BHP.

When ROS production by HepG2 cells was adjusted for viability of HepG2 cells incubated with 0.4 and 0.8 mM t-BHP for up to 150 minutes, results showed that no differences between untreated and treated HepG2 cells with 0.4 and 0.8 mM of t-BHP during incubations for up to 120 minutes but statistical differences was observed between untreated and treated cells with 0.8 mM t-BHP at 150 minutes (Fig 4.19c). statistical analysis results from Friedman test followed by Dunn's Multiple Comparison Test showed significance between control and treated cell with 0.8 mM t-BHP at 150 minutes and P value equals 0.0278 ($p \leq 0.05$).

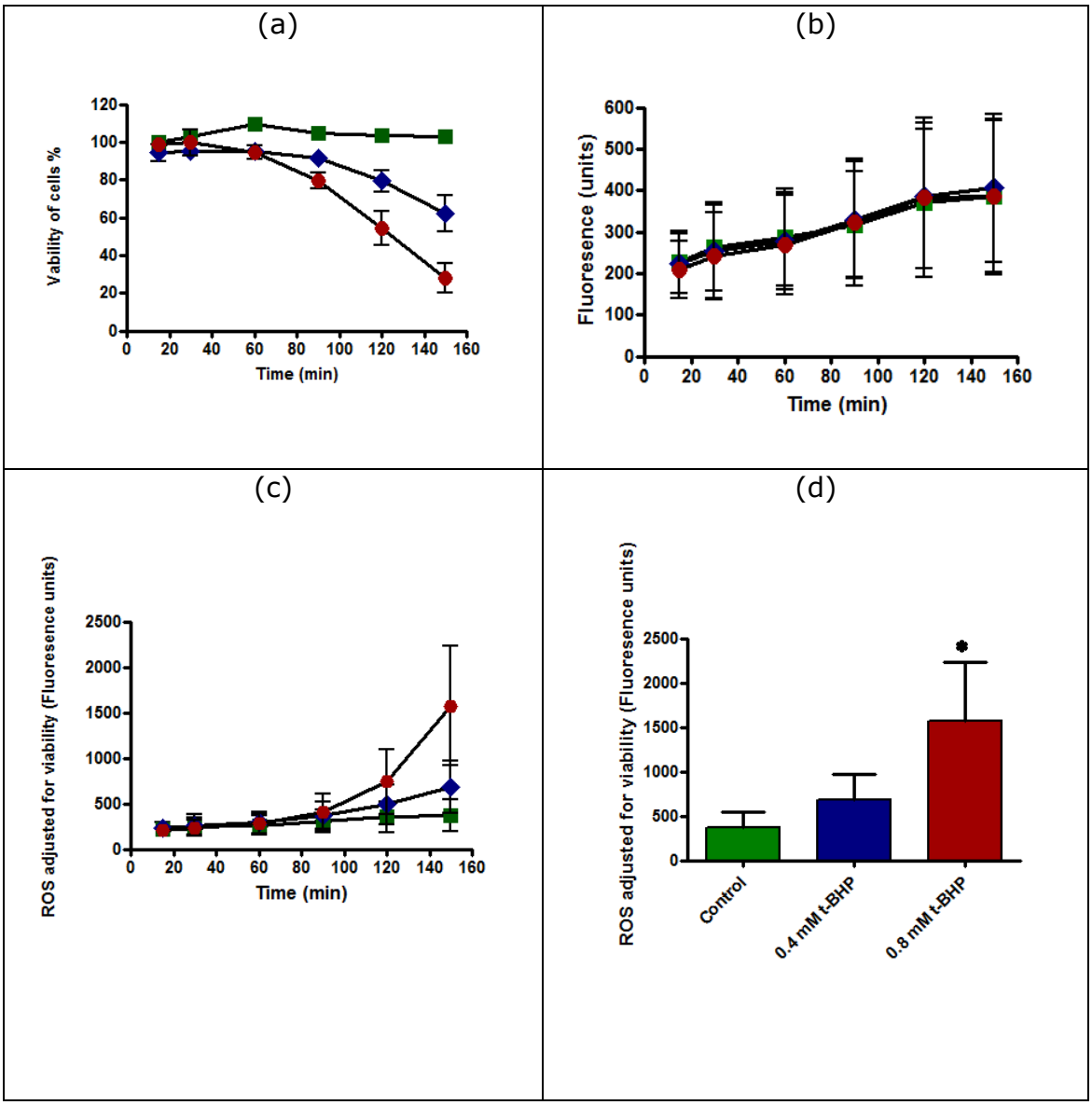

Figure 4.19 ROS production by HepG2 cells (a) Viability of untreated ( ) and treated HepG2 cells with 0.4 ( ) and 0.8 mM t-BHP ( ) for different periods of time (b) ROS production by untreated ( ) and treated HepG2 cells with 0.4 ( ) and 0.8 mM t-BHP ( ) for different periods of time (15, 30, 60, 90, 120, and 150 minutes) (C) ROS production by untreated ( ) and treated HepG2 cells with 0.4 ( ) and 0.8 mM t-BHP ( ) and adjusted for viability. (d) ROS production by untreated (green bar) and treated HepG2 cells with 0.4 (blue bar) and 0.8 (red bar) mM t-BHP at 150 minutes. *Significant value could be obtained where P≤ 0.05 and equal 0.0278 according Friedman test and Dunn's Multiple Comparison Test. Values are Mean±SEM of 3 independent experiments.

4.2.4 Glutathione (GSH)

Results of standard curve for GSH demonstrated linear response (Fig 4.20). Viability of untreated HepG2 cells remained unaffected during incubation for different periods of time (0, 30, 60, 90, 120, and 150 minutes) whereas number of dead cells treated with 0.4 and 0.8 mM of t-BHP was increased when incubation time increased and viability of cells was declined after 2.5 hours of incubation from 80% to about 60% and to 25% for cells treated with 0.4 and 0.8 mM t-BHP respectively (Fig 4.21a).

Glutathione (GSH) content for untreated HepG2 cells remained approximately constant when cells incubated for up to 150 minutes in the absence of t-BHP while the amount of GSH for treated HepG2 cells with 0.4 and 0.8 mM of t-BHP was decreased after 2.5 hours of incubation from 70 fluorescence units to reach about 25-30 fluorescence units for both treatments (Fig 4.21b).

When GSH production by HepG2 cells was adjusted for viability of HepG2 cells that incubated with 0.4 and 0.8 mM of t-BHP for 15, 30, 60, 90, 120, and 150 minutes, results showed that no differences between untreated and treated HepG2 cells with 0.4 and 0.8 mM t-BHP during incubations for up to 150 minutes (Fig 4.21c). Results from Friedman test and Dunn's Multiple Comparison Test showed that no significance at 150 minutes where P value equals 0.0608 ($p \geq 0.05$).

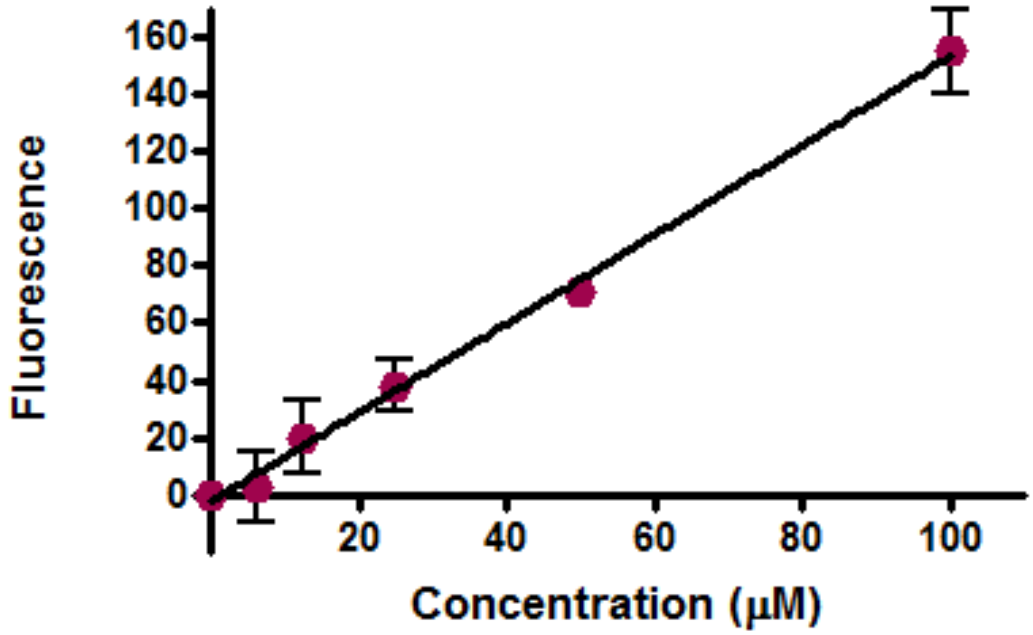

Figure 4.20 Standard curve of GSH.

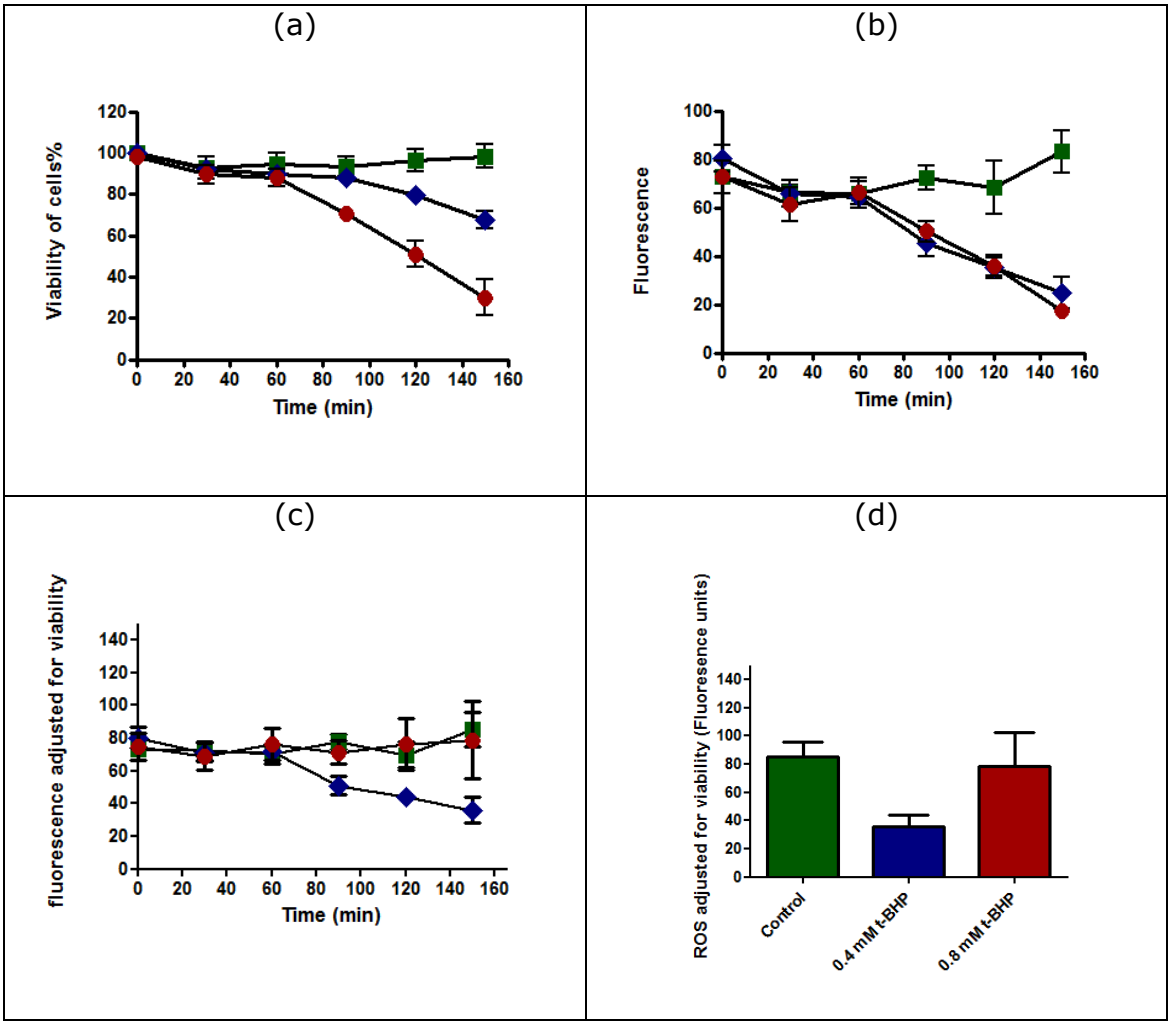

Figure 4.21 GSH production by HepG2 cells (a) Viability of untreated ( ) and treated HepG2 cells with 0.4 ( ) and 0.8 mM of t-BHP ( ) for different periods of time (15, 30, 60, 90, 120, and 150 minutes) (b) GSH production by untreated ( ) and treated HepG2 cells with 0.4 ( ) and 0.8 mM t-BHP ( ) for up to 150 minutes (C) GSH production by untreated ( ) and treated HepG2 cells with 0.4 ( ) and 0.8 mM t-BHP ( ) and adjusted for viability (d) GSH production by untreated (green bar) and treated HepG2 cells with 0.4 (blue bar) and 0.8 (red bar) mM t-BHP at 150 minutes where no significance results could be obtained according to Friedman test where p equals 0.0608 ($p \geq 0.05$) and Dunn's Multiple Comparison Test. Values are Mean±SEM of 3 independent experiments.

4.2.5 Lipid peroxidation (LPO)

Results of viability of untreated and treated HepG2 cells with 0.4 and 0.8 mM of t-BHP for different periods of time 15, 30, 60, 90, 120, and150 minutes showed that the untreated cells were alive during all the incubation time whereas viability of both treated cells with 0.4 and 0.8 mM of t-BHP were declined after 2.5 hours of incubation from 80% to about 60% and 30% for 0.4 and 0.8 mM of t-BHP respectively (Fig.4.22a).

Lipid peroxidation (LPO) increased gradually for untreated HepG2 cells and HepG2 cells treated with 0.4 and 0.8 mM t-BHP for up to 150 minutes. LPO produced by untreated cells increased from 150 fluorescence units to about 180 fluorescence units while LPO that produced by HepG2 cells treated with 0.4 mM of t-BHP was increased from 200 fluorescence units to about 220 fluorescence units at 120 and declined to 210 fluorescence units again. Similarly with 0.8 mM of t-BHP treated cells, produced LPO which was increased from 220 fluorescence units to about 260 fluorescence units at 120 minutes and declined again to about 250 fluorescence units at 150 minutes and (Fig. 4.22b).

When LPO production was adjusted for viability it displayed different results. Results showed that no differences between all untreated and treated cells during incubation time from 15 to 90 minutes and a very little variation could be noticed between them at 120 minutes of incubation but a big difference and significant variation was observed at 150 minutes of incubation between untreated and treated cells with 0.8 mM t-BHP where p equals 0.0046 ($p \leq 0.05$) (Fig 4.22c and d).

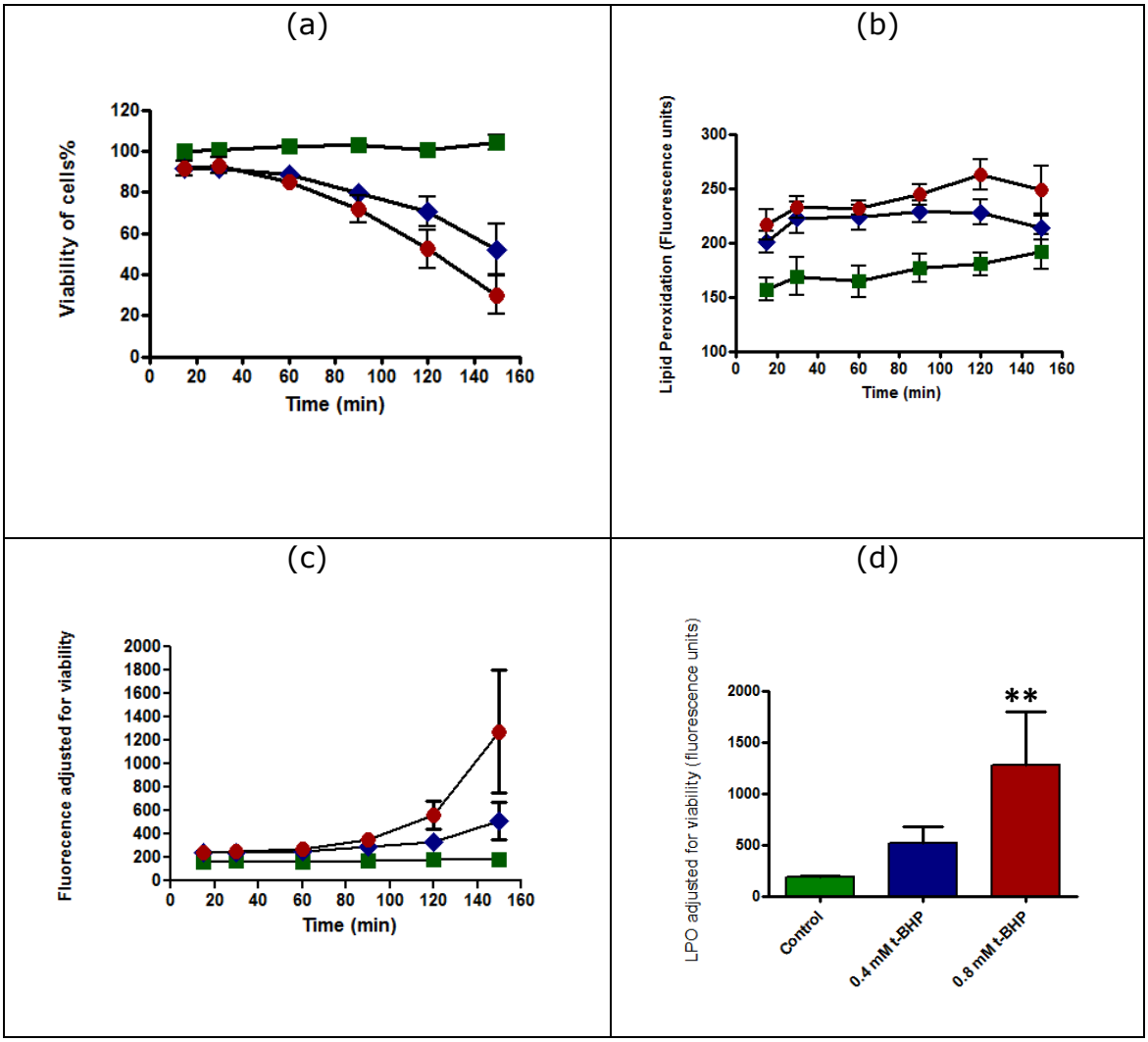

Figure 4.22 Production of LPO by HepG2 cells (a) Production of LPO by untreated ( )and treated HepG2 cells with 0.4 ( ) and 0.8 ( ) mM t-BHP for different periods of time (15, 30, 60, 90, 120, and 150 minutes) (b) viability of untreated ( )and treated HepG2 cells with 0.4 ( ) and 0.8 ( ) mM t-BHP for up to 150 minutes (c) LPO production by untreated ( )and treated HepG2 cells with 0.4 ( ) and 0.8 ( ) mM t-BHP and adjusted for viability (d) LPO production by untreated (green bar) and treated HepG2 cells with 0.4 (blue bar) and 0.8 (red bar) mM t-BHP at 150 minutes. **Indicates control significantly different to treated HepG2 with o.8 mM t-BHP at 150 minutes where p=0.0046 according to Friedman test and Dunn's Multiple Comparison Test. Values are Mean±SEM of 3 independent experiments.

4.3 Discussion

4.3.1. Toxicity effect of t-BHP

The choice of t-BHP was based on the fact that it is one of the most common pro-oxidant agents used in cell lines such as HepG2 cells as a model to study the effect of oxidative stress in cell systems and cell death pathways (Alia et al. 2006b; Sardao et al. 2007). It can decompose to free radicals, (Flora *et al.* 1998) increase of which might be a primary factors for hepatotoxicity (Sohn *et al.* 2007).

In the present study, t-BHP elicited cell death at concentrations of t-BHP greater than 0.2 mM, with distinctly different time course of toxicity and effectiveness of phytochemicals at concentrations of 0.4 and 0.8 mM t-BHP, even though the overall cytotoxic effect of the two concentrations of t-BHP was similar after 5 hours exposure. These findings led us to explore the mechanism (s) of toxicity of t-BHP at these two concentrations, preliminary ideas postulating that 0.4 mM might elicit more of an apoptosis mechanism of damage inspired to a more necrotic mechanism at 0.8 mM, as similar concentrations-dependent shifts in mechanism of toxicity have been proposed for other chemicals (Piret *et al*. 2004; Sardao *et al.* 2007).

4.3.1.1 Effect of phytochemicals

Under same conditions, Q and EGCG significantly protected HepG2 cells against oxidative damage at any concentration of t-BHP and this may be attributed to the up-regulation of proteins by those antioxidants which increased protections and

prevented damages. Although I3C and SFN prevented cells damages at concentrations lower than 0.2 mM t-BHP through up regulation of proteins, the full protective effect of I3C and also SFN could not be recognized at concentrations higher than 0.2 mM t-BHP where the treated cells with I3C were partially protected at 0.4 mM t-BHP and avoidable high cell damage were visualized at 0.8 mM t-BHP. The reason for this can be supportive by what found previously where the up-regulated proteins of treated HepG2 cells with I3C and SFN are considerably overwhelmed by free radicals of high concentrations of t-BHP, 0.8 mM t-BHP in particular. Moreover, the toxicity concentrations of I3C and SFN that were used may significantly interact with t-BHP in cell death in the absence of serum. The omission of albumin represents the absence of its antioxidant properties (Kanno *et al.* 2011) which probably increased oxidative damages induced by t-BHP, high concentration of I3C and SFN.

4.3.1.2 Time dependence and effect of serum

With regards to the time of incubation with t-BHP, oxidative cell damage caused by 0.8 mM t-BHP were higher than 0.4 mM at the extended time of incubation either in the presence or absence of serum. The generated amounts of free radicals by t-BHP are increased as the concentration of the t-BHP increased such that the amounts of free radicals produced by 0.8 mM are higher than the amounts in 0.4 mM. Therefore, cells were considerably damaged when they were exposed to 0.8 mM t-BHP comparing with 0.4 mM and these damages were significantly proportional with the time of incubation. Moreover, the effect of serum to reduce the number of dead cells either in 0.4 or 0.8 mM with the time was obvious where it decrease cell death according to its antioxidant protective effects (Kanno *et al.* 2011).

4.3.2 Detection of caspase-3 activation

When cells undergo apoptosis, proteases (caspases) are cleaved (Nagata 2000). Participation of this caspase family, in particular caspase-3, in signalling and apoptosis represents the crucial importance of these enzymes in this form of cell death (Thornberry 1999). This study analysed caspase-3 cleavage by western blotting and detected its activity to ascertain if t-BHP initiated apoptosis in HepG2 cells.

Approximately 40 to 70% cell death occurred after 150 minutes of incubation with 0.4 and 0.8 mM t-BHP respectively (Fig 4.1). 60, 90 and 120 minutes of incubation time were selected to analyse caspase-3 and determine apoptosis of untreated and treated HepG2 cells with t-BHP. The reason for this was because apoptosis and activation of caspase-3 is expected during the early stages of the cell death cascade. Caspase-3 are activated and apoptosis could be confirmed in treated HepG2 cells with glucose and in treated Jurkat cells but not in HepG2 cells treated with t-BHP for selected times. This may be because that HepG2 cells were exposed to high concentrations of glucose for long times which allowed activating caspases-3 and consequently apoptosis was induced. We cannot rely on the same explanation for treated HepG2 cells with t-BHP because we have cell death without apoptosis where bands of activated caspase-3 were not markedly elevated or remained low as compared with positive and/or negative control. Moreover, in cell-western and immunocytochemistry results we could not find strong visual evidence in caspase-3 activation either in untreated or treated HepG2 cells with t-BHP for 60 and 120 minutes. It is possible that calpains may play a role in down-regulation of caspase-3 activation during cell death pathway due to chronic mitochondrial defects (Bizat *et al.* 2003b).

The intervention studies have suggested inactivation of caspase-3 by calpain. Although predominance of calpain over caspase-3 in spite of a transient activation of caspase-9 has remained speculative assumptions (Bizat *et al.* 2003a), a cross-talk between caspases and calpains has been discovered (Blomgren *et al.* 2001; Chua *et al*. 2000; Lankiewicz *et al.* 2000; Wolf *et al.* 1999). The mechanism of this cross-talk includes abnormal activation of calpain due to increase in the intracellular Ca^{+2}, the caspase-9/-3 apoptotic pathway then may be partially blocked by calpain which leads to the cell death as well as inactivation of caspase-3 (Bizat *et al.* 2003b).

4.3.2.1 Caspase activity

Although little evidences of caspase-3 activation with western blotting, in-cell western, and immunocytochemistry were obtained, caspase-3 activity increased significantly in treated HepG2 cells with 0.8 mM at 150 minutes which may suggest that there was activation for caspase-3 and apoptosis induction. The reason for the little bands in western blotting may due to the role of calpain which may prevent apoptosis due to direct or partial blocked for caspase-3 activation (Bizat *et al.* 2003b).

4.3.2.2 Effect of Q and I3C on caspase-3 activation

Cytoprotection results of Q (Fig 3.4a) and I3C (Fig 3.2c) against toxicity effect of t-BHP encouraged us to test them in western blotting and confirmed their potency in preventing the slight partial activation of caspase-3 induced by t-BHP. We chose the optimum cytoprotection conditions of Q and I3C from concentration,

cytoprotection pattern, and effect of serum according to previous results that showed their antioxidant activity in order to achieve high protection.

Q directly contributed in defence of HepG2 cells against t-BHP where cells retained 100% viable during all the time. The protection was provided by hydroxyl groups that belong to Q through attacking free radicals that produced by t-BHP and inactivated them. Western blot results of Q were identical to viability where no evidence for presence of bands of cleaved caspase-3 in untreated cell with and without Q and t-BHP could be formed. This confirmed that concentration of Q was not toxic to the cell at those selected time due to no evidence for caspase-3 activation and it was capable to prevent the partial activation of caspase-3 which was slightly seen previously in treated HepG2 cells with 0.4 mM t-BHP at 60, 90, and 120 minutes and 0.8 mM t-BHP at 60 minutes.

We chose 25 µg/ml of I3C to study the cytoprotection effect of this compound in HepG2 cells treated with t-BHP because it was not toxic to the cell during incubation. Moreover, since I3C displayed indirect antioxidant activity when it was incubated with cells for 20 hours in medium 2% serum, HepG2 cells were incubated with I3C and t-BHP using these conditions.

I3C was capable of providing full cytoprotection against 0.4 mM t-BHP during 150 minutes of incubation and cells were remained alive 100%. The reason for this changing in cytoprotection behaviour against 0.4 mM t-BHP this time because low concentration of I3C was being used which in turn prevented cell death by I3C. Western blot results were identical with viability where no evidence for activated caspase-3 bands which means that I3C was not toxic at that concentration and it even protected cells from 0.4 mM t-BHP probably by up-regulating proteins, prevented caspases activation and stopped apoptosis induction. On the other hand and although I3C was partially protected treated HepG2 cells with 0.8 mM t-BHP at 120 and 150 minutes there was no evidence for apoptosis in western blot accompanying cell death. The reason for this, the low of concentration of I3C was

unable to protect cells against 0.8 mM t-BHP for long time which caused low activation of caspase-3. This imperceptible activation did not display as bands in blot may be because the role of calpain which blocked even that slightly activation and stopped apoptosis.

4.3.3 Reactive oxygen species

Although ROS generated in small amounts in untreated and treated HepG2 cells with 0.4 and 0.8 mM t-BHP for up to 120 minutes, increased the apparent cell death was observed at 150 minutes in HepG2 cells treated with 0.8 mM t-BHP where ROS amounts increased significantly. ROS initiate in early stages of apoptosis and before the completely death of cells they are just signals, and, secondly, when there are impaired mitochondrial oxygen reduction this causes alterations in mitochondrial permeability leading to production of ROS. Finally, the produced ROS induce the depolarization of the mitochondrial membrane and lead to further mitochondrial dysfunction (Oh and Lim 2006; Segovia and Berges 2009). Moreover and because the complete cell death occurred after 2.5 hours, ROS may be generated after 90 minutes of incubation which then impaired and consequently being low or observed later than the exact time, this impairing may be attributed to NAC, the scavenger of ROS which may abolish the increased amounts of ROS (l'Hoste *et al.* 2010).

4.3.4 Glutathione (GSH)

GSH plays a fundamental role in protecting cells from oxidative stress by scavenging ROS (Reed 1990) and therefore, GSH decreases when ROS increases (l'Hoste *et al.* 2010). Our findings indicated no significant decrease in GSH content, this imperceptible decreased in GSH was accompanying with increased cell death which may be explained by either no ROS generated or the amount of ROS are not much to cause depletion of GSH content. Our previous finding represented that t-BHP induced apoptosis because caspase-3 activity and ROS were increased significantly in treated HepG2 cells with 0.8 mM t-BHP at 150 minutes. Moreover and relying on our suggestion about ROS, the remaining of ROS after NAC involvement were not too much to consume large amount of GSH which consequently led to decrease GSH slightly.

4.3.5 Lipid peroxidation

Great attention has been paid to free radicals and peroxidation of lipids-containing lipoprotein system of cells due to their implication in oxidative stress and damaging cell components (Wallin *et al.* 1993).

Increased lipid peroxidation (LPO) level and cell death in treated HepG2 cells with 0.4 and 0.8 mM t-BHP comparing with control indicated that t-BHP can provoke cell death in HepG2 cells due to lipid peroxidation. According to this beside statistical

results of LPO, ROS and caspase-3 activity we can suggest that apoptosis can be induced in HepG2 cells due to lipid peroxidation initiation.

4.4 Conclusion

In conclusion, apoptosis can be induced in treated HepG2 cells treated with t-BHP but the exact time for induction could not be determined successfully because much of confusing factors may contribute in apoptosis signalling disorder such as calpain in caspase-3 activation and NAC in ROS which led to induce apoptosis late.

Phytochemicals completely or partially reduced the cytotoxicity effect of t-BHP and may be involved in cytoprotection for future studies.

Chapter 5

Evaluation of direct cellular assay for NQO1

Chapter 5

Evaluation of a direct cellular assay for NQO1

5 Introduction

Experimental studies in cell culture and animal models have pointed that cancer may be prevented by the protective effects of natural agents in food (Shokouhi et al. 2008). Recently, evidences of the protective effects of phytochemicals such as SFN have increased due to awareness of induction of phase ll detoxifying enzymes, NQO1 in particular (Tarozzi *et al.* 2009). On the other hand, it has been found that diet enriched with catechins may affect the ability of the organism to detoxify free radicals and environmental xenobiotics through a significant decrease in NQO1 activity due to an unclear reason. Therefore further studies are needed to established cell-based assays for NQO1 as part of screening programmes for modulation of NQO1 activity (Wiegand *et al*. 2009).

A recent cell-based assay for NQO1 was described by Bongard *et al.* (2011), based on modulation of duroquinone. NQO1 is a phase ll enzyme that catalyses the linked intracellular conversion of $NADPH_2$ to NADP and duroquinone (DQ) (Fig 5.2) to hydroduroquinone (DQH_2) in cell. The DQH_2 product leaves the cell and is converted to DQ, during which ferricyanide [$Fe(CN6)^{-3}$] is reduced to ferrocyanide [$Fe(CN6)^{-4}$]. This enzymatic reaction can be prevented by dicumoral, the inhibitor of NQO1 (Fig 5.1).

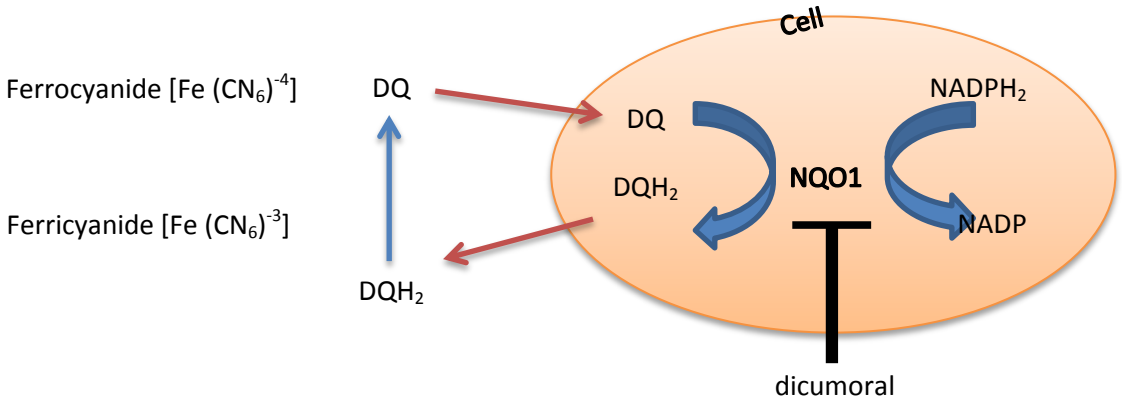

Figure 5.1 reduction of Ferricyanide to Ferrocyanide according to NQO1 activity in the cell.

Different methods have been used to determine NQO1 activity. The classic NQO1 enzyme assay is the usual method for measuring NQO1 activity in cell lysates whereas the intact-cell activity has largely been inferred from functional results such as antioxidant activity or toxicity of NQO1-catalyzed activation of the compound under study (Lee *et al.* 2005). Therefore, we chose to determine the intact-cell activity in order to investigate the effect of Q, EGCG, I3C, and SFN in stimulating NQO1 activity. In brief, DQ-mediated reduction of the cell-membrane-impermeative secondary electron acceptor, ferricyanide, was used to quantify intact-cell NQO1 activity. This approach involves adding quinone duroquinone to the cells and then measuring the appearance rate of the two-electron reduction product, durohydroquinone by its reduction of ferricyanide (Bongard *et al.* 2011).

5.1 Materials and methods

5.1.2 Chemicals

All chemicals were purchased from Sigma Chemical Company, Pool, and Dorset, U.K.

5.1.3 Treatment of cells

HepG2 cells were cultured in 24-well plates for 24 hours and then incubated with phytochemicals (100 µg/ml of Q, 100 µg/ml of EGCG, 25 µg/ml of I3C, and 1 µg/ml of SFN) for 20 hours in complete culture medium prior to incubation with 1 ml HBSS/Hepes solution per well by itself or containing 600 µM ferricyanide or 600 µM ferricyanide + 50 µM DQ, these concentraions being those employed in the original paper by Bongard *et al.* (2011). HBSS/Hepes solution contained Hanks' Balanced Salt Solution with 10 mM HEPES buffer pH 7.4 and 5.5 mM glucose. Incubation at 37ºC was for up to 60 minutes. Culture wells were washed extensively three times with PBS after incubation with phytochemicals in order to prevent the interference between these compounds and ferricyanide. In a related experiment, HepG2 cells were incubated as described above with no prior treatment with phytochemicals. The absorbance of ferricyanide in the medium was measured spectrophotometrically at 412 nm using spectrophotometer: Multiskan Ascent manufactured by Thermo Electron Corporation.

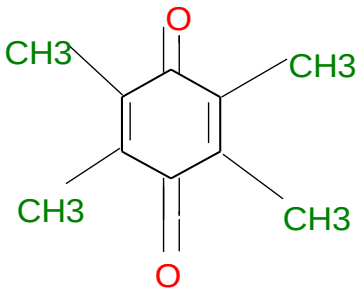

Figure 5.2 duroquinone = (2, 3, 5, 6-Teramethyl-1,4-benzoquinone, Tetramethyl-*p*-benzoquinone $C_{10}H_{12}O_2$)

5.2 Results

Results demonstrated that ferricyanide was not reduced on incubation with HepG2 cells alone (Fig 5.3, green square) but was efficiently reduced in the presence of duroquinone (Fig. 5.3, red circle), absorbance falling from about 0.14 to about 0.02 after incubation for one hour ((Fig. 5.3).

In, cells pre-treated with Q for 20 hours, ferricyanide was reduced to ferrocyanide in the absence of duroquinone (Fig. 5.3a), the extent of which was greater in the presence of duroquinone (Fig. 5.3a).

Similar results were obtained with cells pre-incubated with EGCG, although the rate of ferricyanide reduction in the absence of duroquinone was rather less than observed in cell pre-treated with Q (Fig 5.3b).

On the other hand, no direct (i.e in the absence of duroquinone) reduction of ferricyanide was observed in cells pre-treated with either I3C or SFN (Fig. 5.3c and d) respectively, and the reduction of ferricyanide in the presence of duroquinone in cells pre-treated with I3C or SFN was no greater than that in cells not treated with these compounds.

Taken together, these results suggested that the rate of duroquinone-mediated reduction of ferricyanide in HepG2 cells was not enhanced after treatment with any one of four phytochemicals known to induce NQO1 activity. The results also indicated that the two direct antioxidants of the four phytochemicals tested (i.e. Q and EGCG) interfered in the assay in that they mediated ferricyanide reduction in the absence of duroquinone.

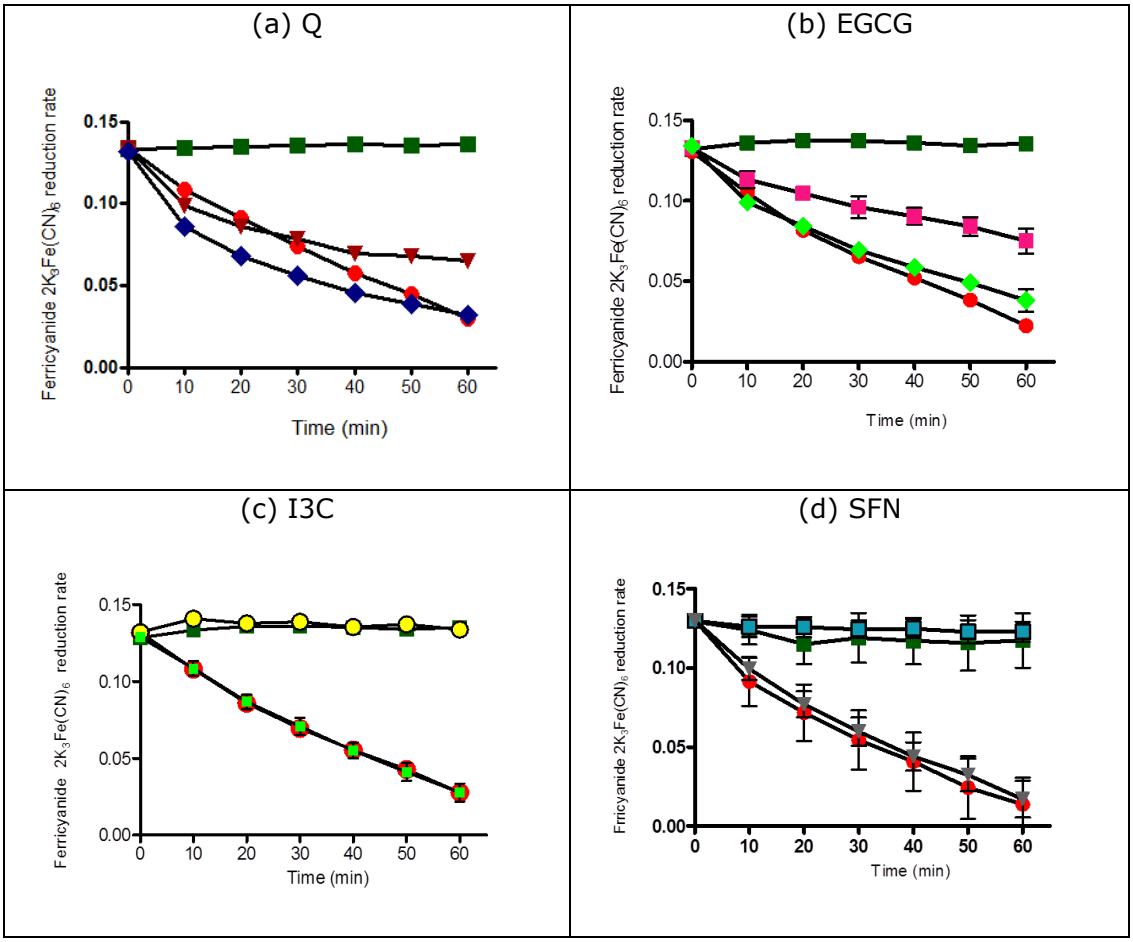

Figure 5.3 NQO1 activity expressed by Ferricyanide $2K_3Fe(CN)_6$ reduction and achieved by incubation of HepG2 cells with Fe+3 (600 µM) ( ), Fe+3 (600 µM) +DQ (50 µM) ( ) in (a), (b), (c), (d), and with:

(a) Fe^{+3} (600 µM) +Q (100 µg/ml) ( ), and Fe^{+3} (600 µM) + DQ (50 µM) + Q (100µg/ml) ( ).

(b) Fe^{+3} (600 µM) +EGCG (100 µg/ml) ( ), and Fe^{+3} (600 µM) + DQ (50 µM) +EGCG (100µg/ml) ( )

(c) Fe^{+3} (600 µM) +I3C (25 µg/ml) ( ), and Fe^{+3} (600 µM) + DQ (50 µM) +I3C (25 µg/ml) ( ).

(d) Fe^{+3} (600 µM) +SFN (1 µg/ml) ( ), and Fe^{+3} (600 µM) + DQ (50 µM) +SFN (1 µg/ml) ( ).

Values are Mean±SEM of 3 independent experiments.

5.3 Discussion

In the present study, we aimed to use the four compounds as antioxidants against oxidative stress for further studies into stimulation of NQO1 enzyme mechanism, as measured by the DQ-mediated reduction of ferricyanide as described by Bongard *et al*, 2011.

It was very important to determine that Fe^{+3} was reduced according to the NQO1 activity with no external interference with the enzymatic reaction, therefore we incubated HepG2 cells with ferricyanide alone (i.e in the absence of phytochemicals).

Neither I3C nor SFN had any effect on Fe^{+3} reduction which means that any reduction to Fe^{+2} may be attributed to the activity of NQO1 which catalyses the conversion of duroquinone to hydroduroquinone, with linked conversion of ferricyanide reduced to ferrocyanide. On the other hand, ferric ion was reduced to ferrous ion in both cells treated with Q and EGCG, thus the reduction reaction may due to the effect of these two compounds and not to NQO1 activity.

The possible mechanism for Q and EGCG action can be suggested as being linked to their direct antioxidant activity; accordingly, ferric ion can be reduced to ferrous ion by Q and EGCG (not duroquinone) through accepting electron from them and by thus Q and EGCG interfered with enzymatic reaction. Additionally, Q and EGCG possess quinone structure which is missing in SFN as well as in I3C and this similarity in structure with duroquinone allowed them to be reduced by NQO1 due to two-electron transfer instead of duroquinone which consequently led to interference NQO1 action. Moreover, the long incubation time of HepG2 cells with Q and EGCG (20 hours) allowed them to penetrate the cell and remain inside and act

as substrate of NQO1 and involved reduction reaction even through the treated cells were extensively washed by PBS.

On the other hand, it seems possible that the cell-based assay can be used to study modulation of NQO1 activity by compounds such as I3C and SFN which act by indirect antioxidant mechanisms.

5.3 Conclusion

NQO1 activity assessed by DQ-mediated reduction of ferricyanide cannot be measured in intact cells following treatment with Q or EGCG because they interfere in the enzymatic reaction. This suggests a major limitation to use of this assay to screen for induction of NQO1 activity by plant-derived chemicals and extracts.

Chapter 6

General discussion

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6 General discussion

The daily consumption of vegetables and fruits rich with antioxidants such as onion, garlic, cruciferous vegetables, citrus fruits, and green tea has a clear impact in improving the health of the individual and disease resistance (Riboli and Norat 2003). Recently, scientists have interested to study the effects of plant-derived extracts containing compounds that possesses antioxidant properties (Stanner *et al.* 2004). Polyphenols are considered one class of these compounds and most of their components have been proved effective in treating diseases, especially the intractable ones such as cancers (D'Archivio *et al.* 2007). However, their bioavailability are affecting by several factors such as plasma protein where hydroxyl group in the B-ring of flavonoids enhanced the binding affinities to proteins (Xiao and Kai 2012) which may cause metabolism delay.

Therefore we are interested in these compounds and believed that they or at least most of them may be effective, and open in the future the prospects of medical and to answer on how to treat medical conditions that have to date proven resistant to therapeutic solutions.

We selected two polyphenol compounds, Q from flavonol and EGCG from flavanol (catechins), and two isothiocyanate compounds, I3C and SFN, to test their effect on

the HepG2 cells. In this regard, liver was chosen as a model for toxicity because it is particularly susceptible to toxins as well as oxidative insults and the portal vein brings blood to this organ after intestinal absorption (Martin *et al.* 2008). Although HepG2 cells are classified as cancer cells we used these cells as a model to identify the protective effect of phytochemicals against oxidative stress induced by t-BHP by culturing cells in high density to be confluent and non-dividing. Our aim was to identify these compounds and their chemical properties to employ them later on for further cellular protection against oxidative stress induced by t-BHP. The antioxidant properties of the four selected compound were tested by using free radical scavenging assay or known as DPPH assay which is considered one of the trusted, common, and reliable methods (Clarke *et al.* 2013), especially for vegetables and fruits to assess the antioxidant potency to scavenge free radicals (Magalhaes *et al.* 2008; Moon and Shibamoto 2009; Sanchez-Moreno 2002). The mechanism of free radical scavenging is based on the direct attacking and radicalization of free radicals by the hydroxyl groups on the antioxidant compounds, flavonoids in particular due to multiple numbers of hydroxyl groups (Ben Sghaier *et al.* 2011; Xiao and Kai 2012) on their structure. For this reason, EGCG (Masamune *et al.* 2005) and Q (Chiodo *et al.* 2010) are considered as direct antioxidants and that was compatible with their potent activity in the DPPH assay, EGCG was more active than Q due to high numbers of hydroxyl group on its structure where it possesses 9 hydroxyl groups while Q was less active than EGCG because it has only 5 hydroxyl groups in structure. Moreover it has been suggested that once the EGCG molecule has trapped the free radicals it becomes stable due to *ortho*-di-hydroxyl groups positioned on the B-ring of EGCG which confers a high stability to the trapped EGCG (Bors *et al.* 1990; Salah *et al.* 1995). In relation to Q, we believed in action that this molecule can employ hydroxyl groups on its structure and directly metabolize O_2 through oxygenlysis process (Fiorucci *et al.* 2004) where hydroxyl group on B-ring plays a distinctive role in radical scavenging action (Trouillas *et al.* 2006). On the other hand and according to the inactive behaviours of I3C and SFN

in free radical scavenging, we considered that they are not direct antioxidants. However I3C possesses one hydroxyl group but it may be not enough to inactivate the free radicals. So any defensive activity of I3C and SFN against oxidants may be attributed to a mechanism other than the direct one.

The main characteristic of antioxidant studies is the possibility of employing them for cellular protection against oxidative stress; therefore we derived our work in this direction in the intertie of providing new information on the time dependence of cytoprotection and the influence of serum in the culture medium. With regard to serum albumin, we used medium containing 10% serum in direct and indirect cytoprotection to reach the approximated exposure of cells to interstitial fluid (IST) in most tissues. Medium made up with 10% (v/v) serum contains approximately 0.35 g/100ml of albumin where human plasma at normal concentrations between 35 and 50 g/L (Rothschild *et al.* 1988), a value that is not far away to that found in interstitial fluid (IST); various authors provided values for IST albumin level to be between 0.15 and 0.21 g/100ml (for references see chapter 3). Moreover, since serum had no effect on cell viability in the absence of phytochemicals and t-BHP during short or long incubation time, we suggested that any variations in cytoprotection may due to the presence of phytochemical and oxidant due to binding of these compounds to serum albumin. Earlier studies have shown that binding of substances such as sulfadiazine, salicylate or phenylbutazone to human plasma and human serum albumin is inversely related to protein concentration (Boobis and Chignell 1979) and D'Archivio *et al.* (2007) have mentioned that the activity of flavonoids may decreased because of binding to serum albumin. Therefore and in order to avoid the interference of serum with chemicals which may affect results (Alia et al. 2006b), we decreased the amount of albumin to 2% and free-serum medium later on.

With respect to direct cytoprotection, structure of antioxidant and serum albumin played an essential role in evaluation of antioxidant activity. Although EGCG

(Masamune *et al.* 2005) and Q (Chiodo *et al.* 2010) are potent direct antioxidants due to multiple number of hydroxyl groups on its structure that participate in free radical scavenging (Ben Sghaier *et al.* 2011; Xiao and Kai 2012), their activities during cellular protection were low. The reason for this decline in antioxidant activity may be attributed to the effect of albumin because activity of EGCG and Q increased when serum decreased or omitted. We suggested that albumin may bind to hydroxyl groups on phytochemicals and prevent them from being free for radicals attack and hydrogen donation which consequently lead to a decrease in the antioxidant activity. Similar suggestion has been made by Otagiri (2005) and Zsila and Iwao (2007) who have studied binding of serum to different components of polyphenols and reported that the bioactivities of polyphenol were often executed in complex biological system such as blood where various interactions take place. Moreover Xiao et al. showed in 2009 that increasing the hydroxyl groups on ring B of EGCG increases the affinity to serum while Rohn et al. (2004) reported that the total antioxidant activity of Q decreased by interaction between quercetin-BSA in comparison to an equivalent amount of free quercetin. Although we generally excluded I3C and SFN from direct cytoprotection experiments because of lack of any direct antioxidant properties, we did use them in one experiment including medium 2% serum and they were inactive.

Similarly, serum albumin had a crucial effect on the results of indirect cytoprotection pattern where the activity of Q and EGCG increased when serum decreased or omitted. This probably because serum binds to Q and EGCG which lowered the activity of these compounds (Rohn *et al.* 2004; Xiao *et al.* 2009). On the other hand, partial protection provided by I3C against the toxicity effect of t-BHP during 20 hours exposure to HepG2 cells in medium 10% serum may attributed to the up-regulating proteins. According to the literature, I3C and its acid condensation products (I3C-A) have been classified as indirect antioxidants where upregulation of QR has been attributed to the synergistic co-activation of the ARE

and the XRE by I3C-A and crambene, another phytochemical (Nho and Jeffery 2004). On the other hand and during indirect cytoprotection in lack of serum, the cell death caused by higher concentrations of I3C suggested that this compound is totoxic but its totoxic effect was much reduced in the presence of serum. Although we did not know what is the non-toxic concentration of I3C used in literature under our conditions that were used but it was obvious that serum has cytoprotective effects. Serum albumin possesses antioxidant properties and the cytotoxicity effect of DHA on HCC cell line can be modulated by serum albumin (Kanno *et al.* 2011). With regards to SFN, it is known to act as indirect antioxidant (Malaguti *et al.* 2009) but we could not prove this because SFN was cytotoxic and caused cell death may be due to high concentrations that were used. It has been strongly recommended that this compound should be used in low concentration otherwise the significant inhibitory effect would occurr (Yeh and Yen 2005). Moreover, a relevance between cytotoxicity of SFN and omission of albumin suggested that serum albumin may play an essential role in binding with SFN and transporting it inside cell and causing cell death. It is well known that the transportation, metabolisim, deposition, and efficacy of drugs are strongly affected by their binding to serum albumin (Trnkova *et al.* 2011).

With regard to cytotoxicity, we utilized t-BHP as a model of an oxidant compound in cellular reaction to induce oxidative stress in cell system by producing free radicals, which might be the main factor for hepatotoxicity and caused cell death. t-BHP is an organic hydroperoxide, which can decompose to free radicals (Rush *et al.* 1985). Once inside cells, the free radicals generated from t-BHP induce several physiological alterations alongside loss of cell viability via necrosis or apoptosis (Sardao *et al.* 2007). Our finding represented that complete cell death occurred by two different concentrations of t-BHP and initial proposition was made to hypothesis that 0.4 mM t-BHP might elicit more of an apoptosis as opposed to more necrosis at 0.8 mM. Reports have suggested that high concentration of t-BHP may induce

necrosis. Additionally, the type of cell death may be varied according to the conditions of cellular treatment including type of cell line, concentration of t-BHP and time exposure to cell. For example, oxidative stress induced in H9c2 myoblasts after one hour treatment with 50 μM t-BHP whereas after 6 hours treatment with 50 and 100 μM t-BHP detachment and cell death occurred (Sardao *et al.* 2007). Moreover, studies have demonstrated that treatment of cardiomyocytes with 1 mM t-BHP induced loss of cell shape, depletion of ATP, and formation of adenosine (Andersson *et al.* 1996) while others reported that this concentration caused significant increase intracellular calcium concentration in cardiomyocytes isolated from rat (Daly *et al.* 1991).

Apoptosis, a form of programmed cell death, is considered one of the most potent defence processes against diseases such as cancer where potentially deleterious mutated cells are eliminated (Martin 2006a). Moreover, activation of caspases is considered one of the major features of apoptosis and can be used to distinguish it from other types of cell death (Fink and Cookson 2005). Although our findings presented a significant increase in caspase-3 activity in treated HpG2 cells with 0.8 mM t-BHP at 150 minutes, little visual evidence for apoptosis by western blotting, in-cell western, or immunocytochemistry due to slightly indications for activated caspase-3 was observed and this may because that caspase-3 confused and delayed by calpains. It is possible that calpains may play a role in down-regulation of caspase- 3 activation during cell death pathway due to chronic mitochondrial defects (Bizat *et al.* 2003b). Suggestions have been made by scientists about inactivation of caspase-3 by calpain. Although predominance of calpain over caspase-3 in spite of a transient activation of caspase-9 has remained speculative assumptions (Bizat *et al.* 2003a), a cross-talk between caspases and calpains has been discovered (Blomgren *et al.* 2001; Chua *et al.* 2000; Lankiewicz *et al.* 2000; Wolf *et al.* 1999). The mechanism of this cross-talk includes abnormal activation of calpain due to increase in the intracellular Ca^{+2}, the caspase-9/-3 apoptotic

pathway then may be partially blocked by calpain which leads to the cell death as well as inactivation of caspase-3 (Bizat *et al.* 2003b). Our hypothesis to induce apoptosis was confirmed by other parameters in such that significant increase in ROS level at late time of incubation 150 minutes by 0.8 mM t-BHP. We expected that the significant increase in ROS level would occur before that time because generation of ROS is initiated at the early stages of apoptosis and before death of cells, as demonstrated in the literatures (Shih *et al.* 2004). We can assume that amounts of generated ROS may be removed quickly by NAC, scavengers of ROS, which they abolish the increased amount of ROS (l'Hoste *et al.* 2010). With regards to GSH, it plays a fundamental role in protecting cells from oxidative stress by scavenging ROS (Reed 1990) and therefore, GSH decreases when ROS increases (l'Hoste *et al.* 2010). Our concerning findings about ROS indicated that they increased significantly in HepG2 cells treated with 0.8 mM t-BHP at 150 minutes therefore; we tend to suggest that the generated amounts of ROS may be removed by NAC, the scavenger of ROS, resulting in consumed small amounts of GSH which consequently led to decrease GSH slightly. Studies have reported that depletion of GSH may be involved in apoptosis induction in HepG2 cells treated with *T.arjuna* (Sivalokanathan *et al.* 2006). The last parameter assessed was LPO, is a form of cellular damage beside nucleic acid and protein breakdown induced by ROS (Esterbauer and Cheeseman 1990). During LPO formation, a large number of toxic by-products (Devasagayam *et al.* 2003) are produced, derived from the breakdown of polyunsaturated fatty acids; and these include malonaldehyde (MDA) and 4-hydroxy-2(E)-nonenal (4- HNE) (Esterbauer and Cheeseman 1990). These products behave as a toxic 'second messenger' because they affect at sites distance from the place of their generation which subsequently cause highly functional cellular damage (Raha and Robinson 2000). In our results, the significant increased level of LPO accompanying with increased cell death at later time of treatment was reported and this suggest that apoptosis is occurred. A rising level of malondialdehyde

following oxidative stress induced by iron ions in corneal endothelial cells and increased apoptosis was observed (Serbecic *et al.* 2005).

After identifying the mechanism of toxicity induced by t-BHP, the next step was the utilizing of phytochemicals to stop apoptosis induction through inactivation of caspase-3. We selected Q and I3C which are differed in their antioxidant properties to provide protection under the optimum cellular conditions from, concentration, cytoprotection pattern, and presence of serum. Inactivation of caspase-3 by Q suggested that this compound is an effective cytoprotectant, and it can down-regulate caspase activation and prevent apoptosis. The proposed mechanism based on the pattern of cytoprotection where Q scavenged free radicals initiated from decomposition of t-BHP immediately. Earlier studies have shown that Q exhibited inhibitory effect on caspase-3 activation and prevent apoptosis in rat glioma C6 (Chen *et al.* 2006). Moreover, the slight bands of activated caspase-3 were disappeared from blot when HepG2 cells treated with I3C which suggested that I3C stopped apoptosis induction. To the best to our knowledge no publications about the cytoprotective effect of I3C in HepG2 cells under the cellular conditions used to inactivate caspase-3 and stop apoptosis induction have been reported. Numerous reports have pointed the role of I3C to induce apoptosis in many type of cancer cells and angiogenesis (Kunimasa *et al.* 2010) and (Choi *et al.* 2010). We suggested the mechanism of I3C action in such that, I3C is a good cytoprotctant under condition that we used and it may activate the suppressors of apoptosis such as Bcl2, Bcl-XL, and Mcl-1 or down-regulate the activator of apoptosis such as Bax, Bok, and Hrk which subsequently resulting in stop apoptosis induction (Tan *et al.* 2009).

In related study of phytochemicals with NOQ1 , the induction of phase ll enzyme, in particular NQO1 prevents the generation of ROS and toxic semiquinone radicals by reducing compounds with a quinone structure due to two-electron transfer (Wiegand *et al.* 2009). We attempted to assess NQO1 activity in intact cells in the

absence and/ or presence of phytochemicals by determining the reduction of ferric ion. As the principle of this assay is based on the ability of NQO1 to catalyse the conversion of duroquinone to hydroduroquinone where this enzymatic reaction is linked with reducing of ferricyanide to ferrocyanide (Bongard *et al.* 2011) therefore, lower the reduction reaction rate of iron is considered important sign of NQO1 activity. In the absence of duroquinone, evaluation of Q and EGCG in inducing NQO1 in intact HepG2 cells seemed to be impossible because each of them interfere with the reduction reaction resulting in effect on NQO1 assessment whilst I3C and SFN are not. The overlapping caused by Q and EGCG in enzymatic reaction may be attributed to the fact that each of them possesses quinone structure where I3C and SFN are missing that. Therefore, the long incubation for Q and EGCG with cells may allow them to penetrate inside the cells and being reduced by NQO1 as quinone compounds which subsequently leading to reduced ferric to ferrous.

In this study, our aim was to develop a cellular protection assay for identification of antioxidant activity in plant extracts which may be used for further therapeutic reasons. We used four compounds Q, EGCG, I3C, and SFN classified within polyphenols groups; including flavonols, flavones, flavanones, catechins (flavanols), anthocyanidins, isoflavones, dihydroflavonols and chalcones (Iwashina 2000), which are considered to be the most abundant antioxidants in human diet (D'Archivio *et al.* 2007). We examined the activity of these compounds *in vitro* and found that duration of incubation time and presence of serum has impact on their activity. From all compounds, Q was the active compound under any cellular conditions. However we do not know what is the effect of Q and/or other compounds *in vivo.* Intervention studies have shown that polyphenols are absorbed after consumption of food rich with polyphenols where an increase in polyphenol plasma concentration are recorded (Cao et al. 2010) such that significant increase Q plasma concentration has observed after Q consumption and the Q plasma concentration has increased from 332 ±21 µg/L to 516 ± 30.8 µg/L when the amount of Q

administrated increased from 500 mg/day to 1000 mg/day respectively (Jin et al. 2010). From this, it seems that increased consumption of Q may lead to increase Q plasma concentration; however Jin *et al* (2010) did not investigate the toxicity of Q when the large amount of Q are consumed, although the lack of any report of toxicity could indicate a lack of obvious toxicity. These amounts of Q plasma concentration in µg/ml equal 0.3 and 0.5 respectively which are less than by 10 times of the lower concentration that we were used in cytoprotection studies 6 µg/ml. The big difference in concentrations that we were used and Q plasma concentration can be explained by fact that the experimental studies are demonstrating the protective effect of phytochemicals against high concentrations of the pro-oxidant such as t-BHP for short time; therefore high concentrations of polyphenols are required to display the protective effects of these compounds. Whereas *in vivo* the damages that caused by different factors such as smoking, drinking alcohol, or fat foods are slow and may take years to be observed. In addition, to reach the concentration of phytochemicals, Q in particular or their metabolites in plasma we can suggest for further cytoprotection studies to decrease the concentration of t-BHP in order to mimic the oxidative stress which may display the protective effect of Q in low concentrations. With regard to our result, I3C and SFN produced concentration-dependent cell death while Q and EGCG were not toxic under cellular conditions used. Accordingly, and depends on the intervention studies which elucidated an increase in polyphenol plasma concentration after administration (Cao *et al,* 2010; Jin *et al*, 2010), we may assume the possibility of using high doses of Q and EGCG to achieve high Q and EGCG-plasma concentration (100 µg/ml) as they did not appear toxic, although more caution is urged for I3C and SFN in *in vivo* studies in light of their cytotoxicity.

The *in vivo* intervention studies have shown that the bioavailability of polyphenols may be affected by several factors after administration of diet rich with flavonoids, such as binding to plasma protein where hydroxyl groups in the B-ring enhanced

the binding affinities to proteins and these bindings may have consequence for the delivery of free polyphenols and/or their derivatives to tissues and cells (Xiao and Kai 2012). Therefore, in future *in vivo* studies with Q and EGCG we may expect that free Q and EGCG plasma concentration will be lower than expected because of this plasma protein binding issue.

The inverse association between the incidence of various diseases and the intake of foods rich in polyphenols (D'Archivio *et al.* 2007) strongly suggests the desirability of following a polyphenol-rich diet. Clearly, the fate of such ingested polyphenols needs to be considered. Glucuronidation, methylation, and sulphation reactions are important metabolic processes that occur to diet polyphenol constituents after administration (Xiao and Kai 2012). These processes are considered the common detoxification pathways that lead to an increased solubility and a higher molecular weight and addition of either glucuronic acid, methyl or sulfate group respectively to the xenobiotic substrate (Day *et al.* 2000).

Epidemiological studies have shown the metabolism of polyphenols and formation of their derivatives, such that about 66% of ingested olive oil phenols were observed in the small intestine and their methylated and metabolites glucuronidated have been identified (Vissers *et al.* 2002). Moreover, Vad der Woude *et al.* (2004) have confirmed the metabolism of Q in the small intestine and human liver hepatocytes by methylation, Glucuronidation, and sulphation reactions. Q is also metabolised in HepG2 cells by similar metabolic routs although the exact site of conjugation differ from those in normal human hepatocytes. Overall, these observations suggest that Q and other polyphenols exist as both the parent compound and metabolites under *in vivo* conditions and during circulation with HepG2 cells.

On the other hand, strong arguments have risen about the duel effect of polyphenol consumption, for example green tea consumption is associated with a reduced risk of liver cancer incidence (Ui *et al.* 2009) whereas no inverse association existed

with risk of pancreatic cancer in humans (Lin *et al.* 2008) or risk of stomach cancer death (Hoshiyama *et al.* 2002).

From all of the above, it is apparent that the antioxidant activity, both direct and indirect, of phytochemicals can be determined by their ability to protect HepG2 cells from oxidative stress elicited by t-BHP, with due allowance for the role of binding to serum albumin and the concentration of t-BHP. Moreover, additional mechanistic insights into the mode of toxicity of t-BHP to HepG2 cells have been gained and fundamental problems in use of a cell-based assay to detect direct antioxidant inducers of NQO1 revealed.

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