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Protective effect of dietary antioxidants and plant extracts on acute inflammation and hepatotoxicity *in vitro*

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Dedication

Dedicated to my Mother´s soul, to my Father, to my dearest Wife, to Hazem and Renad

Mohamed

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
AAE	Ascorbic acid equivalent
ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
AP-1	Activated protein-1
APP	Acute phase protein
APR	Acute phase response
BSA	Bovine serum albumin
CRP	C-reactive protein
CYP	Cytochrome P450s
DEM	Dimethylnitrosamine
DEN	Diethylnitrosamine
Dex	Dexamethazone
DHLA	Dihydrolipoic acid
EC	(-)-epicatechin
ECG	(-)-epicatechin gallate
EGC	(-)-epigallocatechin
EGCG	(-)-epigallocatechin gallate
ELISA	Enzyme-Linked Immunosorbent Assay
FRAP	Ferric reducing antioxidant power
FW	Fresh weight
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2	Human hepatocarcinoma cell line
IL-1	Interleukin-1
IL-6	Interleukin-6
LA	Lipoic acid
LIF	Leukemia inhibitory factor
NAC	N-acetylcysteine
NF-κB	Nuclear factor-kappaB
NR	Neutral red

OPD	O-phenylenediamine dihydrochloride
RBP	Retinol binding protein
PBS	Phosphate buffer saline
ROS	Reactive oxygen species
SAA	Serum amyloid A
SELDI-TOF MS	Surface Enhanced Laser Desorption/Ionisation Time of Flight Mass Spectrometry
TAC	Total antioxidant capacity
TEAC	Trolox equivalent antioxidant capacity
TNF	Tumor necrosis factor
TOC	Tocopherol
TPC	Total phenolic content
Trolox	6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid
TTR	Transthyretin

1. INTRODUCTION

Dietary antioxidants are believed to play an important role in the prevention and treatment of a variety of diseases associated with oxidative stress (Kaur and Kapoor 2001). Although there is a wide range of dietary antioxidants, the bulk of the research to date has been focused on the nutrient antioxidants vitamin E, C, and carotenoids and their beneficial effects in the protection against cancer and cardiovascular diseases (Diplock 1994). Certain relatively uncommon antioxidants such as lipoic acid (LA), N-acetylcysteine (NAC) and phenolic compounds have not been extensively investigated although numerous studies have conclusively shown that their antioxidant potential may be actually greater than that of carotenoids and vitamins (Manach et al. 2004).

Phenolic compounds (e.g., flavonoids and phenolic acids) are present in many plant sources, in a great variety of structures and specificities. They have basic molecular similarities in that all have at least one aromatic ring and a hydroxyl group (Manach et al. 2004). Phenolic compounds together represent an array of antioxidants which may act by different mechanisms to confer an effective defense system against free radicals attack. Catechins or flavanols are one of the six classes of the flavonoids. Tea has been reported as a rich source for these catechins including (+)-catechin, (-)-epicatechin (EC), (+)-gallocatechin, (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG) (Arts et al. 2000). A number of epidemiological, animal and *in vitro* studies have demonstrated that catechins may exert anticarcinogenic, antimutagenic, antimicrobial, and antiarteriosclerotic effects (Shon et al. 2004; Zhang et al. 2006).

In the last decade intense interest has been directed towards the antioxidant lipoic acid especially after numerous observations demonstrated its unique, multifunction properties (Packer et al. 1995a). Unlike all other antioxidants, LA is soluble in both water and fat and therefore, it is easily transported across cell membranes, enabling it to confer free radical protection both inside and

outside cell. Moreover the oxidized and reduced forms of LA together constitute an effective redox couple with unique properties including; regeneration of exogenous and endogenous antioxidants such as vitamins C and E, and glutathione; quenching of reactive oxygen species; chelation of metal ions, and modulating transcription factor activity, especially that of Nuclear factor-kappaB (NF-κB) (Biewenga et al. 1997; Packer 1998; Wollin and Jones 2003). Several studies demonstrated its preventive and therapeutic potency in a broad range of diseases such as diabetes, strokes and heart attack, HIV, atherosclerosis, cataract and acute and chronic neurological disorders. LA therefore approaches the 'ideal' or 'universal' antioxidant (Moini et al. 2002b; Packer et al. 2001; Packer et al. 1997).

It was reported that antioxidants in a mixture or combinations exhibit higher effect then that of single antioxidant. This may be explained by the synergistic effect between antioxidants (Murakami et al. 2003). Many plant sources specially fruits, vegetables, herbs, nuts, spices and many plant byproducts, are rich sources for antioxidants. Extracts of such plant may represent good examples for such antioxidants combination although it may contain several unknown components. Although solvent extraction may yield higher amount of an active compound than water extraction, the later is also considered since most of plants are eaten raw or processed in water.

Despite increasing evidence on the potential of antioxidants in modulating the etiology of certain diseases, little is known about their role in acute phase responses and inflammatory diseases. Inflammation is the response of the host against infection and injury (Gruys et al. 2005). The early, unspecific and highly complex reactions of the host occurring immediately after any inflammatory stimuli, is collectively called acute phase response (APR) (Baumann and Gauldie 1994; Ebersole and Cappelli 2000; Gabay and Kushner 1999; Koj 1996). Adaptation of liver cells to the APR is characterized by changes in synthesis of liver derived plasma proteins which lead to significant

changes in plasma protein concentrations thus called the acute phase proteins (APPs). The synthesis of one class of plasma proteins, including fibrinogen, haptoglobulin, serum amyloid A, C-reactive protein and other anti-inflammatory or procoagulatory proteins, is upregulated and termed as positive APPs, whereas the expression of certain constitutively expressed proteins, so-called negative APPs, most notably albumin, transthyretin (TTR), retinol binding protein (RBP) and transferrin, are down regulated (Baumann and Gauldie 1994; Schweigert 2001). Regulation of the synthesis of APPs is modulated by a very complex network of cytokines, which can act independently or in concert with each other and with endocrine hormones and glucocorticoids to inhibit or stimulate the production of an acute phase protein. Various cytokines are involved including interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor (TNF) and leukemia inhibitory factor (LIF) (Mackiewicz et al. 1991). The most potent and broadly effective stimulant to the APP production by human hepatocytes appears to be IL-6 (Castell et al. 1989b).

Attention has been mainly focused on C-reactive protein (CRP) as a positive APP in contrast, there are no studies so far that have looked at negative APPs. TTR is one of negative APPs, synthesized mainly by the liver and also produced in the choroid plexus of the brain and the eye (Lim et al. 2002). TTR under physiological conditions, functions as a carrier for both thyroxine (T3 and T4) and retinol (vitamin A), in the latter case through binding to RBP (Eneqvist et al. 2003). With regard to nutrition, TTR plasma levels have thus been proposed as sensitive biochemical parameters of subclinical protein malnutrition, because both the adequacy and levels of protein as well as energy intakes are reflected in plasma levels. Plasma levels of TTR, however, are as well affected by acute and chronic diseases associated with an APR. Changes in TTR microheterogeneity may lead to instability in its tetramer structure or to amyloidosis (Bergen et al. 2004; Saraiva 2001). A truncated variant of TTR has been described as a biomarker for ovarian cancer, indicating a close interplay

between nutritional status, inflammation and possibly the occurrence of cancer (Gericke et al. 2005; Schweigert et al. 2006).

The functional relationship between inflammation and cancer has been postulated since several decades. Recently, several evidences demonstrated the rise of many cancers from sites of infection, chronic irritation and inflammation. It has been proposed that active oxygen species and some cytokines generated in inflamed tissues can cause injury to DNA of target cells and result in survival of damaged cells that ultimately leading to carcinogenesis (Calmels et al. 1997). Epidemiological and laboratory studies indicate that chronic inflammation and hyperplasia induced by viral hepatitis B and C, or nitroso compounds are closely associated with the development of human hepatocellular carcinoma (Ahn et al. 1999; Chuang et al. 2000).

Diethylnitrosamine (DEN) is one of the most important environmental carcinogens, present in a variety of foods, including cured meats, smoked and salt-dried fish, grain products, cheese, soybeans and alcoholic beverages. It was also detected in tobacco smoke, in baby pacifiers and baby bottle nipples as contaminant in the rubber, and it can be synthesized endogenously (Lijinsky 1999; Tricker and Preussmann 1991; Yurchenko and Molder 2006). DEN is primarily used as a hepatocarcinogen to induce hepatocellular carcinoma. Moreover it can induce carcinogenesis in other organs like kidney, trachea, lung, esophagus, fore stomach, and nasal cavity (Enzmann et al. 1995; Verna et al. 1996).

In the present work, I studied the protective effect of water and solvent extracts of eight plant and plant byproducts including green tea, artichoke, spinach, broccoli, red onion, eggplant-, orange- and potato peels and eight antioxidants agents including EC, EGC, ECG, EGCG, ascorbic acid (AA), NAC, α-LA, and alpha tocopherol (α-TOC) toward inflammation induced by IL-6 and hepatotoxicity induced by diethylnitrosamine (DEN). TTR, as a negative APP

was used as an inflammatory biomarker where neutral red assay was used for evaluation of the cytotoxicity. All experiments were performed *in vitro* using human hepatocarcinoma cell line (HepG2). Additionally the antioxidant activity, phenolic content and TTR microheterogeneity were also determined.

2. AIM OF STUDY

- Establishment of an acute inflammatory model of HepG2 cells, using different pro-inflammatory cytokines. The negative acute phase proteins TTR and RBP were used as negative acute inflammatory biomarkers.
- Study the antioxidant activity and phenolic contents of some selected plants and plants byproducts extracts using different assays.
- Study the anti-inflammatory effects of such plant extracts as well as other antioxidants agents on IL-6 inflammated cells.
- Study the effect of plant extracts and antioxidant agents on the microheterogeneity of TTR secreted by HepG2 using SELDI-TOF-MS assay.
- Study the hepatotoxicity of diethylnitrosamine (DEN) as well as plant extracts and antioxidants agents on HepG2 cells using neutral red assay.
- Study the protective effects of specific concentrations of plant extracts and antioxidants on the hepatotoxicity induced by DEN on HepG2 cell.

3 REVIEW OF LITERATURE

3.1 Inflammation

3.1.1 Historical Perspectives

The earliest documented reference to inflammation was in the Egyption smith papyrus from around 1650 B.C. In it, the ancient Egyptian associated inflammation with heat via symbol of flame, whereas the ancient Greeks used a term meaning the hot thing refering to inflammation. The word inflammation comes from the Latin 'inflammare' (to set on fire). Cornelius Celsus on 1st century A.D. was credited as the first literature documented the four cardinal signs of inflammation: calor (warmth), dolor (pain), tumor (swelling) and rubor (redness and hyperaemia). The fifth sign: loss of function was added later at 2nd century A.D by Galenos (Borchardt 1999; Rather 1971).

3.1.2 Overview

Inflammation is an important, protective response of the host against infection and injury, directed towards neutralizing the invading pathogen and wound healing and plays a central role in many chronic diseases (Gruys et al. 2005). The magnitude of the inflammatory response is crucial: insufficient response results in immunodeficiency, which can lead to infection and cancer. Whereas excessive response causes morbidity and mortality in several diseases such as rheumatoid arthritis, Crohn's disease, atherosclerosis, diabetes, Alzheimer's disease, multiple sclerosis, cerebral and myocardial ischemia (Cohen 2002). If inflammation spreads into the blood stream, as it occurs in a septic shock syndrome, sepsis, meningitis and severe trauma, the inflammatory responses can be more dangerous than the original stimulus. Homeostasis and health are restored when inflammation is limited by a coordinated sequence of systemic and metabolic changes collectively referred to as the APRs (Tracey 2002).

3.2 Acute phase response

The term acute phase response (APR) refers to the early, unspecific and highly complex reaction of the host occurring immediately after any inflammatory stimuli, trauma, bacterial, viral, or parasitic infection, surgery, fracture, burns, tissue necrosis, or in presence of chronic diseases such as cancer and immunologically mediated inflammatory disease (Baumann and Gauldie 1994; Ebersole and Cappelli 2000; Gabay and Kushner 1999; Koj 1996). APR is almost a primarily liver-specific phenomenon characterized by a rapid and dramatic alteration in plasma concentrations of the APPs. APR considers as a part of the non-specific immune response, and its various components are relatively consistent despite the large variety of conditions that induce it. The APR is followed by specific immune response, which in contrast is selective (Ananian et al. 2005; Baumann and Gauldie 1994).

The purpose of the APR is to neutralize the inflammatory agent, prevent further injury, isolate and destroy the infective tissues, remove the harmful molecules and debris, and activate the repair processes that are necessary to return the organ to its normal function (Baumann and Gauldie 1994; Ebeler et al. 2002; Gabay and Kushner 1999). Bacterial infections usually lead to a strong APR while viral infections induce milder APR (Urbach et al. 2002). The APR occurs within a few minutes or hours after initiation, and either resolves within a few days upon recovery from illness or trauma or becomes a chronic APR as it occurs in chronic diseases such as cancer (Mackiewicz 1997; Wigmore et al. 2001).

3.3 Acute phase initiation

The APR is usually initiated at the site of disturbance or injury by local inflammatory cells, neutrophils and macrophages. These mononuclear cells release a broad spectrum of mediators including free radicals and reactive oxygen species (peroxide, nitric oxide), derivatives of lipids (prostaglandin E2,

thromboxane A2, platelet activating factor), and a variety of regulatory cytokines (Cavaillon 1994; Koj 1996; Laskin and Pendino 1995). Both local and systemic APR is thought to be manly initiated, regulated, amplified, and terminated by a complex activated cytokine cascade. The local response involves also an increase in capillary permeability, and infiltration of leucocytes to the place of inflammation. The increased capillary permeability allows the transport of different molecules between circulation and the area of tissue injury. These molecules contain several plasma proteins, like proteinase inhibitors, transport proteins, and other binding proteins, as well as some ions such as Na^+ and Cl^-. Leucocytes and capillary endothelial cells express adhesion surface receptors in response to inflammatory mediators. Phagocytic cells, neutrophilic granulocytes and macrophages play a key role in eliminating foreign antigens. Their function is based on phagocytosis, lysosomal hydrolases, and oxygen radicals. (Heinrich et al. 1990; Heinrich et al. 1998; Koj 1998; Mackiewicz 1997; Martin et al. 1999; Paape and Capuco 1997; van Miert 1995).

3.4 Role of cytokines in acute phase response

Cytokines are soluble, extracellular proteins that regulate innate as well as immunologically regulated inflammatory reactions. This implies that cytokines are involved in many different processes including cell growth and differentiation, development, and repair processes leading to the restoration of homeostasis. Cytokines are released by activated leukocytes (interleukins) and other cells and in small quantities can regulate cell function non-enzymatically (Burger and Dayer 2002; Ebersole and Cappelli 2000; Gabay and Kushner 1999; Mackiewicz 1997). Their synthesis is initiated by several inflammatory mediators, which induce the cascade of signal transduction, transcription of cytokine genes, translation into cytokine polypeptide, and its processing and secretion (Koj 1996). There are three main groups of cytokines corresponding to effect pathways can be distinguished; (1) cytokines that primarily act as positive or negative growth factors for a variety of cells (IL-2,

IL-3, IL-4, IL-7, IL-10, IL-11, IL-12 and granulocyte-macrophage colony stimulating factor), (2) cytokines with pro-inflammatory properties (TNF-α/β, IL-1α/β, IL-6, IFN-α/γ, IL-8, and macrophage inhibitory protein-1), and (3) factors with anti-inflammatory activity (IL-1 receptor antagonists, soluble IL-1 receptors, TNF-α binding protein and IL-1 binding protein) (Gruys et al. 2005; van Miert 1995).

Pro-inflammatory cytokines mainly IL-1, IL-6, and TNF-α play a key role in the hepatic APR. They activate hepatocytes receptors, and synthesis of different APPs. (Baumann and Gauldie 1994; Heinrich et al. 1990; Heinrich et al. 1998; Ingenbleek and Young 1994; Koj 1996; Kushner 1993; Le and Vilcek 1989; Murtaugh et al. 1996). It has been found that these cytokines modulate intermediary metabolism of carbohydrate, fat, and protein, regulate hypothalamic-pituitary secretion, act in the brain to reduce food intake and may directly affect the process of bone growth (Johnson 1997; Stephensen 1999). At the local reaction site, these cytokines activate fibroblasts and endothelial cells, to initiate the secondary release of cytokines (Baumann and Gauldie 1994). This secondary wave and the appearance of these early cytokines in the circulation are responsible for the start of the systemic inflammatory response (Burger and Dayer 2002).

3.4.1 Interleukin-1

Interleukin-1 (IL-1) is a family including 11 distinct members that are related in their genomic and protein structures. The initial members of this family, IL-1α and IL-1β, were discovered over two decades ago and are now considered as prototypic pro-inflammatory cytokines (Dumont 2006). Monocytes, macrophages, keratinocytes and dermal fibroblasts produce IL-1 (primarily IL-1α), often in response to environmental stress. IL-1 induces the production of cytokines and chemokines and increases the expression of adhesion molecules on endothelial cells. IL-1 has both local and systemic effects on cell metabolism, immune and inflammatory reactions. IL-1 is

considered an important mediator of inflammation, based on its presence at inflammatory sites and its ability to induce many of the hallmarks of the inflammatory response (Dinarello 2006). *In vivo*, IL-1 elicits systemic inflammatory reactions, such as fever, activate lymphocytes, upregulate prostanoid synthesis and stimulate binding of blood neutrophils to the vessel walls (Merhi-Soussi et al. 2005). *In vitro* IL-1 induces prostaglandin E2 release from many cell types, production of proteases, catabolism of cartilage and bone, and growth of fibroblasts, all of which could contribute to the pathogenesis of chronic inflammation. IL-1 may causes these effects by inducing an NF-κB and CCAAT/enhancer-binding proteins beta (C/EBPβ) dependent autocrine IL-6 loop (Ebersole and Cappelli 2000; Kramer et al. 2008). IL-1 stimulates an increase in whole body amino-acid flux, and activation of the pituitary-adrenal system. IL-1 is also an important macromolecule in the linkage of the neuroendocrine and immune response systems (Engelsma et al. 2002).

It was shown that IL-1 stimulates hepatic APR and APPs. Induction of IL-6 by IL-1 partly explains its role in APP production; however, it has been established that IL-1 alone increases the transcription of some APPs and decreases transcription of other hepatic proteins. IL-1 induces serum amyloid A, fibrinogen, factor B (serine protease member), metallothioneins, and serum clotting factors, complement components and IL-6. IL-1 with other cytokines also induces liver production of type 2 nitric oxide synthase. In contrast, it decreases albumin, cytochromes, transferrin and lipoprotein lipase transcription (Burger and Dayer 2002; Ebersole and Cappelli 2000)

3.4.2 Interleukin-6

Interleukin-6 (IL-6) is a pleotropic cytokine, related to IL-1 and tumor necrosis factor-α in that all three are coordinately released from activated monocytes and furthermore one can induce production of another (Ebersole and Cappelli 2000). Most, nucleated cells have been shown to express and synthesize IL-6

in vitro. The prominent source appears to be stimulated monocyte/macrophages, and cytokine stimulated stromal cells (Cox et al. 1997). IL-6 is a multifunctional cytokine that plays an important role in host defense. it induce the growth and differentiation of B lymphocytes, differentiation and/or activation of T lymphocytes and macrophages, expression of liver APPs, maturation of megakaryocytes, and enhancement of multipotential hematopoietic colony formation. IL-6 plays a key role in the regulation of APR and is considered to be the major modulator of most APPs. It stimulates hepatic mRNA levels of most positive APP and decreases those of negative APPs (Castell et al. 1989a; Heinrich et al. 1990). IL-6 has also an important role in eliciting cellular immune responses to affected cells and mucosal humoral responses directed against reinfection (Gabay 2006).

During inflammation the sequential activation and cytokine cascade leads to raised IL-6 levels. Thus IL-6 expression is enhanced at the site of inflammation. Nevertheless, plasma levels of IL-6 rise rapidly in response to bacterial infection and sepsis and can remain elevated depending on the severity and duration of the infection. IL-6 exhibits two contrasting features, in acute inflammation IL-6 exhibits anti-inflammatory properties (Xing et al. 1998), whereas in chronic inflammation such as collagen-induced arthritis, murine colitis, or experimental autoimmune encephalomyelitis, it act as pro-inflammatory cytokine (Alonzi et al. 1998). Therefore IL-6 plays an important role in inflammatory process because it can convert acute inflammation to chronic inflammation. It was shown that IL-1, TNF, growth factors, lipopolysaccharide, other bacterial products and neuropeptides stimulates expression of the IL-6 gene (Ebersole and Cappelli 2000). IL-6 exerts a broad spectrum of effects on various target cells and is involved in regulating various elements of the endocrine system and hematopoiesis. IL-6 has been shown to have pyrogenic activity (Dinarello et al. 1991) and clinically correlated with elevated body temperature in patients with severe burns (Nijsten et al. 1987). Elevated levels of IL-6 have been detected in the body fluids of patients with

a variety of systemic autoimmune diseases such as rheumatoid arthritis and system lupus erythematosus. Towards the end of pregnancy, the detection of high levels of IL-6 in the amniotic fluid may be a strong indicator of intrauterine infection (Casart et al. 2007). IL-6 may also play a role in islet function for insulin production and thus be related to the development of diabetes. (Ebersole and Cappelli 2000; Yu et al. 2007).

3.4.3 Tumor necrosis factor

Tumor necrosis factor (TNF) is an important pleotropic cytokine with multiple biological effects, including cell growth, differentiation, apoptosis, immune regulation and induction of inflammation. Many of the responses attributed to TNF are not the direct result of TNF, but rather its ability to regulate the expression of TNF-responsive genes whose products directly mediate the response including transcription factors, cytokines, growth factors, and cell surface antigens (Simeonova et al. 2001; ten Hagen et al. 2008). It contributes to the induction of different APR and induction of several APPs in human hepatoma cell lines include increased biosynthesis of complement proteins factor B and C3, as well as α1-anti-chymotrypsin and decreased biosynthesis of albumin and transferrin (Baumann et al. 1993).

TNF is also responsible for activating white blood cell precursors in the bone marrow, growth of inflammatory tissue fibroblasts and macrophages as well as a broad spectrum of synergistic or antagonistic effects that influence the specific immune response of the stressed organism against foreign antigens and invading microorganisms. TNF causes muscle catabolism that is also mediated by glucocorticoids, as well as glucagon-induced hyperglycemia and amino acid uptake by the liver. It has the direct ability to stimulate other cytokines such as IL-6 and IL-8, as well as nitric oxide, hypothalamic prostaglandin E2 synthesis, thus stimulating fever which is a primary characteristic of the APR. The effects of TNF are enhanced in the presence of IL-1 or interferon-γ. After acute phase, circulating TNF is cleared by binding to

specific receptors in lungs, spleen, liver, skin, kidneys and other organs. Very high tumor necrosis factor levels appearing in the bloodstream represent an unusual event in nature, associated only with extreme threat and or pathophysiological crisis (Ebersole and Cappelli 2000; Mackiewicz et al. 1991; ten Hagen et al. 2008).

3.5 Acute phase proteins

Within a few hours after the acute phase initiation, the pattern of protein synthesized by the liver is drastically altered resulting in an increase or decrease of some plasma proteins. Proteins whose plasma concentration increases by at least 25% or more, such as C-reactive protein (CRP), serum amyloid A (SAA), haptoglobin, fibrinogen, ceruloplasmin and α-globulin with antiprotease-activity, are termed as positive APPs, whereas other normal blood proteins whose plasma concentration decreases including, transthyretin (TTR), retinol binding protein (RBP), cortisol binding globulin, transferrin and albumin, represent the negative APP (Abraham et al. 2003; Koj 1985; Schweigert 2001). The decrease in negative APPs may reflect the need to use available amino acids to increase production of positive APPs and to decrease production of proteins less useful in host defense (Gabay 1999). During starvation, protein-malnutrition and anorexia, there is no full positive response, and a general depression of hepatic protein synthesis occurs. Both malnutrition and the anorectic effects of pro-inflammatory cytokines in the brain result in a negatively changed hepatic synthesis (Gruys et al. 2005; Lyoumi et al. 1998).

3.5.1 Classification of acute phase proteins

There are essentially three systems to classify APPs in man: the first is based on the degree of change in concentration, in which APPs classified to positive and negative (Table 3.1). The positive APPs are subdivided into three groups, group I, whose concentration increases by half (e.g. ceruloplasmin, complement factor C3 and factor C4); group II, whose concentration increases

two to five fold (e.g. fibrinogen and haptoglobin); and group III, whose levels increased to as much as 1000-fold the normal value (e.g. CRP and SAA) (Gruys et al. 2005). The second classification is based on concentration kinetics of change; in it APPs classified as either first or second phase proteins. The first phase refers to APPs which their levels raise as early as four hours after inflammatory initiation, peak within one to three days, and return to normal quickly. Second phase APPs, such as fibrinogen and haptoglobin, increase one to three days after APR initiation, peak in 7-10 days, and decline to normal after two weeks or more; and the third classification system is based on cytokine group responsible for inducing gene expression of the APPs. based on this classification, APPs are classified either to Type I APPs which induced by IL-1-like cytokines, including IL-1α, IL-1β, TNF-α, and TNF-β, and synergistically by IL-6 like cytokines, while type II APPs are induced by IL-6 like cytokines alone, including IL-6 and IL-11. CRP, SAA, and C3 (complement protein-3) are examples of type I proteins, while type II proteins include fibrinogen and haptoglobin (Baumann and Gauldie 1994; Mackiewicz 1997; Moshage 1997).

Table 3.1: Acute phase plasma proteins in human and rat (Heinrich et al. 1990).

Species	Increase			No change	Decrease
	10-100-fold	2-10-fold	≤ 2-fold		
Human	C-reactive protein Serum amyloid A	α_1-Proteinase inhibitor α_1-Acid glycoprotein α_1-Antichymotrypsin Fibrinogen Haptoglobin	Caeruloplasmin C3 of complement α_2-Antiplasmin C_1-inactivator	α_2-Macroglobin Haemopexin Serum amyloid P Prothrombin	Inter- α-antitrypsin Transferrin α_1-Lipoprotein Prealbumin Albumin
Rat	α_2-Macroglobulin α_1-Acid-glycoprotein	Fibrinogen Haptoglobin Cysteine proteinase inhibitor	α_1-Proteinase inhibitor Caeruloplasmin Prekallikrein Haemopexin C-reactive protein	α_1-Macroglobulin Antithrombin III Serum amyloid P Prothrombin	α_1-Inhibitor 3 Transferrin Prealbumin Albumin

3.5.2 Negative acute phase proteins

3.5.2.1 Transthyretin

Transthyretin (TTR), formerly called prealbumin because it migrates on front of albumins on electrophoresis gels, is a plasma protein synthesized mainly by the liver and also produced in the choroid plexus of the brain and the eye (Lim et al. 2002). TTR is a homotetramer of 55 kDa, each monomer of ~14 kDa contains 125–130 amino acids residues. The monomer contains two β-sheets that interact to form a dimer (Schreiber and Richardson 1997). TTR plasma half life is 1.9 days. TTR under physiological conditions functions as a carrier for both thyroxine (T3 and T4) and retinol (vitamin A), in the latter case through binding to RBP (Eneqvist et al. 2003). TTR binds virtually all of serum retinol-binding protein and about 15% of serum thyroxine. TTR is the main thyroxine transport protein in cerebrospinal fluid (Vekey et al. 1997), and might contribute to the transport of serum thyroxine across the blood–brain and blood–choroid-plexus–CSF barriers (Saraiva 2002). The liver, kidney, muscle and skin are thought to be major sites of TTR degradation in the rat, but its cellular uptake is poorly understood (Figure 3.1). Approximately 1–2% of plasma TTR circulates bound to high-density lipoproteins (HDLs) and the association of TTR with the HDL vesicle occurs via apolipoprotein AI (apoAI) (Sousa et al. 2000).

In plasma, TTR is extensively modified with thiol adducts. The unmodified TTR only accounts for ~5%–15% of the total TTR, where the other 85%–95% is posttranslationally modified (Ando et al. 1997b; Kishikawa et al. 1996; Lim et al. 2003a; Terazaki et al. 1998). TTR in conditions not yet completely understood, may aggregate, forming the fibrillar material associated with TTR amyloidosis. This amyloid cause organ dysfunction and ultimately can lead to death (Bergen et al. 2004; Saraiva 2001).

With regard to nutrition, TTR plasma levels have thus been proposed as sensitive biochemical parameters of subclinical protein malnutrition, because

both the adequacy and levels of protein as well as energy intakes are reflected in plasma levels. Plasma levels of TTR, however, are as well affected by acute and chronic diseases associated with an APR. A truncated variant of TTR has been described as a biomarker for ovarian cancer, indicating a close interplay between nutritional status, inflammation and possibly the occurrence of cancer (Gericke et al. 2005; Schweigert et al. 2006).

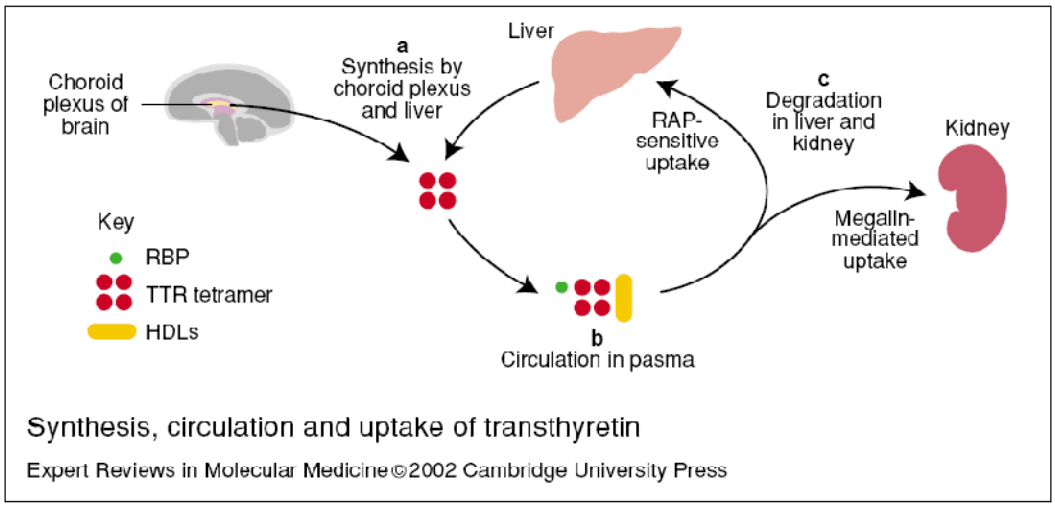


Figure 3.1: Synthesis, circulation and uptake of transthyretin.
(a) TTR tetramer (red circles) is synthesised by the choroid plexus of the brain and by the liver. The mechanisms underlying delivery and uptake of TTR within and out of the brain are not known. (b) The tetramer circulates in plasma bound to RBP (green circle), thereby providing a transport function for vitamin A; and a small proportion of TTR binds high-density lipoproteins (HDLs; yellow bar). (c) TTR is degraded in kidney and liver, as well as muscle and skin (not shown). In the kidney tubules, TTR is taken up by megalin [a member of the low-density lipoprotein (LDL) receptor family]; in liver, an as yet unidentified receptor that binds receptor-associated protein (RAP, a binding characteristic of LDL receptors) is responsible for TTR uptake.

3.5.2.2 Retinol binding protein

Retinol Binding Protein (RBP) is a single chain polypeptide, belongs to the α-globulin family of human plasma proteins, consists of 183 amino acids and with a molecular weight ~21,060 Da (Naylor and Newcomer 1999). RBP is

synthesized primarily in the liver, where it requires the binding of retinol to trigger its secretion. Other sites of synthesis are known and include the kidney, peritubular and sertoli cells of the testis, the retinal pigment epithelium, and the choroid plexus of the brain. RBP is the primary plasma transport protein for retinol (vitamin A). It binds retinol in a ratio of 1:1 stoichiometry resulting holo-RBP, serving not only to solubilize retinol but also to protect it from oxidation (White and Kelly 2001). In the plasma, holo-RBP binds to the larger protein, TTR. *In vitro* one tetramer of TTR can bind two molecules of RBP. However, the concentration of RBP in the plasma is limiting such that the complex isolated from serum is composed of TTR and RBP in a one to one stoichiometry with a resultant molecular mass of about 80,000 Da. The binding of RBP to TTR prevents extensive loss of the low molecular weight RBP through glomerular filtration and may also restrict free partitioning of RBP into the intercellular space outside the vascular system (Newcomer and Ong 2000). This complex then delivers retinol to specific receptors of the retina, skin, gonads, lungs, salivary glands, and other tissues. Immunoassays for serum levels of RBP are useful in the detection of liver disease, protein-calorie malnutrition, and vitamin A deficiencies. In addition, because vitamin A is important in the maintenance of differentiation and rate of proliferation of epithelial tissue, the determination of RBP serum levels have been shown to be important in the mediation of anticancer effects (Schweigert et al. 2006).

The molar ratio of serum RBP to TTR has been proposed as a simple indirect method to assess vitamin A status during infection and inflammation (Rosales and Ross 1998). When vitamin A deficiency is present, the RBP: TTR ratio is believed to fall as a result of differential reduction in RBP. Both RBP and TTR are synthesized and secreted by the liver; however, RBP secretion is dependent on hepatic vitamin A stores, whereas that of TTR is negligibly affected. Thus, in vitamin A deficiency, RBP accumulates in the liver and serum retinol and RBP concentrations decrease, while serum TTR remains within normal range. Although during infection or inflammation hepatic synthesis of

both proteins is reduced. Inflammation seems to enhance the usefulness of the ratio for the assessment of vitamin A deficiency. As regards whether the reduction in serum retinol is due to vitamin A deficiency, to inflammation or to the combination of both, the reduction in both serum retinol and RBP:TTR molar ratio during inflammation seems to indicate vitamin A deficiency, whereas a reduction in plasma retinol but not in RBP:TTR ratio suggests inflammation-induced reduction in plasma retinol (Rosales and Ross 1998; Schweigert et al. 2006; Zago et al. 2002).

3.5.3 Positive acute phase proteins

3.5.3.1 Serum amyloid A

Serum amyloid A (SAA) is a family of small apolipoproteins that associate with a fraction of high-density lipoprotein (HDL) during APR (Cooper 1990). The human SAA have been subclassified according to their magnitude of fluctuation during the APR in two families. One family, acute phase SAA (A-SAA), comprising human SAA1 and SAA2, is well known as an APP, which, like CRP, is dramatically upregulated by pro-inflammatory cytokines. The other family is constitutive SAA (C-SAA), comprised of a single isoform, SAA4, which is constitutively present in serum in concentrations independent of the APR (Whitehead et al. 1992). The three SAA isoforms, SAA1, 2 and 4, A-SAA and C-SAA, are mainly synthesized by the liver. A third A-SAA isoform (SAA3) is expressed in animals and not in human. The dramatic fluctuation in concentration of circulating serum A-SAA, up to 1000 times of its normal levels, is a reflection of pro-inflammatory cytokine concentration. Thus, serum A-SAA concentration is considered to be a main and useful marker to monitor inflammatory disease activity (Kumon et al. 2001).

Chronic systemic elevation of A-SAA has been linked to metabolic disease and is a well-established risk factor of atherosclerosis. It also appears to correlate with insulin resistance and body mass index in humans. Interestingly, although liver is the organ believed to be most important for A-SAA secretion in the

APR, in insulin resistance-related human studies SAA1 and SAA2 expression was higher in adipose tissue than in liver. Thus, it appears that the regulation of A-SAA in the context of obesity and insulin resistance has some unique features as compared to the APR. A-SAA induction is triggered primarily by cytokines such as IL-1 and TNF-α, and also by IL-6 and related cytokines (Scheja et al. 2008). It was found that A-SAA in tissues attracts inflammatory cells and inhibits the respiratory burst of leukocytes and modulates the immune response. Recent evidence also suggested a possible involvement of SAA in carcinogenesis (Malle et al. 2008).

3.5.3.2 C-reactive protein

C-reactive protein (CRP) is an APP produced primarily by the liver and by adipocytes. It is a member of the pentraxin family, consisted of 224 amino acids with a monomer molar mass of 25,106 Da (Steel and Whitehead 1994). CRP levels rises up to 1000-fold or even more as a response to infection, inflammation and tissue necrosis (Pepys and Hirschfield 2003). Viral infections tend to give a lower CRP level than bacterial infection. IL-6 considers as the major upregulator of CRP gene expression in the liver. CRP has a half-life of 19 hours, raises within 6 hours, and peaks at 48 hours. Therefore CRP is used mainly as a marker of inflammation and bacterial infection and can be used as a very rough proxy for heart disease risk (Shah et al. 2008). Smoking and obesity are positively correlated with CRP levels, whereas weight loss and cessation of smoking decrease CRP values (Danesh et al. 1999; Yudkin et al. 1999). CRP is also believed to play another important role in innate immunity, as an early defense system against infections (Nicolini et al. 2007). Recent research suggests that patients with elevated basal levels of CRP are at an increased risk of diabetes, hypertension and cardiovascular disease (Danesh et al. 2004). Elevated circulating markers of inflammation, in particular CRP, could help identify persons at risk for developing colorectal cancer (Erlinger et al. 2004).

3.6 Cancer

Cancer remains the second leading cause of death in the world. According to the American cancer society, 7.6 million people died from cancer a year. Recent toxicological and epidemiological studies indicated that chemicals present in the environment may be responsible for 80 to 90% of all cancer in humans. Dietary patterns, foods, and other dietary constituents are closely associated with the risk for several types of cancer such as gastric and colon cancer. It has been estimated that 35% of cancer deaths may be related to dietary factors (Ames and Gold 1998; Ramos 2008).

There are basically two major classes of environmental carcinogens; the potent carcinogens such as the aflatoxins and nitrosamines, which can produce cancer in laboratory animals even with very low concentrations, and the weak carcinogens such as atmospheric pollutants, a number of pesticides and food additives. In addition to the direct effect of such carcinogens, it was observed that several carcinogens are able to generate reactive oxygen spices and other free radicals that play an important role in the pathogenesis of the disease (Masuda et al. 2000; Yu et al. 2002). Since many different carcinogens occurring in food, water and air even at low concentrations, prevention either by reducing exposure to such carcinogens or by chemoprevention or diet modification such as increasing antioxidants, may be more effective than other traditional method for cancer treatment such as surgery, chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy.

3.6.1 N-nitroso compounds

N-nitroso compounds are a large group of genotoxic chemical carcinogens which occur in the human diet and other environmental media, and can be formed endogenously in the human body (Lin 1990; Tricker and Preussmann 1991). It can be divided into two classes: the class of nitrosoamines and the class of nitrosoamide. Compounds of both groups differ considerably in their chemical formation and biological effectiveness (Table 3.2) (Dietrich et al. 2005).

Table 3.2: Properties and differences between N-nitrosamines and N-nitrosamides (Dietrich et al. 2005).

Properties	**N-nitrosamines**	**N-nitrosamides**
Chemical structure	NO-group attached to nitrogen atom	NO-group attached to nitrogen atom Under acidic conditions
Formation	Generally under acidic conditions	
Formation catalyst	Thiocyanates	Citrate and other organic acids
Formation inhibitor	Redox compounds (ascorbate, tocopherols)	Redox compounds (ascorbate, tocopherols)
Stability/decomposition	Relatively stable in water Most are stable under neutral conditions Thermally stable Decompose under UV light	Unstable in aqueous solvents Unstable under neutral and alkaline conditions Thermally unstable (decompose readily at >100 °C) Decompose under normal light and UV light
Carcinogenicity	Require enzymatic activation by cytochrome P-450 enzymes to act as carcinogen	Do not require enzymatic activation to act as carcinogen; often act at site of occurrence
Tumor induction in rodents	Lung, liver, bladder, esophagus, pancreas, nasal cavity, kidney, trachea	Lymphatic system, central nervous system stomach, gastrointestinal tract, bone
Organ specificity	Yes	Suggested
Neurocarcinogens in animal studies	No	Yes
Chemical determination	Gas chromatography/thermal energy analyzer(GC/TEA)	HPLC/photolytic interface/thermal energy analyzer (HPLC/TEA) combined with gas chromatography/mass spectrometry (GC/MS) for structure confirmation

3.6.2 Formation of N-nitroso compounds

Both groups of N-nitroso compounds are characterized by a nitroso group (—N=O) attached to a nitrogen atom (—N—N=O). Both are formed by the reaction of a nitrite compound with amines or amides. N-nitrosamines are derived from nitrosation (reaction with nitrite) of secondary amines containing dialkyl, alkylaryl and diaryl substituents. N-nitrosamides are derived from nitrosation of amides such as N-alkylamides, N-alkylureas, and N-alkylcarbamates (see Equations in Figure 3.2).

Equation 1: Formation of the nitrosating species

$NO^-_2 + H^+ \rightleftharpoons HNO_2$ (nitrous acid)

$2HNO_2 \rightleftharpoons N_2O_3 + H_2O$ (nitrous anhydride)

$HNO_2 + H^+ \rightleftharpoons (H_2NO_2)^+$ (nitrous acidium ion)

Equation 2: Formation of *N*-nitrosamines

$RR'NH + N_2O_3 \rightleftharpoons RR'NNO + HNO_2$

Equation 3: Formation of *N*-nitrosamides

$RNHCOR' + (H_2NO_2)^+ \rightleftharpoons RN(NO)COR' + H_2O + H^+$

Figure 3.2: N-nitroso compounds formation (Dietrich et al. 2005).

Under acidic conditions, nitrite forms intermediate nitrosating species such as nitrous anhydride and the nitrous acidium ion (N_2O_3, $(H_2NO_2)^+$, respectively) (Mirvish 1995). In vitro, the nitrosation of amines is accelerated by thiocyanates, the nitrosation of amides is catalyzed by citrate and other organic acids (Dietrich et al. 2005). Organic acids and thiocyanates are compounds occurred naturally in foods and also used as food additives, and thus could be present to enhance nitrosation (Yamamoto et al. 1988). Under neutral conditions, nitrosation can take place via bacteria catalyzed processes. Bacterial nitrosation can for example occur at neutral pH in the hypochlorhydric stomach in individuals with chronic gastritis (Mirvish 1995). Hypochlorhydria facilitates the bacterial overgrowth in the stomach with nitrate-reducing bacteria. The formed nitrite can then promote the formation of N-nitroso compounds, catalyzed by bacterial strains with nitrosating activity. vitamins C and E are considered as nitrosation inhibitors by reducing nitrite to nitric oxide (NO), which is not directly a nitrosating agent (Tricker 1997). Certain types of vegetables are high in nitrate which can be reduced to nitrite by bacteria in the saliva. However, only about 5% of ingested nitrate is reduced to nitrite via this pathway (Dietrich et al. 2005). Oxides of nitrogen which formed during food processing, preservation and preparation, could act as direct nitrosating agents, with amino compounds and other nucleophiles to produce N-, C-, O-, and S-nitroso compounds (van Maanen et al. 1998). The major two sources of nitrosating agents in food are resulting from; the addition of nitrate and/or nitrite to foods, and the heating and/or drying of foods in combustion gases in which molecular nitrogen can be oxidized to oxides of nitrogen (Tricker and Preussmann 1991).

3.6.3 Occurrence of N-nitroso compounds

Diets, drinking water, smoking as well as cosmetics and pesticides consider the main sources for N-nitroso compounds (Tricker and Preussmann 1991). Moreover, they can be formed in the organism by endogenous reaction of amines with nitrite. Different type of foods (Table 3.3) contain high levels of these compounds specially those preserved by addition of either nitrate and/or nitrite, mainly processed meat (Larsson et al. 2006); food preserved by

smoking such as fish and meat products (Ologhobo et al. 1996; Tricker and Preussmann 1991); food subjected to drying by combustion gases (containing oxides of nitrogen) such as malt for the production of beer and whiskey, low-fat dried milk products and spices; pickled and salt-preserved foods, in particular plant-based products (pickled vegetables) in which microbial reduction of nitrate to nitrite occurs; food stored under humid conditions favoring fungal contamination, particularly the growth of fusarium moniliforme; migration and formation of nitrosamines from food contact materials. Nitrosamines present in rubber formulations can migrate into baby foods and drinks; N-nitrosated derivatives of certain pesticides (Masuda et al. 2000). Several studies reported that the exposure to nitrosamines from diet is generally ranged from 0.3-1.0 mg/day (Tricker and Preussmann 1991; van Maanen et al. 1998). N-nitrosamine especially dimethylnitrosamine (DEM) and diethylnitrosamine (DEN) may also be found in drinking water as a contaminant resulting from reactions occurring during chlorination or via direct industrial contamination (Frierdich et al. 2008). Because of the relatively high concentrations of DEM formed during wastewater processing with choler and boron the reuse of municipal wastewater is a particularly important area of concern (Schreiber and Mitch 2006). Nitrosamines could also occur in tobacco smoke via the interaction of nitrogen oxides and tobacco amines (Levy et al. 2004).

Table 3.3: Nitrosamines in foods and their carcinogenicity (Lijinsky 1999)

Nitrosamine	**Food and highest concentration recorded (µg kg^{-1})**	**Relative carcinogenicity**
N-Nitroso- Diethylamine	beer (8), bacon (17), cured meats (22), corn bread, seafood China (4.8), sausages (10), cheese(20).	++++
Dimethylamine	sausage (12), Thai fish (25), smoked pickled fish (32), dried milk(4.5), broiled squid Japan(300), salted meat Russia(54)	++++
Pyrrolidine	fried bacon (100), sausages China, Germany (45) broiled squid Japan(10), smoked meat (10), ham(36).	+++
Piperidine	bologna, sausages (50), spiced smoked meat(9), Chinese pickles (14), Thai fish (23), Thai pork (6), Tunis stew base (43),	+++
Methylbenzylamine	corn bread, China (100)	++++
Thiazolidine	smoked pork (5), sausage (5), smoked oysters (109), fried bacon (240), cured meats (27), smoked fish (2).	-
Proline	fried bacon (68), cured meats (400), smoked pork (2100), sausage (940), smoked oyster (167)	-
Thiazolidine	fried bacon (14000), cured meats (3900)	-
Carboxylic acid	smoked fish (1600)	-

3.6.4 Carcinogenicity of N-nitroso compounds

Although the carcinogenicity of N-nitrosoamines cannot be tested in humans, over 300 N-nitroso compounds have been shown to be carcinogenic in different animal species (Tricker 1997; Tricker and Preussmann 1991). However several studies observed that tumors induced by N-nitroso compounds in experimental animals showed similar morphological and biochemical properties to tumors found in the corresponding human suggested a possible link to the incidence of various cancers in humans (Eichholzer and Gutzwiller 1998; Pegg et al. 2000). Several nitrosamines for example are able to produce tumors in many species of animals and in a variety of organs (Lijinsky et al. 1992). Nitrosamines, in order to induce tumors, require metabolic activation through P450-catalyzed α-hydroxylation, generating unstable metabolites that will alkylate the DNA at the site of activation (Pinto 2000). It has been demonstrated that nitrosamine compounds can be activated by redox cycling producing derivative radicals and reactive oxygen species (ROS) which are able to induce mutagenicity leading to carcinogenicity (Boveris 1998).

3.6.5 Mechanisms of action

N-Nitrosamines are stable under physiological conditions and require metabolic activation by cytochrome P450. While N-Nitrosamides and their related compounds are chemically active under physiological pH conditions and decompose to form the same alkyldiazohydroxide species as an intermediate step in the production of an electrophilic alkyl diazonium ion, the ultimate carcinogen, which can react at nucleophilic sites of various cellular constituents. The alkylation of DNA is generally considered to be the critical cellular target for carcinogens in the initiation of cancer. The results of several *in vitro* studies suggest that N-nitroso compounds exhibit similar biological activity in human and animal tissues (Tricker and Preussmann 1991).

3.6.6 Diethylnitrosamine

Diethylnitrosamine (DEN), called also N-Nitrosodiethylamine (NDEA), is one of the most important environmental carcinogens, present in a variety of foods, including cured meats, smoked and salt-dried fish, grain products, cheese, soybeans and alcoholic beverages. It also detected in tobacco smoke, in baby pacifiers and baby-bottle nipples as contaminant in the rubber, and it can be synthesized endogenously (Lijinsky 1999; Tricker and Preussmann 1991; Yurchenko and Molder 2006). DEN is primarily used as a hepatocarcinogen to induce hepatocellular carcinoma. Moreover it can induce carcinogenesis in other organs like kidney, trachea, lung, esophagus, fore stomach, and nasal cavity (Enzmann et al. 1995; Verna et al. 1996). DEN is biologically and chemically inert unless activated in the biological systems, primarily in the liver by cytochrome P450s (CYP) which generate unstable metabolite with a very short half-life called ethyl-acetoxyethyl-nitrosamine (Anis et al. 2001; Yoo et al. 1990). This intermediate can be conjugated by the phase II enzymes to a nontoxic compound or it can produce ethyl-diazonium ion that directly interacts with cellular macromolecules and DNA producing mutation and further oncogenesis (Figure 3.3).

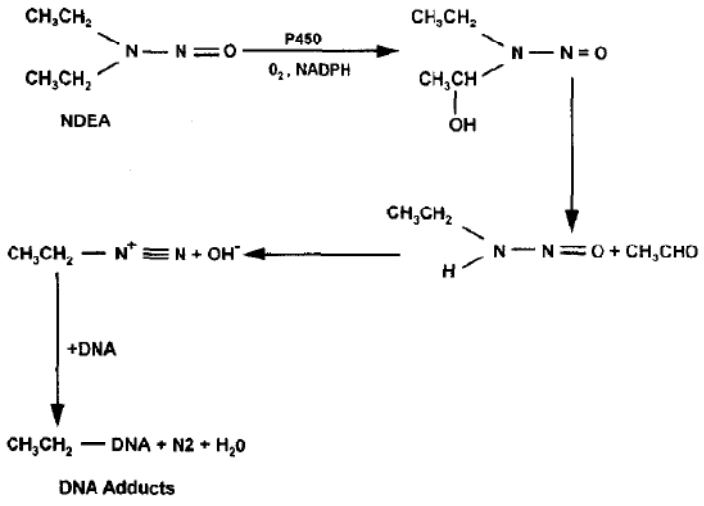


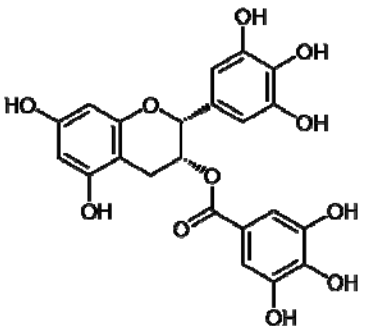
Figure 3.3: Biotransformation of DEN and mechanism of DNA-adduct formation (Verna et al. 1996).

Metabolic activation of DEN by cytochrome P450 enzymes is responsible for its cytotoxic, mutagenic and carcinogenic effects (Enzmann et al. 1995; Pinto et al. 2001; Verna et al. 1996) Administration of DEN to experimental animals has been reported to generate lipid peroxidation products and activated oxygen species during hepatocarcinogenesis. These free radicals may augment an oxidative stress by the formation of H_2O_2 and superoxide anions which contribute to the development of a diseased state, such as nitrosamine-induced malignancy or induce other oxidative stress diseases. Transportation of DEN through blood may also induce oxidative effects in the erythrocytes and other organs (Anis et al. 2001).

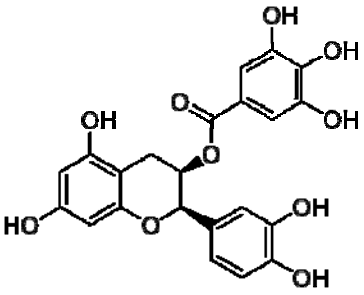
3.7 Antioxidants

3.7.1 Definition, classification and sources

Antioxidants can be defined as any substance that when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate. They neutralize free radicals by donating one of their own electrons and do not become free radicals by donating electrons because they are stable in either form (Young and Woodside 2001). There are two classes of antioxidants, endogenous and exogenous. The human body has its own endogenous antioxidant system consisting of antioxidant enzymes (e.g. superoxide dismutase, catalase and glutathione peroxidase) and relatively small molecule antioxidants (e.g. glutathione, ubiquinol (coenzyme Q), uric acid, melatonin, bilirubin etc.). It appears that endogenous antioxidants have evolved to meet the very basic requirement of overcoming oxidative stresses occurring during normal metabolic processes. Diet containing fruits, vegetables, nuts, whole grains, spices and herbs normally provides an exogenous source of antioxidants including fat soluble antioxidants, such as vitamin E, α, β-carotene, coenzyme Q10, lycopene, and water soluble antioxidants including vitamin C (Hu et al. 2000).



EGCG



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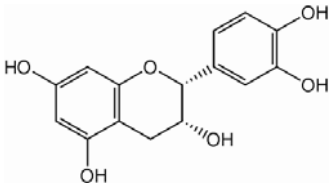
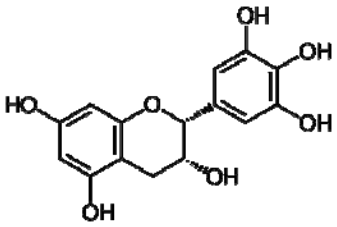
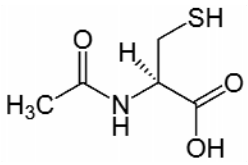


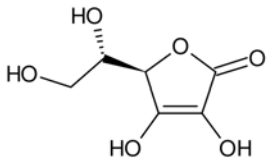
EC



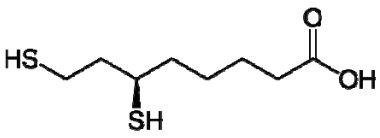
EGC



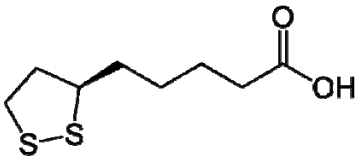
NAC



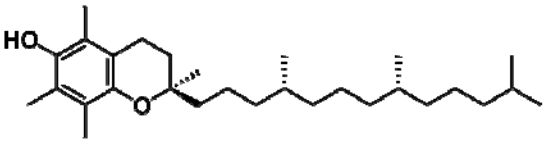
AA



DHLA



α-LA



α-TOC

Figure 3.4: Chemical structure of investigated antioxidants.

Lipoic acid (LA), N-acetylcysteine (NAC) and phenolic compounds specifically catechins (Figure 3.4) are also potent antioxidants, and may exert greater antioxidant potency than that of carotenoids and vitamins (Manach et al. 2004). Catechins or flavanols are one of the six classes of the flavonoids. With regard to their structure, the catechins are distinguished by a number of constitutive carbon atoms built upon a C6–C3–C6 flavone skeleton with different amounts and substituent patterns for hydroxyl groups resulting from interactions with gallic acid. Tea infusions contain high levels of catechins (102-418 mg of total catechins/L) and those reported till now include (+)-catechin, (+)-gallocatechin (GC), EC, EGC, ECG, EGCG (Arts et al. 2000). A number of epidemiological, animal and in vitro studies demonstrated a protective effect of such catechins against the development of certain cancers, coronary heart disease, and stroke (Agarwal et al. 1992; Ahmad and Mukhtar 1999; Laughton et al. 1991).

Numerous observations demonstrated unique, multifunction properties of lipoic acid (Packer et al. 1995a). Unlike all other antioxidants, LA is soluble in both water and fat and therefore, it is easily transported across cell membranes, enabling it to confer free radical protection both inside and outside cell. Moreover the oxidized and reduced forms of LA together constitute an effective redox couple with unique properties including; regeneration of exogenous and endogenous antioxidants such as vitamins C and E, and glutathione; quenching of reactive oxygen species; chelation of metal ions, and modulating transcription factor activity, especially that of nuclear factor-kappaB (NF-κB) (Biewenga et al. 1997; Packer 1998; Wollin and Jones 2003). Several studies demonstrated its preventive and therapeutic potency in a broad range of diseases such as diabetes, strokes and heart attack, HIV, atherosclerosis, cataract and acute and chronic neurological disorders (Moini et al. 2002b; Packer et al. 2001; Packer et al. 1997). Animal tissues including kidney 3.67 µg, liver 1.97 µg, heart 2.09 µg and brain 0.59 µg/g wet tissues are considered the main sources of LA where spinach was reported as the highest plant source (Satoh et al. 2008).

Antioxidants in a mixture or combinations may exhibit higher effect then that of single antioxidant. This may be explained by the synergistic effect between antioxidants (Murakami et al. 2003). Many plant sources specially fruits, vegetables, herbs, nuts, spices and many plant byproducts, are rich sources for antioxidants. Extracts of such plant may represent good examples for antioxidants combination although it may contain several unknown components. Despite solvent extraction may yield higher amount of an active compound than water extraction, the later is also considered since most of plants are eaten raw or processed in water.

3.7.2 Antioxidants and inflammation

A number of nutritional components are hypothesized to modulate inflammation, and hence impact on disease risk. The most extensively studied nutrients are the long-chain n^3 polyunsaturated fatty acids. However, limited evidence is also emerging with other antioxidant vitamins (Browning and Jebb 2006; De Lorgeril 2007). Based on the antioxidant activities, α-tocopherol, especially at high doses, has been shown to decrease the release of pro-inflammatory cytokines, the chemokine IL-8 and plasminogen activator inhibitor-1 (PAI-1) levels as well as decrease adhesion of monocytes to endothelium. In addition, α-tocopherol has been shown to decrease CRP levels, in patients with CVD and in those with risk factors for CVD (Singh et al. 2005). Phenolics such as quercetin able to block substances involved in allergies and is able to act as an inhibitor of mast cell secretion, causes a decrease in the release of tryptase, MCP-1 and IL-6 and the down-regulation of histidine decarboxylase mRNA from few mast cell lines (Shaik et al. 2006). Resveratrol also decreased both the inflammatory reaction and the stricture formation in experimental caustic esophageal burns (Uguralp et al. 2008). Results from multiple species support the antioxidant/anti-inflammatory properties of the prototype compound, astaxanthin, establishing it as an appropriate candidate for development as a therapeutic agent for cardiovascular oxidative stress and

inflammation (Pashkow et al. 2008). Extracts of rosemary can be considered an herbal anti-inflammatory and anti-tumor agent (Peng et al. 2007). Further mulberry fruit, a rich source of the major anthocyanin, cyanidin 3-glucoside (C3G), elicited protection against carrageenan-induced inflammation (Kim and Park 2006). Glabridin, a functional compound of liquorice, attenuates colonic inflammation in mice with dextran sulphate sodium-induced colitis (Kwon et al. 2008). Hence reactive oxygen species play a key role in enhancing the inflammation, it was found that curcumin able control undesired effects of oxidative stress and to inhibited inflammation and diabetes-induced elevation in the levels of IL-1β, VEGF and NF-kB (Rodriguez-Lopez et al. 1993).

3.7.3 Antioxidants and cancer induced by diethylnitrosamine

Carcinogenesis is a multi-factorial process that takes place over a considerable period of time. The well-recognized stages of initiation and promotion leading to the establishment of truly transformed cell types involve many stages at which free radical processes might be involved. Recent data, however, show that some dietary antioxidants such as vitamin E, vitamin C, selenium, and some phytochemicals may have potential as adjuvants in cancer therapy. Data concerning the protective effect of dietary component against cancer induced by DEN was limited. However it was found that rats pre-treated with vitamin E prior to DEN, had a reduced degree of oxidative stress (Bansal et al. 2005a). β-carotene inhibits rat liver chromosomal aberrations and DNA chain break after a single injection of diethylnitrosamine (Sarkar et al. 1997). Other studies confirm the fact that selenium is particularly protective in limiting the action of DEN by its antioxidant property (Thirunavukkarasu and Sakthisekaran 2001). Phenolic compounds such as ellagic acid and quercetin were more effective in decreasing the lipid peroxidation and increasing the GSH in mice treated with DEN (Khanduja et al. 1999). On the other hand silymarin exhibits hepatoprotective potential DEN induced hepatocellular damage in rats (Pradeep et al. 2007). Furthermore NAC exhibit attenuation in the esophageal carcinogenesis in rats treated with DEN (Balansky et al. 2002). Evidences

indicated that diallyl sulfide exerts a protective role on liver functions and tissue integrity in face of enhanced tumorigenesis caused by DEN, as well as improving cancer-cell sensitivity to chemotherapy (Ibrahim and Nassar 2008).

3.8 Relation between inflammation and cancer

The functional relation between inflammation and cancers was noticed since several decades. As early as 1863, Rudolf Virchow indicated that cancers tended to occur at sites of chronic inflammation (Balkwill and Mantovani 2001; Schottenfeld and Beebe-Dimmer 2006). However, accumulated epidemiologic studies support that chronic inflammatory diseases are frequently associated with increased risk of cancers (Coussens and Werb 2002). Hence many cancers arise from sites of infection, chronic irritation and inflammation (Table 3.4), it turned out that acute inflammation may contributed to the regression of cancer (Philip et al. 2004). The studies aiming at the relationship between inflammation and cancers first led to the determination whether the reactive oxygen and nitrogen species generated by inflammatory cells, such as leukocytes recruited to the inflammatory foci to kill infectious agents, may cause mutagenic assaults and result in tumor initiation (Okada 2002). Now, it has been realized that the development of cancers from inflammation might be a process driven by inflammatory cells as well as a variety of mediators, including cytokines, chemokines, and enzymes, which altogether establish an inflammatory microenvironment. Although the host response may suppress tumors, it may also facilitate cancer development via multiple signaling pathways (Yang et al. 2005).

Although it is now clear that proliferation of cells alone does not cause cancer, sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma, and DNA-damage-promoting agents, certainly potentiates and/or promotes neoplastic risk (Goswami et al. 2008). In addition, tumor cells have co-opted some of the signalling molecules of the innate immune system, such as selectins, chemokines and their receptors for invasion, migration and metastasis (Bartsch and Nair 2006). There is evidence

that inflammatory cytokines and chemokines, which can be produced by the tumor cells and/or tumor-associated leucocytes and platelets, may contribute directly to malignant progression. Many cytokines such as TNF, IL-1, IL-6 and chemokines are inducible by hypoxia, which is a major physiological difference between tumor and normal tissue (Hussain and Harris 2007). Although the mechanisms underlying the association between inflammation and cancer are still remain unraveled (Figure 3.5). The initial inflammation involves the recruitment of a wide range of immune cells to inflamed sites as well as the release of various proinflammatory cytokines and other agents which function in a coordinative manner to commence an inflammatory cascade (Macarthur et al. 2004). The aberrations in the apoptosis and phagocytosis of in situ inflammatory cells may lead to an unresolved chronic inflammation. In a setting of chronic inflammation, the persistent tissue damage and cell proliferation as well as the enrichment of reactive oxygen and nitrogen species contribute to a cancer-prone microenvironment. Moreover, several transcription factors such as NF-kB and enzymes, may regulate this complicated process (Lu et al. 2006).

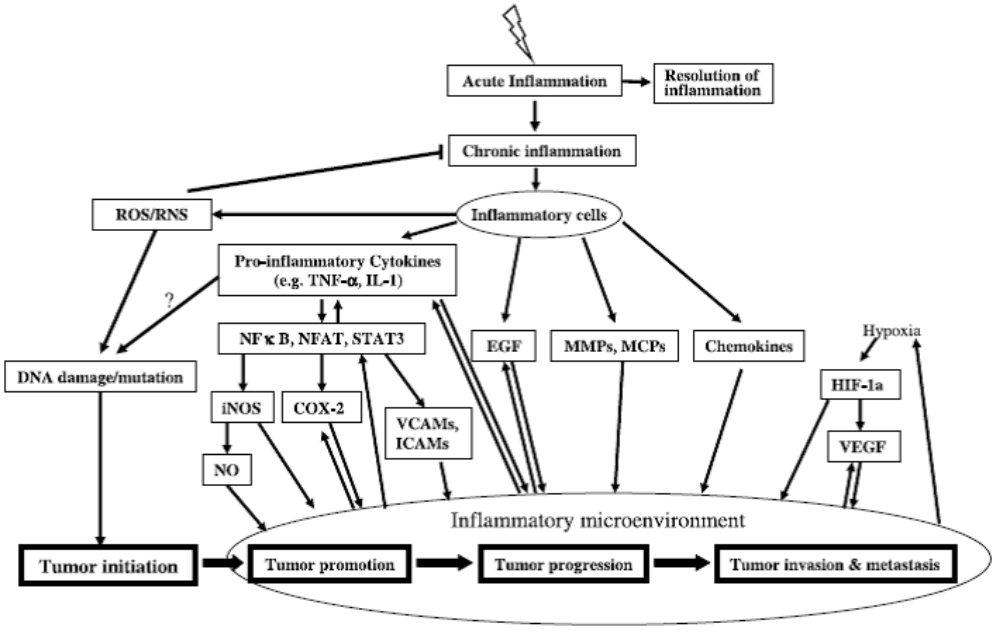


Figure 3.5: Summary of mechanisms for the involvement of inflammation in cancer development (Lu et al. 2006).

Table 3.4: Associations between inflammation and cancer risk (Coussens and Werb 2002).

Pathologic condition	Associated neoplasm(s)	Aetiologic agent
Asbestosis, silicosis	Mesothelioma, lung carcinoma	Asbestos fibres, silica particles
Bronchitis	Lung carcinoma	Silica, asbestos, smoking (nitrosamines, peroxides)
Cystitis, bladder inflammation	Bladder carcinoma	Chronic indwelling, urinary catheters
Gingivitis, lichen planus	Oral squamous cell carcinoma	
Inflammatory bowel disease, Crohn's disease, chronic ulcerative colitis	Colorectal carcinoma	
Lichen sclerosus	Vulvar squamous cell carcinoma	
Chronic pancreatitis, hereditary pancreatitis	Pancreatic carcinoma	Alcholism, mutation in trypsinogen gene on Ch. 7
Reflux oesophagitis, Barrett's oesophagus	Oesophageal carcinoma	Gastric acids
Sialadenitis	Salivary gland carcinoma	
Sjögren syndrome, Hashimoto's thyroiditis	MALT lymphoma	
Skin inflammation	Melanoma	Ultraviolet light
Cancers associated with infectious agents		
Opisthorchis, *Cholangitis*	Cholangiosarcoma, colon carcnoma	Liver flukes (*Opisthorchis viverrini*), bile acids
Chronic cholecystitis	Gall bladder cancer	Bacteria, gall bladder stones
Gastritis/ulcers	Gastric adenocarcinoma, MALT	*Helicobacter pylori*
Hepatitis	Hepatocellular carcinoma	Hepatitis B and/or C virus
Mononucleosis	B-cell non-Hodgkin's lymphoma, Burkitts lymphoma,	Epstein-Barr Virus
AIDS	Non-Hodgkin's lymphoma, squamous cell carcinomas, Kaposi's sarcoma	Human immunodeficiency virus, human herpesvirus
Osteomyelitis	Skin carcinoma in draining sinuses	Bacterial infection
Pelvic inflammatory disease, chronic cervicitis	Ovarian carcinoma, cervical/anal carcinoma	Gonnorrhoea, chlamydia, human papillomavirus
Chronic cystitis	Bladder, liver, rectal carcinoma, follicular lymphoma of the spleen	Schistosomiasis

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Plant samples

Fresh samples of five food plants and three byproducts under investigation; green tea (*Camellia sinensis),* artichoke (*Cynara scolymus),* broccoli (*Brossica oleracea)*, red onion (*Allium cepa), spinach* (*Spinacia oleracea*) eggplant (*Solanum melongena)*, sweet orange (*Citrus sinensis)*, potato (*Solanum tuberosum),* were purchased from local supermarkets in Berlin, Germany.

4.1.2 Chemicals and reagents

Folin–Ciocalteau reagent, TPTZ (2,4,6-tripyridyl-s-triazine), ethanol and DMSO were purchased from Merck (Darmstadt, Germany). ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) and trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) were from Fluka Chemicals (Buchs, Switzerland). Alpha lipoic acid (α-LA), ascorbic acid (AA), DL- tocopherol (DL-TOC), N-acetylcysteine (NAC), (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCG), gallic acid, caffeine, catechin, chlorogenic acid, caffeic acid, ferulic acid, rutin, quercetin, interleukin-6 (IL-6), interleukin-1 (IL-1β), α-tumor necrosis factor (TNF-α), dexamethazone (Dex), HEPES, sodium bicarbonate and o-phenylenediamine dihydrochloride solution, neutral red (3-amino-7-dimethyl-amino-2-methylphenazine hydrochloride) all were purchased from Sigma (Deisenhofen, Germany). RPMI-1640, fetal bovine serum and penicillin/streptomycin were from Gibco Laboratories (Karlsruhe, Germany). Transthyretin (TTR) and retinol binding protein (RBP) antibodies were obtained from DakoCytomation (Hamburg, Germany). The standard TTR and RPB protein was from Dade Behring (Marburg, Germany). All other chemicals used were purchased from Roth (Karlsruhe, Germany).

4.1.3 Equipment

High performance liquid chromatography

All analysis was carried out with Shimadzu 10A system (Duisburg, Germany) using a Supelcosil - LC18 column (250 mm × 4.6 mm ID, 5 µm).

Surface enhanced laser desorption/ionisation time of flight mass spectrometry (SELDI-TOF MS)

SELDI-TOF-MS-based ProteinChip System (PBS II, Ciphergen Biosystems, Fremont, USA) with ProteinChip arrays was used to determine the TTR microheterogeneity.

Microplate reader

Microplate reader (Bio-Rad, Germany) was used to measure the absorption of 96-well microtitre plates.

Spectrophotometer

Novaspec II (Pharmacia Biotech, Cambridge, England) was used to measure the phenolic content.

Micocentrifuge

Microcenterfuge (Heraeus instrument, Germany) was used for immune-participation procedure.

4.1.4 HepG2 cells

HepG2, human hepatoma cells (ATCC, Rockville, MD) were grown in complete RPMI-1640 with 2 mM L-glutamine supplemented with 1.5 g/l sodium bicarbonate, 20 mM HEPES, 10% fetal bovine serum, and a 1% penicillin/streptomycin mixture. Cells were maintained in a humidified atmosphere of 95% air with 5% CO_2 at 37°C, and sub-cultured at a 1:6 ratio approximately once a week. Confluent cells were harvested and seeded at 5×10^5 cells in 75 cm^2 tissue culture flask. The medium was changed every 48h.

4.2 Methods

4.2.1 Plant extract preparations

4.2.1.1 Preparation of the water extracts

The edible portions of artichoke, broccoli, spinach and onion as well as the peels of eggplant, orange and potato were prepared manually and washed twice with tap and distilled water. Each sample was then chopped to small pieces and soaked in distilled water (1:5) over night at 4 ^{o}C. Samples were then homogenized in a homogenizer (Bosch, Germany) and the resulting homogenates were filtered with a three-layer of cheese cloth. The crude juices were centrifuged at 20,000 x g for 30 min to remove any particulate material. The supernatants were further filtered through two Millipore filters (0.45 and 0.22 µM). The filtrates were lyophilized and the lyophilized extracts were stored in an airtight container at -80 ^{o}C until application. The green tea *was* powdered in a coffee grinder (Bosch, Germany) for 5 min. The ground powder was mixed with distilled water (1:10) and boiled for 10 min. the supernatant was recovered through vacuum filtration using filter paper No.1 then thought two Millipore filters (0.45 and 0.22 µm). The filtrate was also lyophilized and stored at -80 ^{o}C until using.

4.2.1.2 Preparation of the solvent extracts

The edible portions of artichoke, broccoli, spinach and onion as well as the peels of eggplant, orange and potato were prepared manually and washed twice with tap and distilled water. Each sample was then chopped to small pieces and then lyophilized. Fifty grams of finally ground freeze-dried samples were extracted three times with four volumes of 70% ethanol by shaking for 1 hour at room temperature. The obtained residues after filtration were further extracted two times with three volumes of 70% acetone by shaking for 1 hour also at room temperature. All supernatants were combined and centrifuged at 20,000 x g for 30 min to remove any particulate material. The supernatants were further filtered through two Millipore filters (0.45 and 0.22 µM).

The combined filtrates were subjected to vacuum distillation at 45°C and 27 mm Hg (1mm Hg ≈133 pa) to remove ethanol and acetone. The resulting aqueous extract was then freeze-dried. The freeze-dried extracts were stored in an airtight container at -80°C until using.

4.2.2 Determination of total phenolic content

Total phenolic content (TPC) of the samples was estimated using Folin–Ciocalteau procedure (Singleton et al. 1999), with few modifications. Briefly Folin–Ciocalteau reagent was diluted 1:10 using deionized water. Samples were dissolved also in deionized water in concentrations of (0.2-2.0 mg/ml depending on the activity). The diluted reagent (0.5 ml) was mixed with (0.5 ml) sample in test tubes and held at room temperature for 5 min. Then 2 ml of 7.5% sodium carbonate solution was added. The tubes were mixed and allowed to stand for 15 min at room temperature. The absorbance of the solutions was measured at 765 nm by a Novaspec II spectrophotometer (Pharmacia Biotech, Cambridge, England). The total phenolic content was expressed as mg gallic acid equivalents (GAE)/g lyophilized extract.

4.2.3 HPLC analysis

One mg of the water or solvent freeze-dried extracts was dissolved in 1 ml eluent A or 70% methanol respectively. Chromatography was carried out on a Shimadzu 10A system (Duisburg, Germany) using a Supelcosil - LC18 column (250 mm × 4.6 mm ID, 5 µm, flow rate: 1 ml/min, dual wavelength UV-Vis detection at 280 and 254 nm) with a column temperature of 40°C. The eluents were A = 2 % acetic acid pH 1.9 and B = methanol. The gradient was applied under the following conditions: 15 % eluent B, 0 min; 15–35 % eluent B, 27 min; 35–68 % eluent B, 8 min; 68 % eluent B, 5 min; 68–15 % eluent B, 5 min; 15 % eluent B, 15 min (regeneration/equilibration). Run time was one hour and the injection volume was 20 µl. The quantification was performed using an external calibration with diluted stander mixtures containing gallic

acid, caffeine, EGC, catechin, chlorogenic acid, caffeic acid, EC, EGCG, ferulic acid, ECG, rutin and quercetin (10 – 50 µg/ml in 50 % aqueous methanol)

4.2.4 Determination of antioxidants activities

4.2.4.1 Trolox equivalent antioxidant capacity assay

Trolox equivalent antioxidant capacity assay (TEAC) is based on the ability of antioxidant molecules to scavenge the long lived $ABTS^{\bullet+}$ cations. The procedure applied was as described by Re et al. (Re et al. 1999) with a few modifications, where the $ABTS^{\bullet+}$ cation was produced by reacting of ABTS solution (7 mM) with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{\bullet+}$ solution was diluted with 5 mM PBS pH 7.4 to read an absorbance of 0.7 ± 0.2 at 730 nm. A series of dilutions of the water and solvent lyophilized extracts were prepared using 5 mM PBS or with PBS: methanol (3:7 v/v) to yield concentrations ranging from 0.1-1mg/ml. An aliquot of 10 µl of each sample solution was added to 150 µl of ABTS solution and 6 min after initial mixing the absorbance was measured at 730 nm using a microtitre plate reader. TEAC values were obtained by comparing the absorption obtained from tested samples with those obtained from trolox which served as standard. Results are expressed as mM trolox equivalents (TE)/g lyophilized extract.

4.2.4.2 Ferric reducing antioxidant power assay

Ferric reducing antioxidant power assay (FRAP) is based on the reduction of the Fe^{3+}-TPTZ complex to the ferrous form at low pH. This reduction is monitored by measuring the absorption change at 593 nm. The FRAP was measured according to Benzie and Strain (Benzie and Strain 1996) with minor modification. Briefly FRAP reagents included: 250 mM acetate buffer, pH 3.6; 20 mM $FeCl_3$ in the acetate buffer and 10 mM TPTZ (2,4,6-tripyridyl-s-triazine; prepared by mixing 7,8 mg TPTZ in 2.5 ml 40 mM HCl and 2.5 ml $FeCl_3$ solution). A series of dilutions of the lyophilized extracts were prepared using

deionized water or 70% aqueous methanol to yield concentrations ranging from 0.1-1 mg/ml. An aliquot of 10 µl of each dilution was added to 150 µl FRAP reagent solution and 6 min after initial mixing the absorbance was measured at 593 nm using a microtitre plate reader. FRAP values were obtained by comparing the absorption obtained from tested samples with those obtained from ascorbic acid which served as standard. The results are expressed as mM ascorbic acid equivalents (AAE)/g lyophilized extract.

4.2.5 Experimental design to investigate the anti-inflammatory effect of antioxidants and plants extracts using HepG2 cells

To estimate the anti-inflammatory effect of plant extracts, cells were grown in complete medium in 24-well tissue culture plate at 5×10^4 cells per well till 80% confluence. The medium was then replaced with serum free medium for 12 h. The culture supernatants collected and served as control zero time. Plates were then divided in two groups, first group: cells were incubated for 36 h with serum free medium containing serial concentrations (0-500 µg/ml) of the plant extracts (stock solutions of the water extracts were prepared using medium where stock solutions of solvent extracts were prepared using 70% aqueous ethanol). The culture supernatants of this group served as positive controls. Second group: cells were first incubated for 12 h with serum free medium containing serial concentrations of plant extracts (0-500 µg/ml), and then 25 ng/ml IL-6 was added to these cells for further 24 h. In both groups, cells treated with medium or medium containing solvent (did not exceeded 0.5% v/v) served as non-treated controls where cells treated with IL-6 served as negative or treated control. At the end of experiment, culture supernatants of both groups were collected and centrifuged at 10,000 rpm for 5 min at 4°C to remove detached cells then stored at -80°C until assayed for the negative acute phase proteins TTR and RBP as inflammatory biomarker using ELISA. The cells were also harvested after washing twice with ice-cold PBS buffer pH 7.4 and stored at -80°C. All experiments were assayed with triplicate incubations and triplicate determinations.

To estimate the anti-inflammatory effect of selected antioxidants, cells were grown in complete medium in 24-well tissue culture plate at 5×10^4 cells per well to 80% confluence, and then replaced with serum free medium for 12 h. This medium was then collected and served as control zero time. Cells were then stimulated with 25 ng/ml IL-6 for 24 h in serum free medium, then treated for 8 h with different concentrations of the following antioxidants: LA (0–0.8mM in ethanol), EGCG and ECG (0–25 mM in ethanol), EGC and EC (0–50 mM in ethanol), TOC (0–50 mM in DMSO) AA and NAC (0–1mM in medium). A set of non-IL-6 treated cells, exposed to equivalent concentrations of ethanol or DMSO (not exceeding 0.1% v/v) served as culture controls. At the end of experiment, culture supernatants were collected and applied as mentioned above for TTR and RBP ELISA procedure.

4.2.6 Quantitative analysis of TTR by ELISA assay

TTR was quantified in culture supernatants using a sandwich ELISA procedure developed in our laboratory. A 96-well microtitre plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were filled with rabbit anti-human TTR IgG; 50 µl/well of 0.5 mg/ml in 50 mM carbonate buffer (pH 9.6). Plats were then incubated with dry shaking for 1 h at 37°C and then stored overnight at 4°C. The wells were then washed 4 times with PBS/Tween buffer (consisting of 10 mM phosphate buffer with 150 mM NaCl and 0.05% Tween 20 pH 7.4). After washing, 200 µl/well of 0.5% bovine serum albumin (BSA) in PBS buffer was added to block nonspecific bindings and then incubated at 37°C for 1h. After four further washing, the wells were filled with 50 µl/well TTR standard solution (N Protein Standard/Standard SL OQIM 13, Dade Behring GmbH, Marburg, Germany) or diluted culture supernatant (1:5 in PBS/BSA 0.05%), and incubated with shaking for 1 h at 37°C. The plates were then washed and filled with 50 µl/well conjugated HRP anti TTR (diluted 1:3000 in PBS/BSA 0.05%) and incubated with shaking for 1 h at 37°C. After four final washings, color was developed using O-phenylenediamine dihydrochloride (OPD, 100 ml/well of 3.7 mM solution in 50 mM disodium phosphate-25 mM citric acid buffer pH 5.2

containing 0.012% H_2O_2) for 10 min at room temperature. The reaction was stopped by the addition of 1 M H_2SO_4 (50 µl/well) and measured at 490 nm with a microtitre plate reader. Samples were analyzed in triplicate. The standard curve obtained at each plate was used to calculate the TTR concentrations in the samples. The curve obtained by serial dilution of culture supernatant was parallel to the standard curve, indicating that the antigen measured was TTR. The sensitivity of the assay was 10 nM defined as the minimal concentration of TTR that produces an absorbance greater than ten standard deviations of the blank readings. Intra- and inter-assay coefficient of variation (CV) was 8.8% and 8.1%, respectively.

4.2.7 Quantitative analysis of RBP by ELISA assay

RBP was quantified in culture supernatants using a sandwich ELISA procedure developed in our laboratory. A 96-well microtitre plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were filled with rabbit anti-human RBP IgG (50 µl/well of 1.2 mg/ml in 50 mM carbonate buffer (pH 9.6). plats were then incubated for 1 h at 37°C with dry shaking and stored overnight at 4°C. The wells were then washed 4 times with PBS/Tween buffer (consisting of 10 mM phosphate buffer with 150 mM NaCl and 0.05% Tween 20 pH 7.4). After washing, the wells were filled with 50 µl/well RBP standard solution (N Protein Standard/Standard SL OQIM 13, Dade Behring GmbH, Marburg, Germany) or diluted culture supernatant (1:5 in PBS/BSA 0.05%), and incubated with shaking for 1 h at 37°C. After four further washings, the plates were filled with 50 µl/well peroxidase- coupled anti-RBP IgG (diluted 1:2000 in PBS/BSA 0.05%) and incubated with shaking for 1 h at 37°C. After four final washings, colour was developed (using OPD, 100 ml/well of 3.7 mM solution in 50 mM disodium phosphate-25 mM citric acid buffer pH 5.2 containing 0.012% H_2O_2) for 20 min at room temperature. The reaction was stopped by the addition of 1 mol/l H_2SO_4 (50 µl/well) and absorbance at 490 nm was measured with a microtitre plate reader. Samples were analyzed in triplicate. The standard curve obtained at each plate was used to calculate the RBP concentrations in

the samples. The curve obtained by serial dilution of culture supernatant was parallel to the standard curve, indicating that the antigen measured was RBP. The sensitivity of the assay was 10 nM/l, defined as the minimal concentration of RBP that produces an absorbance greater than ten standard deviations of the blank readings. The intra-assay coefficient of variation for samples was 2.9% and the interassay variation was 4.2%.

4.2.8 Quantitative analysis of TTR and RBP by SELDI-TOF-MS

Culture supernatant was analyzed using the SELDI-TOF-MS-based ProteinChip System (PBS II, Ciphergen Biosystems, Fremont, USA) with ProteinChip arrays. Proteins were enriched on three different surfaces: (i) a strong anion exchange, with cationic, quaternary ammonium groups as spot surfaces that interact with the negative charges on the surface of target, (ii) a weak cation exchange, with anionc, carboxylate groups that interact with positive charges as spot surfaces and (iii) a normal phase surface, with active spots containing silicon oxide which allows proteins to bind via serine, threonine or lysine. The strong anion exchange spot surface was first equilibrated by incubating chips in 0.1M Tris/HCl pH 7.5 for 10 min at room temperature with shaking. On the other hand the weak cationic exchange spot surface was equilibrated by incubating chips in 0.1M sodium acetate pH 4.5 for 10 min at room temperature with shaking. Following equilibration, buffer was carefully aspirated without allowing spots to dry. Subsequently, 300 µl of culture supernatant was mixed with 190 µl of binding buffer and added to the spot surface using a bioprocessor. Peptides and proteins were captured on both surfaces after being incubated for 1 h at room temperature with shaking. Unbound proteins and other components were removed from the spot surfaces by washing the spots (twice for 10 min) with 300 µl binding buffer. Thereafter, the arrays were then quickly rinsed with de-ionized distilled water to remove salts. Excess water was subsequently removed. The normal phase chip surface was incubated for 10 min with 300 µl of sample at room temperature and rinsed twice with distilled water for a few seconds. All spot surfaces were then,

treated twice with 1 µl of a saturated energy-absorbing molecule (EAM) solution [sinapinic acid dissolved in 50% acetonitrile and 0.5% trifluoracetic acid (TFA)], and the spots were allowed to dry. Samples were run concurrently and intermingled on the same chip. The instrument was used in a positive ion mode, with an ion acceleration potential of 20 kV and a detector gain voltage of 2 kV. The chips were analyzed manually under the following settings: laser intensity 260/230, detector sensitivity 9 and molecular mass range 0.2-200 kDa, position 20–80 and a 150–190 shot average per sample. All mass spectra were normalized to have the same total ion current. The m/z range from 0 to 3000 was eliminated from the analysis because this area contains adducts possibly from the chip surfaces and artifacts from matrix components.

4.2.9 Experimental design to investigate TTR microheterogeneity

To investigate the effect of antioxidants agents and solvent extracts of plants on the heterogeneity of TTR, a confluent HepG2 cells were incubated for 24h in serum free medium containing two different concentration of each tested substances as following: EC, EGC (50, 100 µM), ECG, EGCG (25, 50 µM) NAC (1, 5 mM), AA , α-LA (0.5, 1mM), α-TOC (100, 200µM), artichoke, eggplant peel, spinach (50, 125 µg/ml), onion (125, 250 µg/ml) orange peel (250, 500 µg/ml), green tea, broccoli (5, 25 µg/ml), and potato peel (25, 50 µg/ml). Cells incubated with medium or medium containing solvent (did not exceeded 0.5% v/v) served as untreated control. Changes in TTR microheterogeneity are detected using immunoprecipitation assay combined with SELDI-TOF-MS technique.

4.2.10 TTR Immunoprecipitation assay

One ml of culture supernatant was incubated with 10 µl of a polyclonal rabbit anti-human TTR or RBP and 10µl of 1mg/mL Sephadex G-15 disolved in HPLC water. The mixture was incubated over night with shaking at 30°C. The mixture was then centrifuged at 13 000 x g for 20 min at room temperature.

The supernatant was carefully removed, and the immunoprecipitated complex of TTR or RBP and antibody was then washed twice with PBS pH 7.4 and one time with HEPES (5mM HEPES pH 7.2) and the final precipitate was redissolved in 10 µl of HPLC water. Each 5 µl was introduced in a spot in two different chips and allowed to dry on air. Spots were then; treated twice with 1 µl of sinapinic acid solution and the spots were allowed to again to dry. The chips were then analyzed using SELDI-TOF-MS under the following settings: laser intensity 250/220, 150-190 shot average per sample, detector sensitivity 9, molecular mass range 0.2 to 200 kDa, and position 20–80.

4.2.11 Experimental design to investigate the cytotoxicity of diethylnitrosamine using HepG2 cells

Approximately 3×10^4 cells in 0.2 ml complete RPMI medium were added to each well of a 96-well tissue culture microtiter plate. The cells were incubated for 24 h and then treated with fresh medium containing various concentrations of DEN (0-100 mM). Each concentration was added to six parallel wells. Cells were then incubated for different time period (24, 48 and 72 h). Cell viability was then measured by neutral red (NR) assay. The initial (IC90) and midpoint (IC50) values, the concentration resulting in 10 and 50% inhibition of cytotoxicity parameters, were calculated from the dose response curves.

4.2.12 Experimental design to investigate the cytotoxicity of antioxidant agents and plant extracts using HepG2 cells

Approximately 3×10^4 cells in 0.2 ml complete RPMI medium were added to each well of a 96-well tissue culture microtiter plate. The cells were incubated for 24 h and then treated with fresh medium containing serial concentrations of antioxidants agents as following: EC, EGC (0-100 µM), ECG, EGCG (0-100 µM), NAC, AA, α-LA (0-10 mM), α-tocopherol (0-0.2 mM), where plant solvent extracts concentrations were (0-1 mg/ml). Stock solutions of the catechins as well as plant extracts were prepared using 70% aqueous ethanol. NAC, LA, and

AA were dissolved in medium where α-tocopherol was dissolved in acetone. Cells treated with medium or medium containing solvent (did not exceeded 0.5% v/v) served as controls. Each tested substance as well as controls was added to six parallel wells. Cells were then incubated for 24 or 48h and prepared for NR assay. The IC90, IC50 values were calculated from the dose response curves.

4.2.13 Experimental design to investigate the protective effect of antioxidant agents and plant extracts on DEN induced hepatotoxicity

To study the protective effects of antioxidants and plant extracts on DEN cytotoxicity, approximately 3×10^4 cells in 0.2 ml complete RPMI medium were added to each well of a 96-well tissue culture microtiter plate. The cells were incubated for 24 h and then treated for 8 h with fresh medium containing serial non-toxic concentration of such antioxidants and plant extracts as following: EC, EGC (0-100 µM), ECG, EGCG (0-50 µM), NAC (0-10 mM), AA (0-2.5 mM), LA (0-1 mM), α-tocopherol (0-0.2 mM), onion, orange peel (0-500 µg/ml), artichoke, broccoli, spinach, eggplant peel (0-250 µg/ml) where green tea, potato peel (0-125 µg/ml). Then a fixed dose of 35 mM DEN (a concentration induces ≈ 40% mortality in the HepG2 cells after 48h as calculated from concentration-response curve) was added to cells for further 48 h. Cells treated with only DEN were served as negative control where cells treated incubated with only medium or medium containing solvent (didn't exceeded 0.5% v/v) served as non-treated control. Each tested substance was tested in six replicates. At the end plats were prepared for neutral red assay.

4.2.14 Neutral red assay

The neutral red (NR) assay (3-amino-7-dimethyl-amino-2-methylphenazine hydrochloride) is a cell viability chemosensitivity assay, based on the ability of viable cells to bind and incorporate a neutral red dye into the lysosomes. The

protocol was adopted and modified from (Babich and Borenfreund 1991) and used to measure the cytotoxicity of test substances. Briefly, the medium was gently removed then the cells rinsed with PBS buffer. Thereafter a 0.2 ml medium (pre-incubated overnight at 37^{o}C) containing 50 µg/ml NR was added to each well. After incubation for approximately 3 h to allow uptake of the dye into viable cells, the cells were immediately rinsed with a fixative (0.1% $CaCl_2$ in 0.5% Formaldehyde) or PBS buffer. The NR dye was then extracted with solubilization solution (1% acetic acid in 50 % aqueous ethanol). After a brief agitation (10 min) on a microtiter plate shaker, the absorbance of the extracted dye was measured using a spectrophotometric plate reader with a 540 nm filter.

4.2.15 Statistics

Results were expressed as mean ± SD, and the significance of differences among deferent treatments was obtained by using one-way analysis of variance and Student's *t*-test. Non-parametric Spearman rank correlation coefficients were used to test the association between relevant parameters. Values of $P < 0.05$ were regarded as statistically significant. The statistical analyses were carried out using SPSS program.

5 RESULTS AND DISCUSSION

5.1 Phenolic content, antioxidant activity and anti-inflammatory potential of selected plant extracts on HepG2 cell

5.1.1 Results

5.1.1.1 Phenolic content

Total phenolic content (TPC) was measured by the Folin–Ciocalteu colorimetric assay, using gallic acid as a standard phenolic compound. A linear calibration curve of gallic acid with R^2 0.99 was obtained in a range of 1–100 µg/ml. TPC of different plant water and solvent extracts are given in Figure (5.1-a). As shown, TPC the of water extracts varied widely from 4.55 to 177.08 mg GAE/g lyophilized extract, where green tea was > eggplant peel > orange peel > artichoke > potato peel > broccoli > spinach > onion. This order has been changed in solvent extracts where green tea was > artichoke > potato peel > orange peel > eggplant peel > spinach > broccoli > onion with TPC ranging from 13.01 to 891.95 mg GAE/g lyophilized extract. Solvent extracts gave higher TPC compared with those of water extracts, except eggplant peel where no significant difference between its water and solvent extract was detected. In both water and solvent extracts, green tea extract gives the highest TPC whereas onion showed the lowest content.

5.1.1.2 HPLC analysis

Individual phenols in both water and solvent extracts were analyzed by HPLC using UV detector at 280 nm and external standard of 12 phenols including gallic acid, chlorogenic acid, caffeic acid, ferulic acid, catechin, EC, ECG, EGC, EGCG, rutin, quercetin as well as caffeine. HPLC chromatogram of the standards applied is shown in Figure (5.2) giving a good separation where Figures (5.3 and 5.4) illustrate the HPLC chromatograms of water and solvent extracts respectively. Further, Tables (5.1 and 5.2) summarize the corresponding concentration of each identified phenols as well as caffeine in mg/g lyophilized extract.

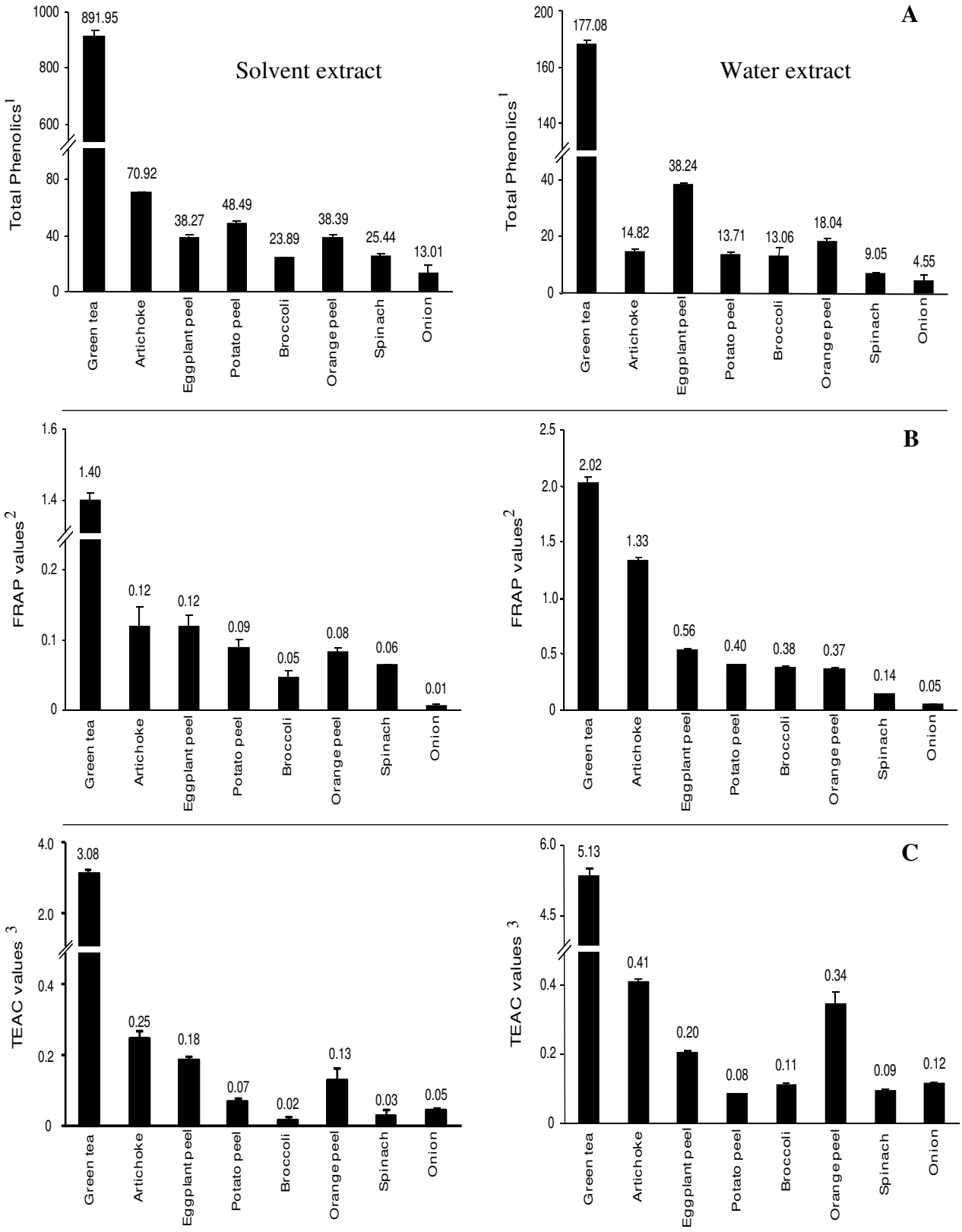


Figure 5.1: Phenolic content and antioxidant activity of plant extracts

Data represent the mean of three replicates ± SD.

A: Phenolic content, B: FRAP values, C TEAC: values.

[1] Data are expressed as mg gallic acid equivalents (GAE)/g lyophilized extract.

[2] Data are expressed as mM ascorbic acid equivalents (AAE)/g lyophilized extract.

[3]Data are expressed as mM trolox equivalents (TE)/g lyophilized extract.

As shown, HPLC results confirmed those obtained by Folin–Ciocalteu colorimetric assay, in which total identified phenols in solvent extracts recorded higher values than water extracts. Further investigation to the data revealed a strong correlation between TPC determined by Folin–Ciocalteu and those determined by HPLC with R^2=0.96 and 0.99 for water and solvent extract, respectively. Green tea extracts recorded the highest identified phenolic compounds whereas broccoli gives the lowest values in both water and solvent extract.

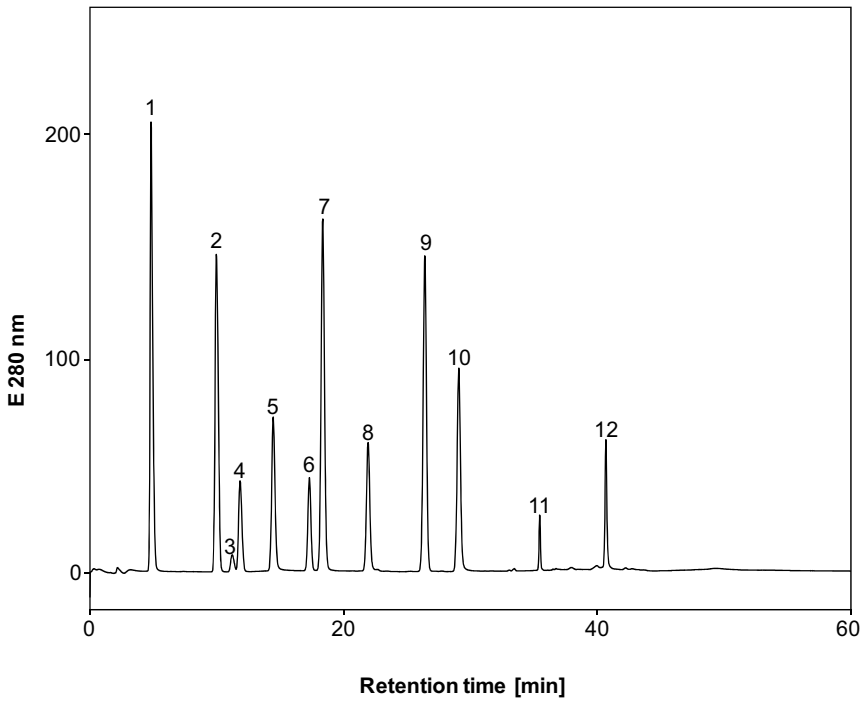


Figure 5.2: HPLC chromatogram of standards phenolic compounds and caffeine. Peaks: 1 = gallic acid, 2 = caffeine, 3 = EGC, 4 = catechin, 5 = chlorogenic acid, 6 = EC, 7 = caffeic acid, 8 = EGCG, 9 = ferulic acid, 10 = ECG, 11 = rutin, 12 = quercetin.

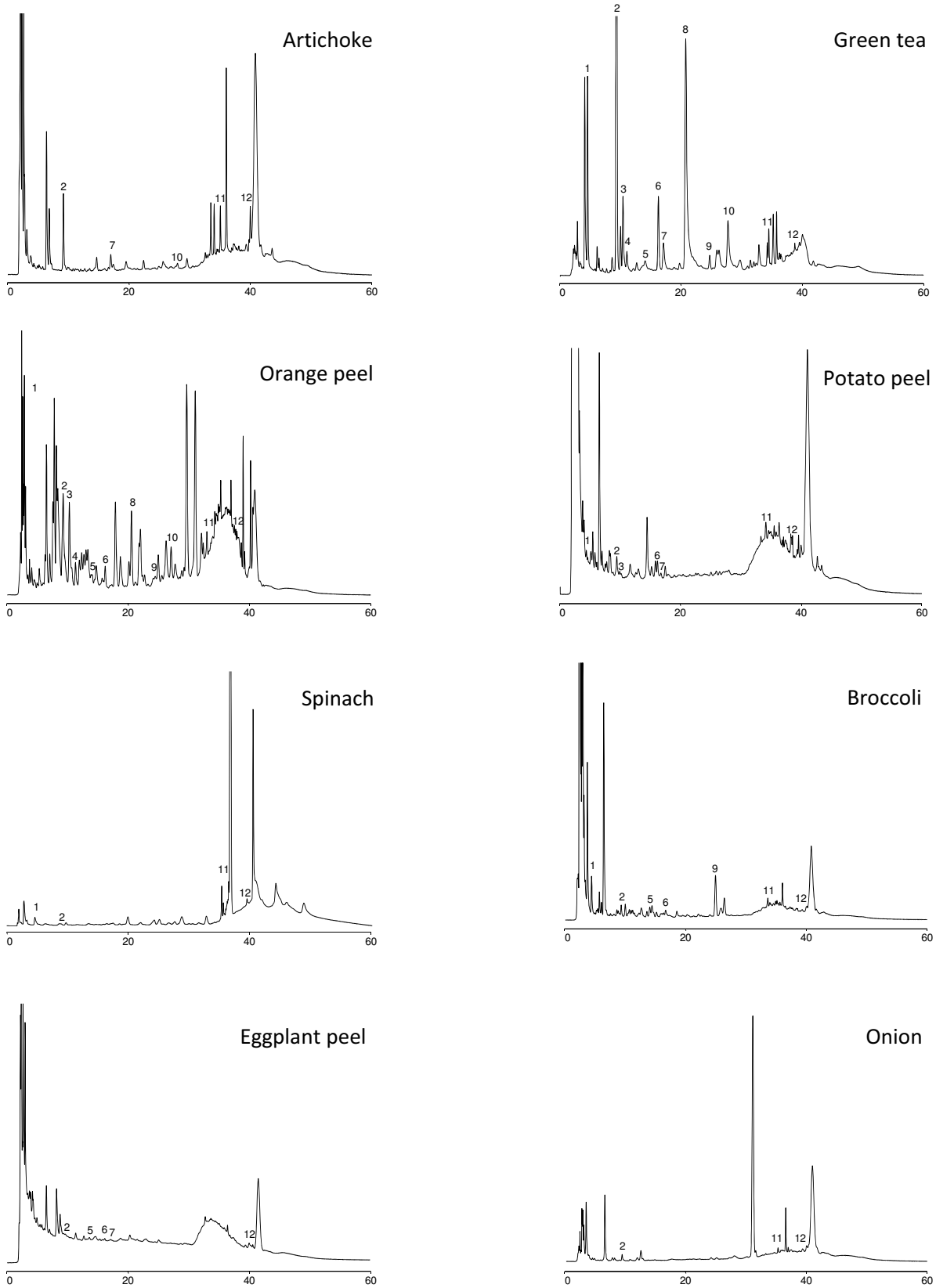


Figure 5.3: HPLC chromatograms of phenolic compounds detected in plants **water** extract Peaks: 1 = gallic acid, 2 = caffeine, 3 = EGC, 4 = catechin, 5 = chlorogenic acid, 6 = EC, 7 = caffeic acid, 8 = EGCG, 9 = ferulic acid, 10 = ECG, 11 = rutin, 12 = quercetin.

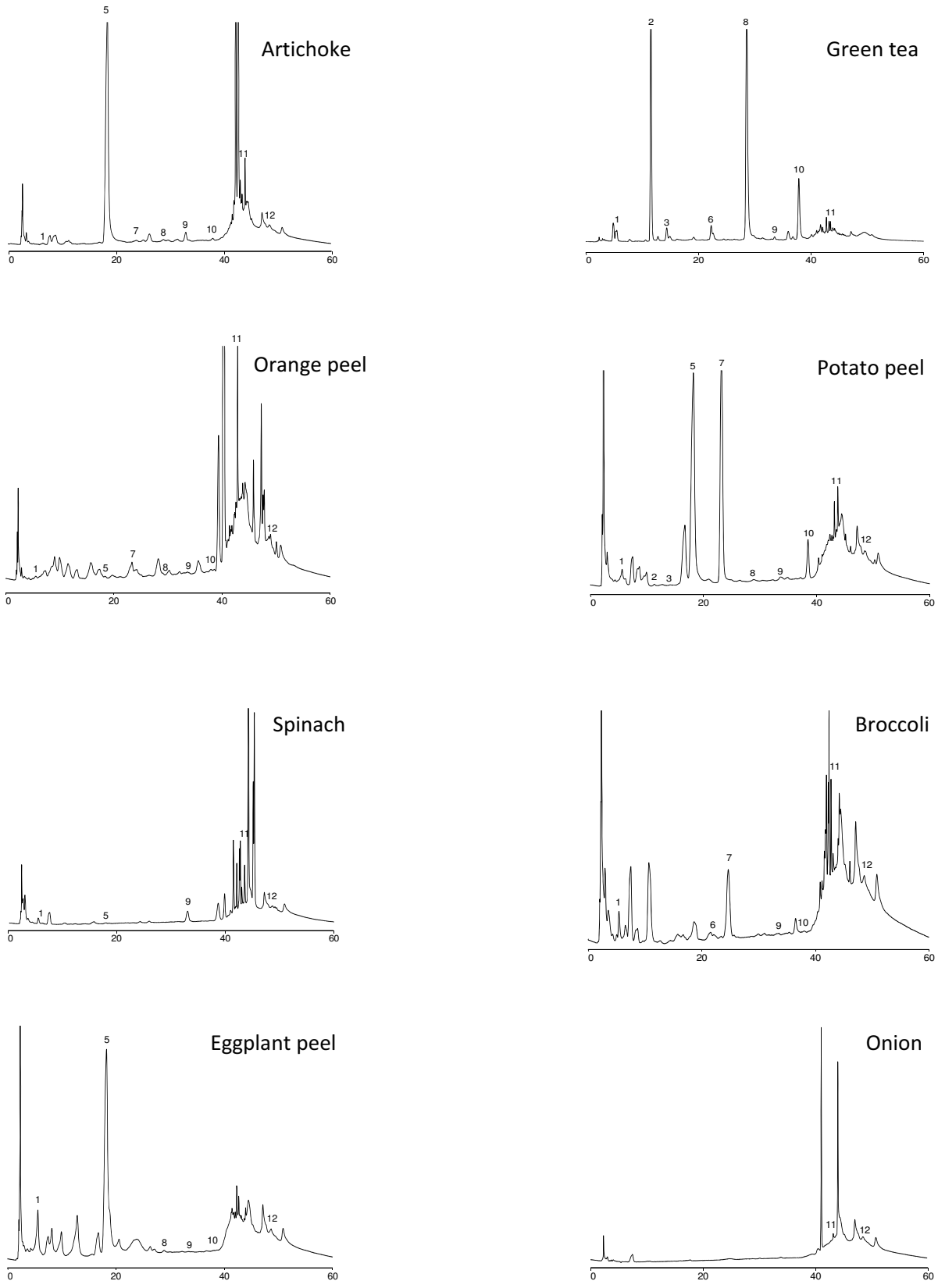


Figure 5.4: HPLC chromatograms of phenolic compounds detected in plants **solvent** extract Peaks: 1 = gallic acid, 2 = caffeine, 3 = EGC, 4 = catechin, 5 = chlorogenic acid, 6 = EC, 7 = caffeic acid, 8 = EGCG, 9 = ferulic acid, 10 = ECG, 11 = rutin, 12 = quercetin.

Table 5.1: Composition and quantities (mg/g lyophilized extract) of the identified phe and caffeine in the **water** extracts as determined by HPLC.

Extracts / Compounds	Green tea	Artichoke	Broccoli	Spinach	Onion	Eggplant peel
Gallic acid	4.81	-	0.04	0.08	-	-
Caffeine	50.87	0.24	-	0.04	0.05	0.17
EGC	79.9	-	-	-	-	-
Catechin	3.52	-	-	-	-	-
Chlorogenic acid	1.61	-	-	-	-	0.18
EC	13.56	-	-	-	-	0.24
Caffeic acid	2.12	0.05	-	-	-	0.06
EGCG	48.55	-	-	-	-	-
Ferulic acid	0.66	-	-	-	-	-
ECG	7.75	-	-	-	-	-
Rutin	5.69	0.84	-	1.03	0.13	-
Quercetin	1.94	3.17	0.14	0.87	2.1	2.25
Total*	220.98	4.3	0.18	2.02	2.28	2.9

* Caffeine not included

Table 5.2: Composition and quantities (mg/g lyophilized extract) of the identified phe and caffeine in the **solvent** extracts as determined by HPLC.

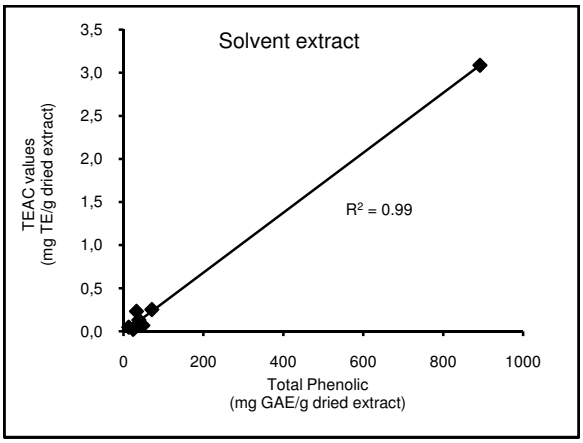
Extracts / Compounds	Green tea	Artichoke	Broccoli	Spinach	Onion	Eggplant peel
Gallic acid	4.73	0.12	0.39	0.91	-	1.83
Caffeine	87.01	-	-	-	-	-
EGC	71.26	-	-	-	-	-
Catechin	-	-	-	-	-	-
Chlorogenic acid	-	41.29	-	0.89	-	20.54
EC	35.99	-	0.35	-	-	-
Caffeic acid	-	0.47	0.03	-	-	-
EGCG	261.45	0.89	-	-	-	0.24
Ferulic acid	0.97	0.72	0.04	0.95	-	
ECG	49.75	0.57	0.05	-	-	0.10
Rutin	24.11	0.45	4.22	3.87	6.46	-
Quercetin	-	3.48	2.93	2.19	2.81	2.54
Total*	535.27	47.99	8.01	8.81	9.27	25.25

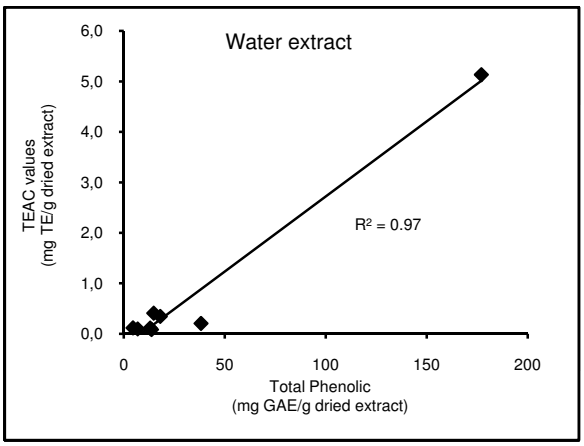
* Caffeine not included

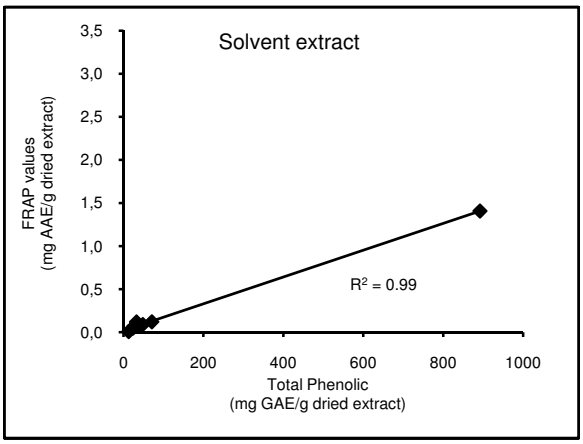
5.1.1.3 Total antioxidant capacity

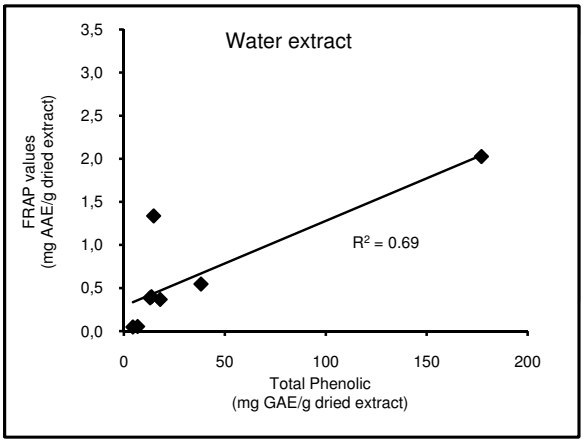
Total antioxidant capacity (TAC) of different plant water and solvent extracts were determined using FRAP and TEAC assays. Data of both assays are given in Figure (5.1-b,c). As shown, a large variation between different plant extracts was observed. TAC of water extract as determined by TEAC assay was widely varied from 0.08 to 5.13 TE/mg lyophilized extract. Green tea was > artichoke > orange peel > eggplant peel > onion > broccoli > spinach > potato peel. This order was changed in solvent extract where green tea was > artichoke > eggplant peel > orange peel > potato peel > onion > spinach > broccoli with TAC values ranged from 0.02 to 3.08 TE/mg lyophilized extract. On the other hand TAC values obtained by FRAP assay of water extract was ranged from 0.05 to 2.02 mM AAE/mg lyophilized extract where green tea > artichoke > eggplant peel > potato peel > broccoli > orange peel > spinach > onion. This order was also changed in solvent extract where TAC values ranged from 0.01 to 1.40 mM AAE/mg lyophilized extract and green tea was > artichoke > eggplant peel > potato peel > orange peel > spinach > broccoli > onion.

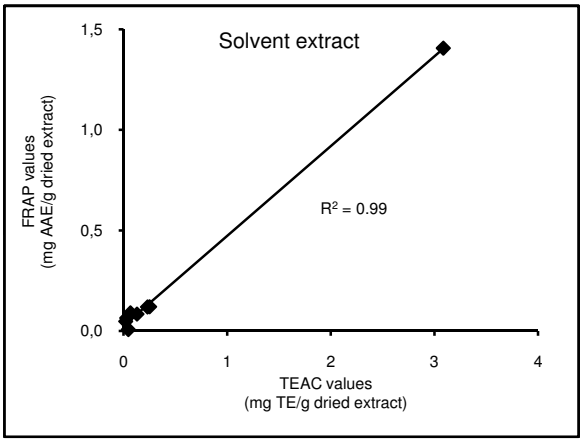
In general, TAC values obtained using FRAP assay were mostly lower than those obtained by TEAC assay. Furthermore TAC values of all water extracts, were significantly higher than those of the solvent extracts using both FRAP and TEAC assays. Green tea followed by artichoke significantly recorded the greatest TAC in both water and solvent extracts as measured by both assays. Water extract of onion and potato peel and solvent extract of onion and broccoli exhibited the lowest TAC values ($p<0.05$). Figure (5.5) shows different relationships between TEAC and FRAP, and between TEAC and/or FRAP and total phenolic, of both water and solvent extracts. As shown, in solvent extracts, strong correlations ($R^2=0.99$) were obtained between FRAP and TEAC and between TEAC and/or FRAP and total phenolic content. A similar strong correlation ($R^2=0.97$) also obtained in water extracts only between TEAC and total phenolic. Whereas other correlations in water extracts between FRAP and TEAC and between FRAP and total phenolic, were quite weak ($R^2=0.69$-0.71).











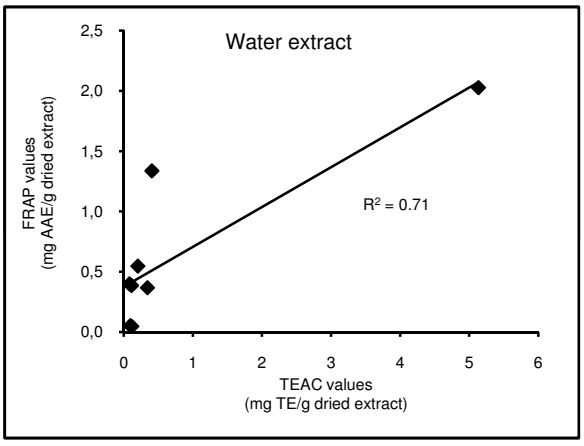


Figure 5.5: Different relations between total phenolic content and antioxidant activity of plant extracts. Each point represents the mean of three replicates

5.1.1.4 Effect of plants extract on TTR secretion in HepG2 cells

To evaluate the effect of plant extracts on TTR produced by HepG2 cells, confluent cells were incubated with serial concentrations (0-500 µg/ml) of plant extracts dissolved in serum free medium, Culture supernatants were collected after 36 h incubation and assayed for TTR using ELISA assay. Figure (5.6) illustrates the influence of different water on TTR secreted by hepG2 cells. As shown, there were significant elevations in TTR production in a dose dependent manner achieved by six plant extracts in the following concentration ranges: green tea (5-25 µg/ml), artichoke (5-500 µg/ml), orange peel (50-500 µg/ml), spinach (125-500 µg/ml), eggplant and potato peels (250-500 µg/ml). Whereas other concentration ranges as well as broccoli and onion extracts induce reduction in TTR compared to non-treated control. Water extracts were ordered upon their potency on TTR as following: green tea > artichoke > potato peel > orange peel > spinach > eggplant peel.

Figure (5.7) summaries the effect of different solvent extracts on TTR secreted by hepG2 cells. All plant solvent extract except broccoli, showed an increase in TTR production in a dose dependent manner in the following ranges: green tea (1-25 µg/ml), artichoke and spinach (5-50 µg/ml), orange peel (50-500 µg/ml), eggplant peel (25-125 µg/ml), onion (5-125 µg/ml), and potato peel (1-50 µg/ml). Other concentrations out of these ranges as well as broccoli extract induce a negative effect in TTR secretion. Solvent extracts were ordered upon their potential effects on TTR as following: green tea ≥ artichoke ≥ potato peel > spinach > eggplant peel ≥ onion > orange peel. With regard to the highest effect achieved by plant extracts on TTR secretion, it was found that water extracts of green tea, artichoke and orange peel, exhibited higher effects than their solvent extracts. Whereas water extracts of eggplant peel, potato peels, spinach, and onion showed lower effect than their solvent extracts.

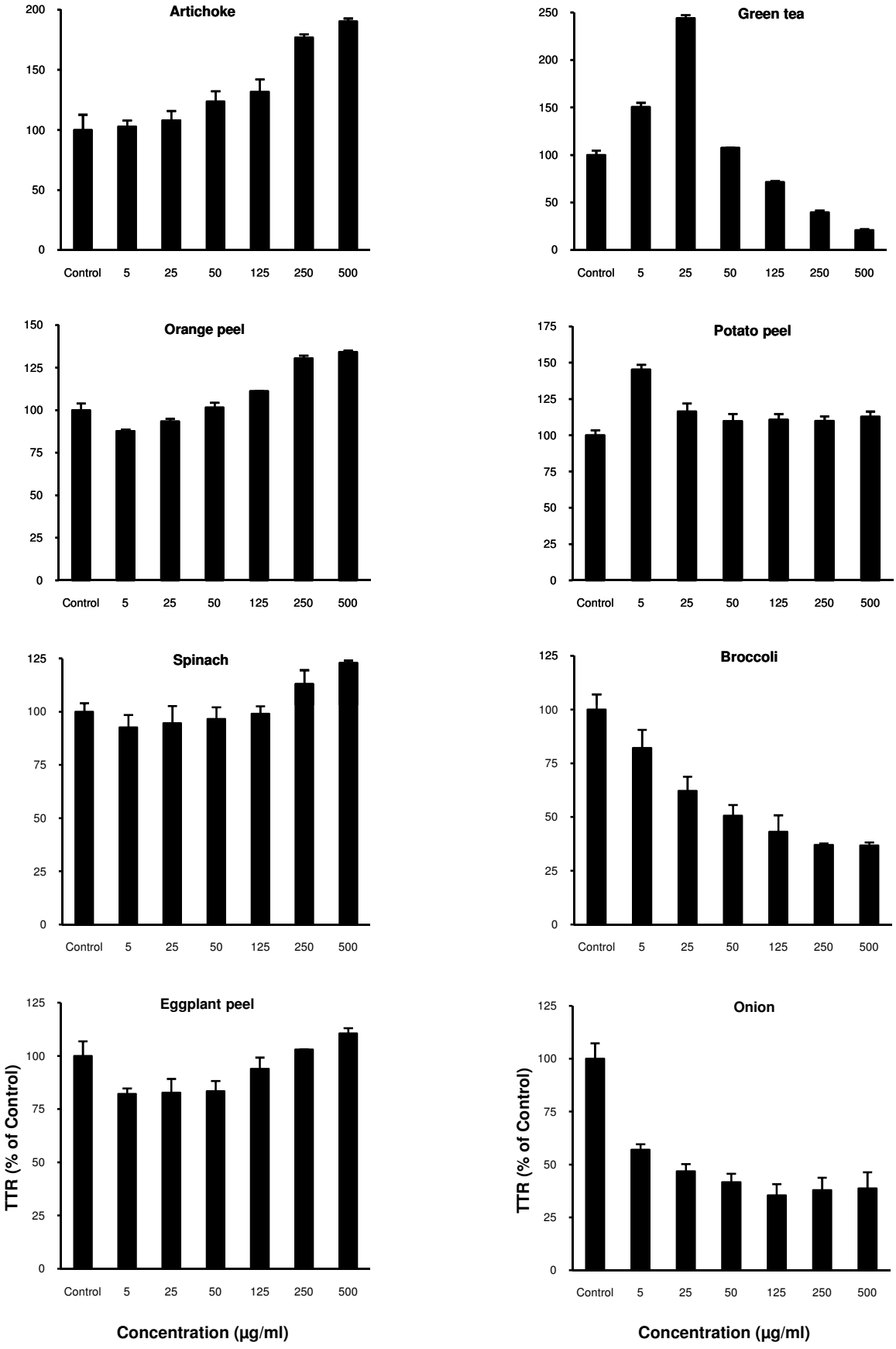


Figure 5.6: Influence of plants **water** extracts on TTR secretion of HepG2 cells.*

* Confluent HepG2 cells were incubated in serum free medium containing serial concentrations of plant extracts. After 36h, culture supernatant was collected and assayed for TTR using ELISA assay. Data represent the mean of three replicates ± SD.

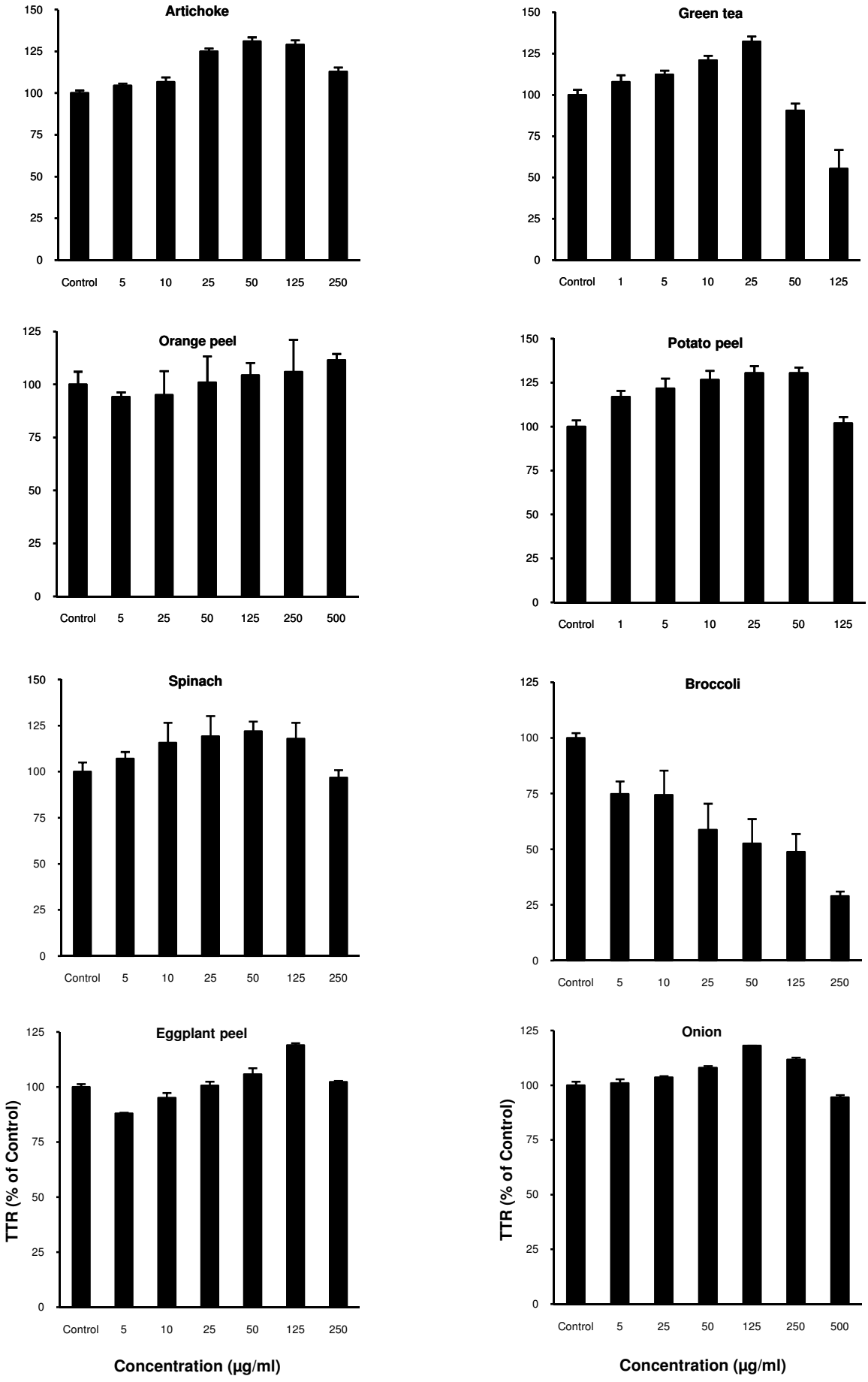


Figure 5.7: Influence of plants **solvent** extracts on TTR secretion of HepG2 cells.*

* Confluent HepG2 cells were incubated in serum free medium containing serial concentrations of plant extracts. After 36h, culture supernatant was collected and assayed for TTR using ELISA assay. Data represent the mean of three replicates ± SD.

5.1.1.5 Effect of plant extracts on TTR secretion in IL-6 treated cells

To investigate the anti-inflammatory effect of plant extracts on IL-6 treated HepG2 cells, they were first incubated for 12 h in serum free medium containing different plant extracts, and then treated with 25 ng IL-6/ml for further 24 h. Culture supernatants were then collected and analyzed for TTR using ELISA assay. Figure (5.8) illustrates the effect of water extract in TTR production as percentage of non-treated control where Figure (5.9) illustrates those of solvent extracts. As shown in both, IL-6 significantly reduces TTR secretion by 50.5±3.69 % compared to non-treated control ($p<0.001$). Six water extracts (Figure 5.8) were able to elevate TTR levels compared to IL-6 treated control in a dose dependent manner at the following concentrations: artichoke, orange peel, spinach, and eggplant peel in a range of 5-500 µg/ml, green tea in a range of 5-25 µg/ml and potato peel at 25 µg/ml. Contrary broccoli, onion as well high concentration of green tea (125-500 µg/ml) induce negative effects. On the other side, solvent extracts (Figure 5.9) were also able to increase TTR levels compared to IL-6 treated controls. With exception of orange peel which showed undetectable effect and high concentrations of broccoli and green tea (over 125 µg/ml), all other solvent extracts were significantly able to increase TTR levels compared to IL-6 treated control in a dose dependent manner of the following concentration: green tea (1-25 µg/ml), artichoke and broccoli (5-50 µg/ml), eggplant peel, onion (5-250 µg/ml), spinach (5-125 µg/ml) and potato peel (1-50 µg/ml). In general, both IL-6 treated and untreated cells water extracts of green tea, artichoke and orange peel, recorded significant elevation in TTR compared to those of solvent extracts ($p<0.05$), where water extracts of eggplant, potato peels, spinach and onion showed lower effect than their solvent extracts. Further, both water and solvent induce lower effect in (IL-6) treated cells than those of untreated. Plant extracts potency in elevating TTR secretion remain as in untreated IL-6 cells, where the order for the water extracts was green tea > artichoke > potato peel > orange peel > spinach > eggplant peel and for the solvent extracts: green tea > artichoke > potato peel > spinach > eggplant peel > onion > orange peel.

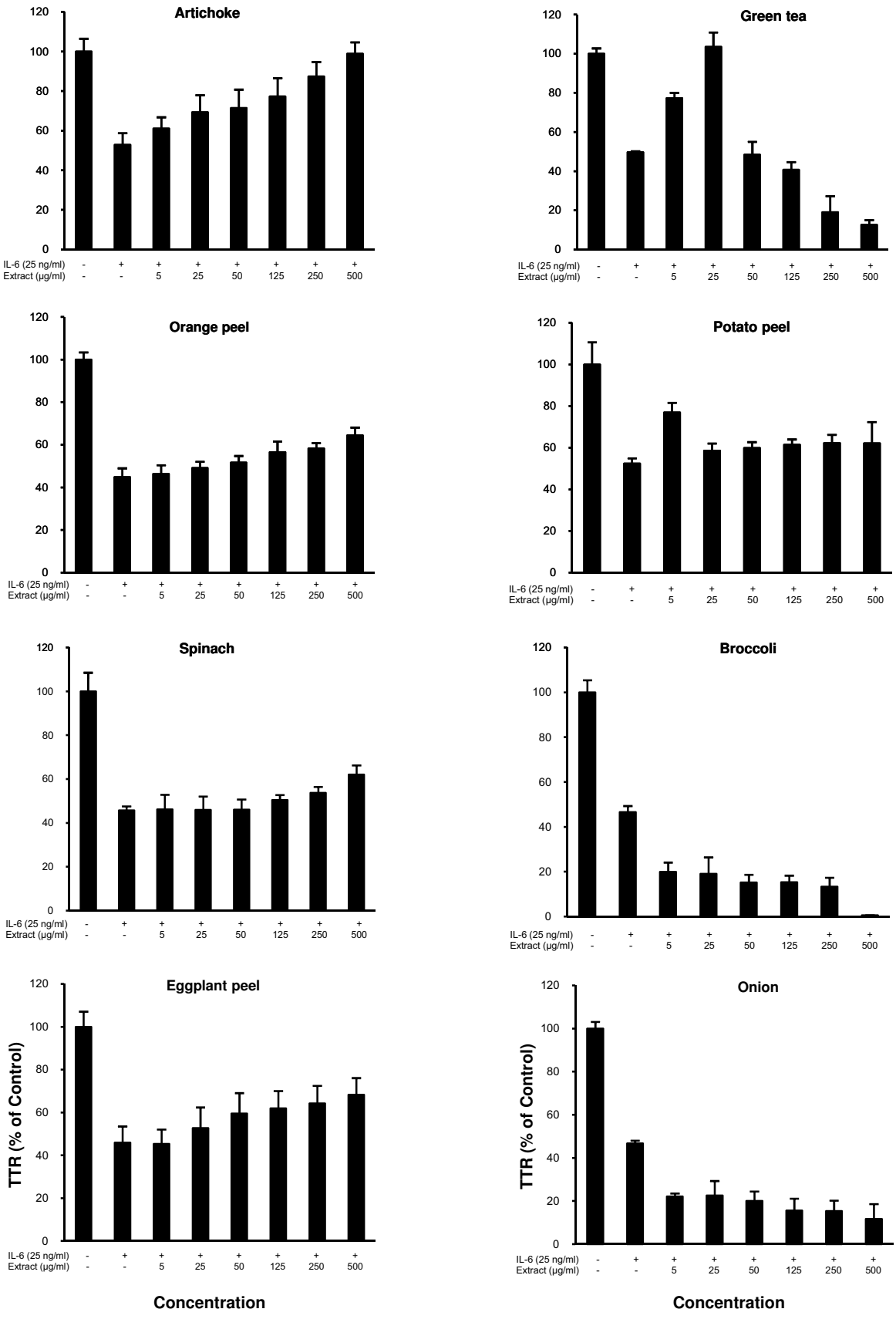


Figure 5.8: Influence of plants **water** extracts on TTR secretion of HepG2 cells treated with IL-6.*

* Confluent cells were first incubated in serum free medium containing serial concentrations of plant extracts for 12h, and then IL-6 (25 ng/ml) was added for further 24h. Culture supernatant was collected and analyzed for TTR using ELISA assay. Data represent the mean of three replicates ± SD.

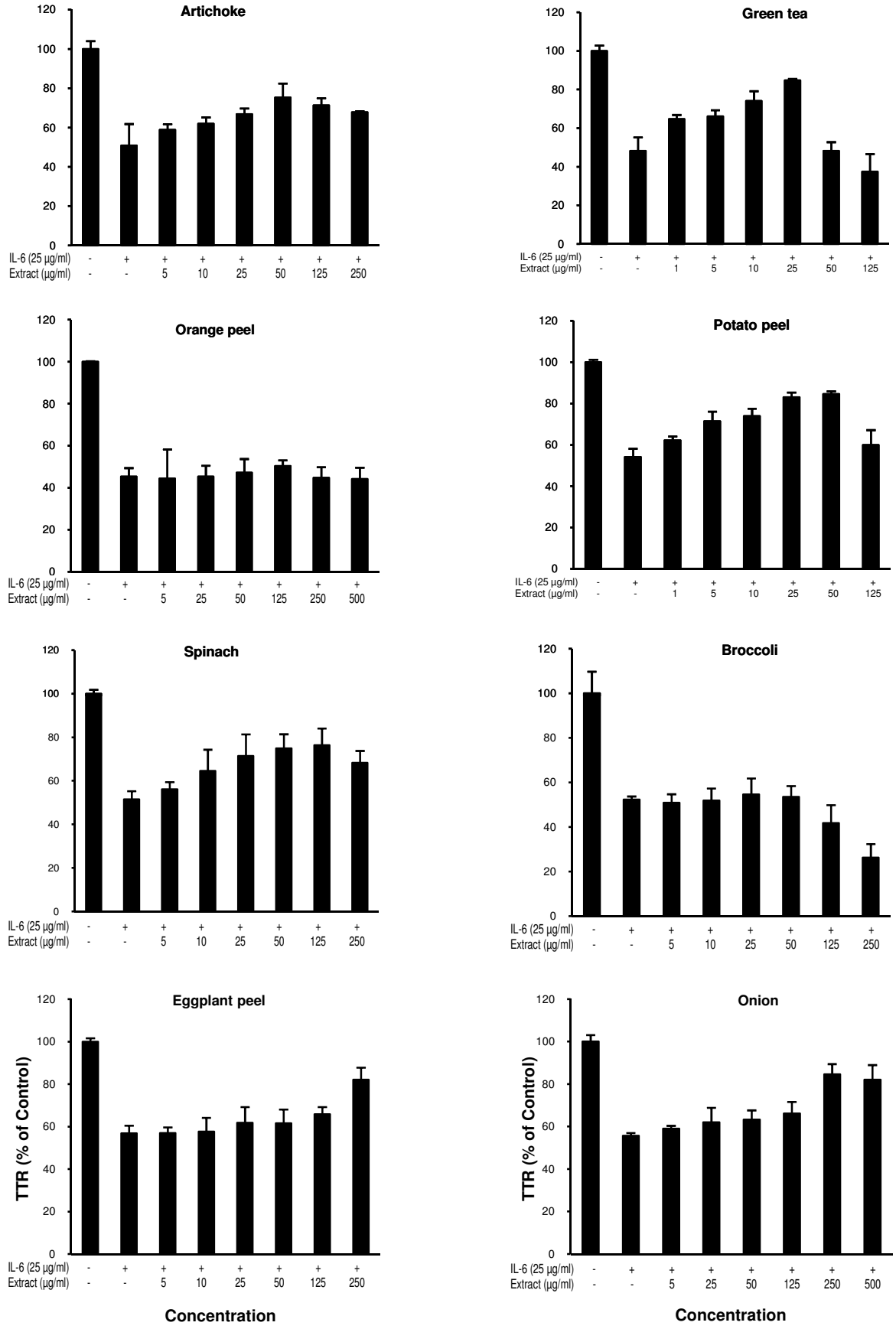


Figure 5.9: Influence of plants **solvent** extract on TTR secretion of HepG2 cells treated with IL-6.*

* Confluent cells were first incubated in serum free medium containing serial concentrations of plant extracts for 12h, and then IL-6 (25 ng/ml) was added for further 24h. Culture supernatant were collected and analyzed for TTR using ELISA assay Data represent the mean of three replicates ± SD.

5.1.2 Discussion

5.1.2.1 Phenolic content

The Folin–Ciocalteu assay has for many years been used as a measure of total phenolics in natural products. It gives a crude estimation of the total phenolic compounds present in an extract. But several interfering substances and non-phenolic compounds may react with the reagent, giving elevated apparent phenolic concentrations (Prior et al. 2005). Moreover, various phenolic compounds respond differently in this assay, depending on the number of phenolic groups they have. Total phenolic content does not incorporate necessarily all the antioxidants that may be present in an extract. This may explain the equivocal correlation between total phenolic content and antioxidant activity of several plant species (Tawaha et al. 2007).

The Folin–Ciocalteu and HPLC results showed that solvent extraction (using 70% aqueous methanol followed by 70% aqueous acetone) gives higher phenolic content than those obtained by water extraction in all plant samples. Solvent extract of green tea for example gives 5 fold higher values than those obtained by its water extract. These results are in agreement with several findings indicating that methanol, acetone as well as ethanol are more effective solvents in extracting phenols from plant than water (Malencic et al. 2007; Pinelo et al. 2005). The physical and chemical properties of each phenolic compound may reflect the quantitative and qualitative variations between individual phenols detected in water and solvent extract of the same plant as observed by HPLC (Tables 5.1 and 5.2) (Pinelo et al. 2005). TPC reported in these plants is widely different in literature, mainly attributed to diversities of the applied analytical methods, variety, maturity, extraction method as well as geographic origin of plants. For example the reported TPC of blueberry ranged widely from 22 to 4180 mg/100 g fresh weight and that in green tea ranged from 8.05 - 86.3 mg GAE/100 g (Prior et al. 2005; Yoo et al. 2008).

It was reported that phenolic compounds mainly catechins including (+)-catechin, EC, ECG, EGC, and EGCG constitute 30–50% of the solids in green tea therefore, green tea extract exhibited higher phenolic content compared to other plant sources (Yao et al. 2005). These findings were consistent with the HPLC data, which identified these catechins as main phenolic components in green tea water and solvent extracts.

Although there was a strong correlations between total phenolic determined by Folin–Ciocalteu and those obtained by HPLC in all plant water and solvent extracts, total phenolics measured by the HPLC were mostly lower than those determined by Folin–Ciocalteu assay. This suggests the contribution of other phenolic compounds that could not be identified due to lack of appropriate standards. For example, in artichoke and eggplant peels, chlorogenic acid and quercetin were the main phenolic compounds in their water and solvent extracts respectively. Although this data are in agreement with several studies (Curadi et al. 2005; Tateyama and Igarashi 2006), other compounds such as cynarin, flavonoids, cynaropicrin, dehydrocynaropicrin, grosheimin, luteolin, inulin as well as luteolin glycosides were also detected in artichoke extract (Jimenez-Escrig et al. 2003; Sanchez-Rabaneda et al. 2003). Whereas in eggplant peel despite there being no available data regarding to their phenolic content, anthocyanins especially nasunin is suggested to be the main phenolic component in their extracts (Noda et al. 2000; Paganga et al. 1999a). Likewise solvent extract of potato peel contained chlorogenic and caffeic acids as major phenolic compounds whereby these phenolic acids were much lower in their water extract (Singh and Rajini 2004).

Several peaks have not been identified with the applied method as in orange peel extracts where rutin, quercetin, chlorogenic and caffeic acid constitute main identified phenolic, but many other compounds have been detected such as *p*-coumaric acid, sinapic, naringin, hesperidin, neohesperidin, kaempferol, sinensetin, luteolin and diosmin (Anagnostopoulou et al. 2005b; Wang et al. 2008).

In the study, water and solvent extracts of spinach, broccoli and onion showed the lowest phenolic content compared to the other investigated plants. Gallic acid , chlorogenic acid, rutin, and quercetin were the major phenols in spinach, moreover many other flavonoids including patuletin, spinacetin, spinatoside, jaceidin were also identified (Cho et al. 2008; Nuutila et al. 2002). In broccoli, ferulic acid and rutin were the main phenolics in the water and solvent extract, respectively. Flavonoids such as quercetin 3-O-sophoroside and kaempferol 3-O-sophoroside, isoquercetin, kaempferol 3-O-glucoside and kaempferol diglucoside are suggested to be a group of key phenolic in broccoli extract (Sun et al. 2007). Additionally, quercetin and its derivatives were identified as the main phenolic compounds in onion extract as observed by us and by others (Lin and Tang 2007; Paganga et al. 1999b; Wach et al. 2007).

5.1.2.2 Antioxidant capacity

It has been observed that there is no simple universal method by which antioxidant activity can be measured accurately and quantitatively. Therefore, many authors strongly suggested the use of at least two methods to measure the TAC due to differences between the test systems (Schlesier et al. 2002). Several assays have been introduced for the measurement of the antioxidant activity of single compound and/or complex mixtures. TEAC assay has attracted much interest because it enables high-throughput screening of potential antioxidant activity of single compounds and biological matrices, such as plasma, as well as food components, and beverages (Gliszczynska-Swiglo 2006; Miller et al. 1993; Proteggente et al. 2002; Re et al. 1999; Rice-Evans and Miller 1994). Likewise FRAP assay is also commonly used for the routine analysis of single antioxidants and total antioxidant activity of plant extracts (Benzie and Strain 1996; Gao et al. 2000; Halvorsen et al. 2002).

Data of present study revealed that plant extracts gave different antioxidant capacities in relation to the extraction method. Moreover the same sample ranking differed depending on the applied assay (i.e TEAC or FRAP). These

variations might be explained by the difference in mechanism of actions of each assay where TEAC assay based on the reaction with $ABTS^{\bullet+}$ radical cation generated in the assay system, and the FRAP assay measures the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). Although TPC of all solvent extracts were significantly higher than those of water extracts, the later were significantly higher in TAC than solvent extract, when measured by both TEAC and FRAP assays. This suggests the contribution of soluble phenolics and other non phenolic compounds presented in the water extract which are able to scavenge the $ABTS^{\bullet+}$ radicals generated in TEAC assay and reduce Fe^{3+} to Fe^{2+} in FRAP assay (Lee and Jang 2004).

It was found that water and solvent extract of green tea followed by artichoke exhibited the greatest TAC compared to other samples as measured by both TEAC and FRAP assay. Despite a comparison with the available data from literature may be possible, it may yet prove to be difficult due to the large variability within the food items and due to the lack of standardization of the assays (Pellegrini et al. 2003), These data were in agreement with (Pietta et al. 1998), who found that TAC of green tea was significantly higher than many plant extracts including artichoke, and with results of (Cao et al. 1996) who ordered the TAC of some plants as following: green tea > whole eggplant > broccoli > onion. Similar observations (Triantis et al. 2005) demonstrated that TAC of broccoli > onion. Further, TAC of potato peel water extract was higher than that from onion (Kaur and Kapoor 2002).

There were strong correlations observed in solvent extracts between TEAC and FRAP and between TEAC or FRAP and TPC, suggesting a dominate contribution of the phenolic compounds to the antioxidant capacity of these solvent extracts. These results were consistent with the findings of many authors (Alali et al. 2007; Cai et al. 2004; Karakaya et al. 2001; Stratil et al. 2006). On the other hand, quite lower correlation coefficients have been observed in the water extracts except between TEAC and TPC which delivered good values. These data suggested that phenolic compounds might not be the main

contributors for antioxidant activity in these water extracts. Further analysis of these data revealed an improvement in the correlation coefficient to 0.99 if the water extracts of artichoke, broccoli and spinach were not considered in the relation.

Hence the investigated water extracts contained several antioxidants including vitamin C, carotenoids, tocopherols and pigments in addition to phenolic compounds, we therefore suggesting that in the low vitamin C containing plant extracts including potato peel, orange peel, eggplant peel, onion and green tea, the phenolic compounds may be main components responsible for the TAC values produced, whereas vitamin C content of artichoke with 10 mg/100 g fresh weight (FW) (Gil-Izquierdo et al. 2001), spinach with 75 mg/100 g FW (Gil et al. 1999) and finally broccoli with 66 mg/100g FW (Sikora et al. 2008) might contribute to their TAC values determined (Souri et al. 2008).

5.1.2.3 Anti-inflammatory effect

Low-grade inflammation has been associated with an increased risk of several acute and chronic diseases (Sepulveda and Mehta 2005). Attention has been mainly focused on the positive APP CRP as an inflammatory marker. In contrast, there is very limit studies which have so far looked at the effect on the negative APP TTR, which has been proposed to be a sensitive biochemical parameter of subclinical malnutrition and inflammation (Beck and Rosenthal 2002; Ingenbleek and Young 1994). Although the HepG2 cells are very similar to primary hepatocytes in terms of biological responsiveness (Knowles et al. 1980; Perlmutter et al. 1989), and it produces most of the APPs (Baumann et al. 1987; Darlington et al. 1986), although some studies have reported that it could not produce either serum amyloid A or C-reactive protein when stimulated with IL-6 (Wigmore et al. 1997a). In the present investigation, the validity of these cells was confirmed as an *vitro* system for studying the regulation of APPs through the determination of negative acute protein TTR which was down-regulated by IL-6 treatment (Castell et al. 1989b; Castell et

al. 1990). Moreover, the study showed for the first time that HepG2 cells also provide a simple and reproducible system for screening complex plant extracts for their anti-inflammatory potential using TTR as biomarker.

In detail, TTR secretion was significantly reduced to half the concentration when HepG2 cells were treated with IL-6 for 36h. In contrary, all plant extracts, except water and solvent extracts of broccoli and water extract of onion, in specific concentrations, significantly increased TTR secretion in HepG2 cells (in both cases of those treated and untreated with IL-6), although in IL-6 treated cells, plant extracts showed lower upregulation in TTR levels compared to untreated cells. Plant water extracts were ordered upon their positive effects as following: green tea > artichoke > potato peel > orange peel > spinach > eggplant peel. Whereas in solvent extracts, green tea > artichoke > potato peel > spinach > eggplant peel > onion > orange peel. Although there was wide variation between plants extracts, water and solvent extracts of green tea, artichoke and potato peel exhibited the highest effects respectively. These findings suggest that these plants extracts act as inducers for TTR secretion and possibility as anti-inflammatory agents. Although there is no comparable study, the results were in agreement with results of (Wigmore et al. 1997b), who observed an attenuation in APPs mediated by IL-6 in isolated human hepatocytes using unsaturated fatty acids.

There was also a strong correlation between phenolic content, antioxidant capacity and the anti-inflammatory effect of green tea, artichoke and potato peel. This suggests a strong contribution of the phenolic content and antioxidant capacity to their anti-inflammatory potential. These findings are in agreement with our study (El-Saadany et al. 2008) in which we have reported that EC, EGC, ECG, EGCG, the main antioxidants catechins in green tea were able to up regulate TTR secretion in HepG2 cells.

On the same line and on basis of the literature data available, many active constitutes were identified in artichoke extract than those identified phenolic

compounds by our HPLC method such as cynarine, flavonoids, cynaropicrin, luteolin, luteolin glycosides, and inulin. These constitutes may contribute to the antioxidative and hepatoprotective potential of artichoke extract, and may not account for the full capacity of their extracts (Gebhardt and Fausel 1997). Whereas in potato peels extracts, several free phenolic acids were also identified, likely to be donating as the active constituents (Singh and Rajini 2004; Singh and Rajini 2008).

In addition to the identified phenolic compounds it was reported that eggplant peel extracts may also contain several anthocyanins, especially nasunin which is reported to act preventively against the oxidative stress (Kimura et al. 1999; Noda et al. 2000). Many other bioactive constitutes were reported in other plants extracts such *p*-coumaric acid, sinapic, naringin, hesperidin, and kaempferol in orange peel, (Anagnostopoulou et al. 2005b; Wang et al. 2008); as well as quercetin-3- O-beta-glucoside, quercetin-4'- O-beta-glucoside, and quercetin-3,4'-di- O-β-glucoside in onion (Zielinska et al. 2008). Although many of these compounds play a beneficial role in disease prevention, or exhibit anti-inflammatory effects, the mechanism of the action of such compounds, however, is in most cases not fully elucidated.

On the other hand water and solvent extracts of broccoli and water extract of onion as well as selected doses of plant extracts such as high doses of green tea showed a negative or proinflammatory effect. It can be suggested that these plants extracts at selected concentrations exhibit a pro-oxidants effects. For example, a recent study showed that the addition of EGCG and other green tea constituents to tissue culture medium generated high levels of hydrogen peroxide (H_2O_2) (Long et al. 2000). It was also reported that at high concentrations (100 µM and above), EGCG functions as a reactive oxygen species producer (Rodriguez-Lopez et al. 1993), whereas at concentrations below 10 µM it exerts radical scavenging activity (Saeki et al. 2002).

Hence IL-6 induces inflammatory responses in the cells via activation of the transcription factor STAT3 (Alonzi et al. 2001). Other transcriptions factors such as nuclear factor-κB (NF-κB) and activated protein (AP-1) seems to be involved in transcription regulation of IL-6. Recent studies suggested several mechanisms for the anti-inflammatory effects of plant extracts containing phenolics. These mechanisms include, the inhibition of nitric oxide production and the expression of nitric oxide syntheses (iNOS); down regulation of cyclooxygenase-2 expression (COX2); inhibition of AP-1 and C/EBPd; inhibition of NF-kB, interferon regulatory factor (IRF)-1 and Akt signaling pathway; inhibition of *in vitro* lipoprotein oxidation; modulation of proinflammatory gene expression; as well as modulation of redox state (Gonzalez-Gallego et al. 2007).

Since the phenolic content and antioxidant potency of the investigated plant extracts are not responsible to fully explain their anti-inflammatory effects, we can therefore conclude that the anti-inflammatory potency of these plant extracts were not only due to their antioxidant potential but might also be due to other mechanisms which still remain to be elucidated. The choice of application of water extracts was considered in this study to be of high importance since most of the food is ingested or processed by using water. In this context, further experiments are planned in characterizing the main active constituents as well as their mechanism of action.

5.2 Anti-inflammatory potential of selected antioxidant agents on HepG2 cells

5.2.1 Results

5.2.1.1 Effect of IL-6 on TTR and RBP levels produced by HepG2 cells

HepG2 cells were cultured under serum free conditions for 32 h and TTR and RBP levels in culture supernatant were measured by ELISA. Measurements demonstrated that HepG2 cells can synthesize and secrete TTR and RBP in absence of any stimulus. To evaluate the effect of IL-6 on TTR and RBP secretion, HepG2 cells were treated with a fixed dose of IL-6 (25 ng/ml) for 32 h under the same conditions. As shown in Figure (5.10), IL-6 significantly reduced TTR and RBP levels as compared with basal production ($p \leq 0.001$).

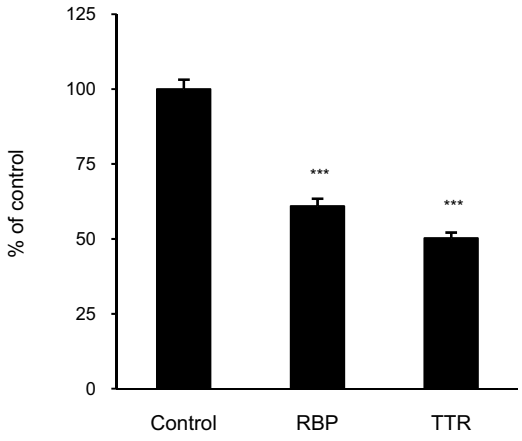


Figure 5.10: The effect of IL-6 (25 ng/ml) on TTR and RBP secretion as measured in culture supernatant after 32 h of incubation. Data represent the mean of eight replicates ± SD. *** = $p \leq 0.001$ *vs* non-treated control.

5.2.1.2 Effect of antioxidant agents on TTR and RBP secretion in IL-6 treated cells

To evaluate the effects of antioxidants on IL-6 induced reduction in TTR and RBP synthesis, HepG2 cells which had been treated with IL-6 were incubated for a further 8 h with varying concentrations of the tested antioxidants. Changes in TTR and RBP levels due to these antioxidants are summarized in Table (5.3) and detailed changes are given in Figures (5.11 and 5.12). The data show that all tested antioxidants increased TTR and RBP levels compared to the IL-6 treated control. This increase in their levels occurred almost in a dose dependant manner, especially for TTR. The strongest effects were achieved with the highest concentration of each antioxidant. LA was the most potent component and AA showed the smallest effect. The values obtained for RBP were even higher than those for TTR for all the tested antioxidants.

Table 5.3: The effect of antioxidants agents on TTR and RBP secretions in HepG2 cells treated with IL-6

Antioxidant	Secretion	
	TTR	RBP
α-LA (0.8 mM)	93.5 ± 13.4	137.8 ± 5.5
EGCG (25 μM)	84.8 ± 14.2	118.6 ± 3.7
ECG (25 μM)	83.5 ± 4.6	115.1 ± 5.7
α-TOC (50 μM)	73.3 ± 2.7	108.1 ± 4.9
EGC (50 μM)	72.2 ± 4.6	107.9 ± 4.8
EC (50 μM)	62.8 ± 11.4	107.9 ± 2.7
NAC (1 mM)	60.9 ± 3.5	104.2 ± 3.3
AA (1 mM)	52.5 ± 14.3	104.1 ± 15.7

Data represent the mean of three replicates ± SD vs. non-treated IL-6 control

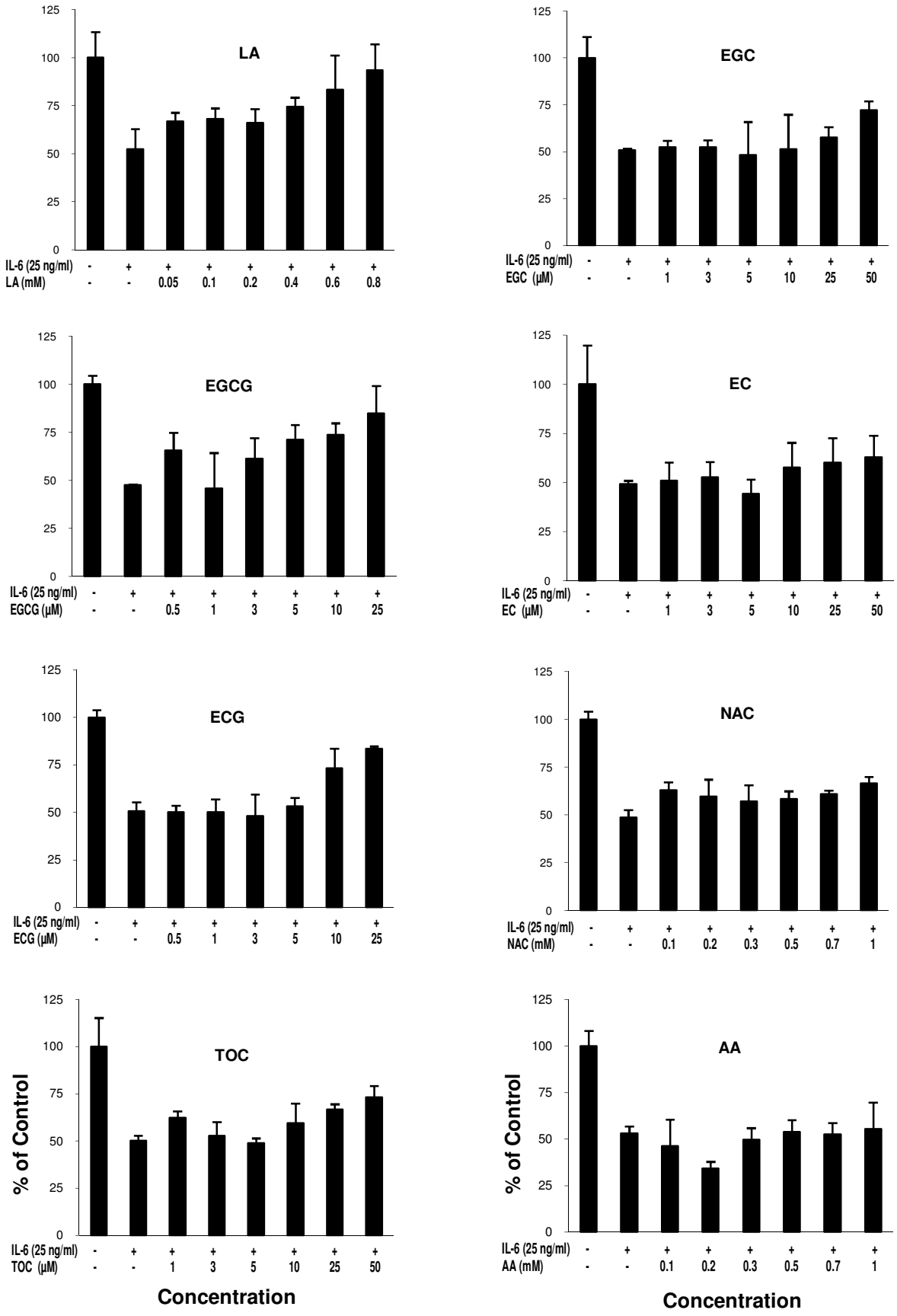


Figure 5.11: Influence of antioxidants on **TTR** secretion of HepG2 cells treated with IL-6.*

* Confluent cells were incubated for 24h in serum free medium containing IL-6 (25 ng/ml) and then serial concentrations of the antioxidants were added for further 8h. Culture supernatant were collected and analyzed for TTR using ELISA assay. Data represent the mean of three replicates ± SD.

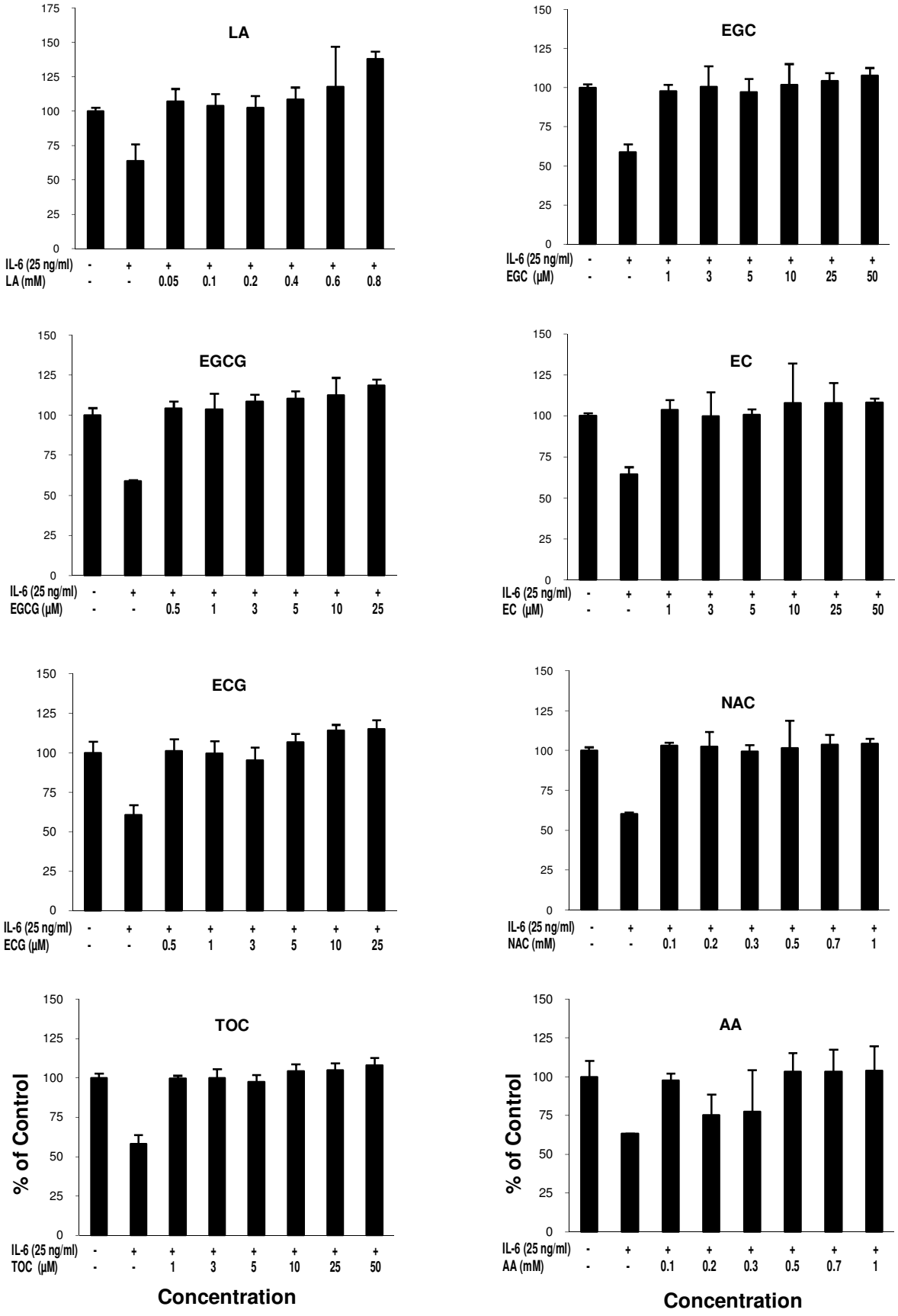


Figure 5.12: Influence of antioxidants on **RBP** secretion of HepG2 cells treated with IL-6.*

* Confluent cells were incubated for 24h in serum free medium containing IL-6 (25 ng/ml) and then serial concentrations of the antioxidants were added for further 8h. Culture supernatant were collected and analyzed for RBP using ELISA assay. Data represent the mean of three replicates ± SD.

5.2.2 Discussion

The human hepatoma cell line HepG2 is very similar to primary hepatocytes in terms of biological responsiveness (Knowles et al. 1980; Perlmutter et al. 1989) and is widely used as a model system for studying the regulation of APP synthesis in the human liver (Baumann et al. 1987; Darlington et al. 1986). On the other hand, however, some studies have shown that HepG2 or Hep3B2 cells could not produce either serum amyloid A or C-reactive protein when stimulated with IL-6 (Wigmore et al. 1997a). *In vitro* studies using hepatocyte cultures and correlations of serum IL-6 concentrations with APP levels in various inflammatory states indicated that IL-6 may be the principal regulator for most of the APP genes (Castell et al. 1989b).

Our results showed that the synthesis and secretion of TTR and RBP in culture supernatant of HepG2 are down regulated by IL-6. TTR and RBP levels decreased significantly in the culture media by 50% and 40%, respectively. These data are in agreement with those reported by (Citarella et al. 1997), who found that TTR levels decreased. Although no *in vitro* data were available concerning RBP, the results confirm those reported by (Gieng et al. 2005) who also recorded a reduction in hepatic RBP concentrations.

The results further demonstrated that the tested antioxidants modulate TTR and RBP secretion in response to IL-6. The effectiveness for the eight antioxidants decreased in the following order LA > EGCG > ECG > TOC > EGC > EC > NAC > NAC > AA, for TTR and RBP. When relating these to the antioxidant activities of catechins, it has been shown that their activity order is as follows: EGCG ≈ ECG > EGC > EC (Rice-Evans et al. 1996). We therefore suggest that the modulatory effects of these antioxidants on TTR and RBP levels could also be dependent on their antioxidant activities. Other possibilities may include properties such as free radical scavenging activity typical for such phenolic compounds (Guo et al. 1996), whereby the inhibition of nuclear

factor-κB (NF-κB) and AP-1 could play the most important role (Sen et al. 2006; Xia et al. 2005).

Although LA has been shown to have less antioxidant activity than EGCG (Lee et al. 2003) it is still found to be the most potent inducer for TTR and RBP production. The specific mechanisms of its action remain unclear although several pharmacological effects of LA could also be responsible including the reported inhibition of NF-κB and AP-1 (Mackenzie et al. 2006; Suzuki et al. 1992).

Despite the most commonly known nutrient antioxidants; TOC and AA, have been reported to have the same antioxidant activity (van den Berg et al. 1999). Their behavior still differs to some extent, whereby TOC exhibits a much greater effect than AA. Both NAC and AA had a low effect on TTR and RBP release in the investigated cell line.

Reduction in TTR and RBP levels has generally been thought to be due to the ability of IL-6 to decrease transcription of negative APP genes. IL-6 did however cause a decrease in mRNA levels for APPs several hours earlier than the changes in secretion (Bartalena et al. 1992). It can be further assumed that the transcription factors nuclear factor-κB (NF-κB) and activated protein (AP-1) could also be involved in TTR and RBP reduction because these transcription factors are essential for the transcription regulation of IL-6. Antioxidants may promote TTR and RBP production through the inhibition of these transcription factors.

We can therefore conclude that the modulatory effects of the tested antioxidants were not only due to their antioxidant activities but also be due to other mechanisms which still remaining to be elucidated. We further assume that NF-κB and/or AP-1 might be involved, since all tested antioxidants could inhibit NF-κB (Barnes and Karin 1997).

5.3 The effect of selected antioxidants and plant extracts on TTR microheterogeneity

To investigate the effect of antioxidants agents and solvent plant extracts on the heterogeneity of TTR, HepG2 cells were incubated for 24h in serum free medium containing two different concentration of each tested substances as following: EC, EGC (50, 100 µM), ECG, EGCG (25, 50 µM) NAC (1, 5 mM), AA , LA (0.5, 1mM), α-TOC (100, 200µM), artichoke, eggplant peel, spinach (50, 125 µg/ml), onion (125, 250 µg/ml) orange peel (250, 500 µg/ml), green tea, broccoli (5, 25 µg/ml), and potato peel (25, 50 µg/ml). The choice of these concentrations was respectively dependant on the concentration gave higher effect on TTR secretion (see part 5.1 and 5.2). Cells incubated with serum free medium served as untreated control. Changes in TTR microheterogeneity were detected using immunoprecipitation assay combined with SELDI-TOF-MS technique.

As illustrated in Figure (5.13), untreated HepG2 cells (control) were able to secrete TTR with molecular structure consisting of four peaks (variants) with the following molecular mass: 13453.8 ± 8.3 Da, 13699.5 ± 9.7 Da, 13821.9 ± 10.2 Da and 14007.2 ± 11.5 Da, suggested to represent unknown, native, S-cysteinylated, and S-glutathionylated TTR forms respectively. The S-cysteinylated variant was the dominant followed by the native variant. For more detailed investigations, the heights of native and S-cysteinylated TTR peaks were measured in each spectra in a valley-to-valley procedure and related to S-cysteinylated TTR (S-cysteinylated TTR served as 100%). Results showed that, native TTR of untreated cells represents approximately 62.2 ± 1.44% of the S-cysteinylated form. Six substances including LA, NAC, onion, AA, EGCG and green tea were able to attenuate this relation by elevating the native TTR form. The potency of these substances to increase the nave form in relation to S-cystinylated form were ordered as following: LA $>$ NAC $\geq$ onion extract $>$ AA $>$ EGCG $>$ green tea with corresponding ratios 130, 110, 110, 104, 90, and 85% respectively, where all other plant extracts and antioxidants showed no detectable effects. The corresponding molecular mass of TTR variants was not significantly changed in all analysis.

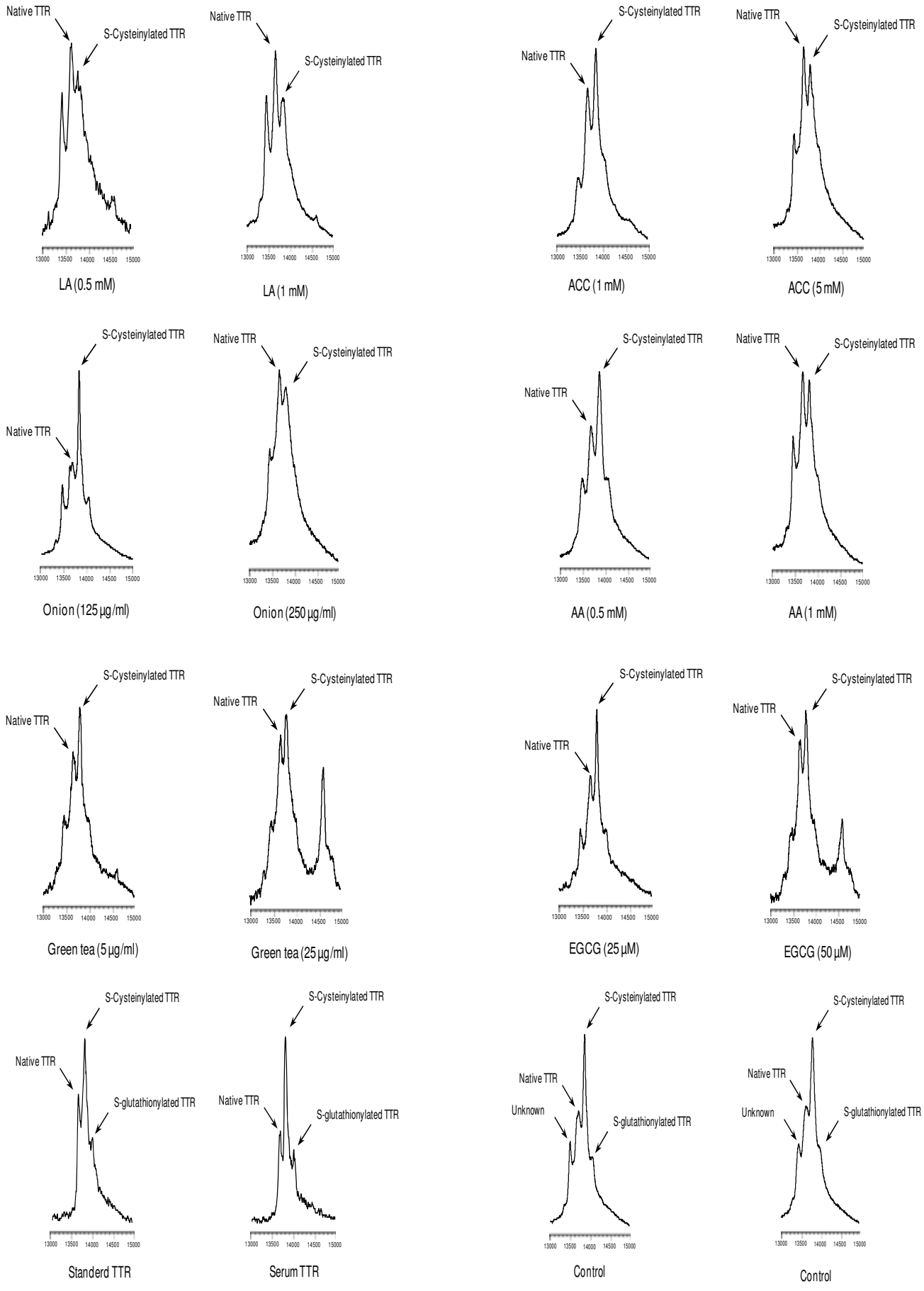


Figure 5.13: Effect of antioxidants and plant extracts on the microheterogeneity of TTR secreted by HepG2 cells as measured by SELDI-TOF-MS assay.

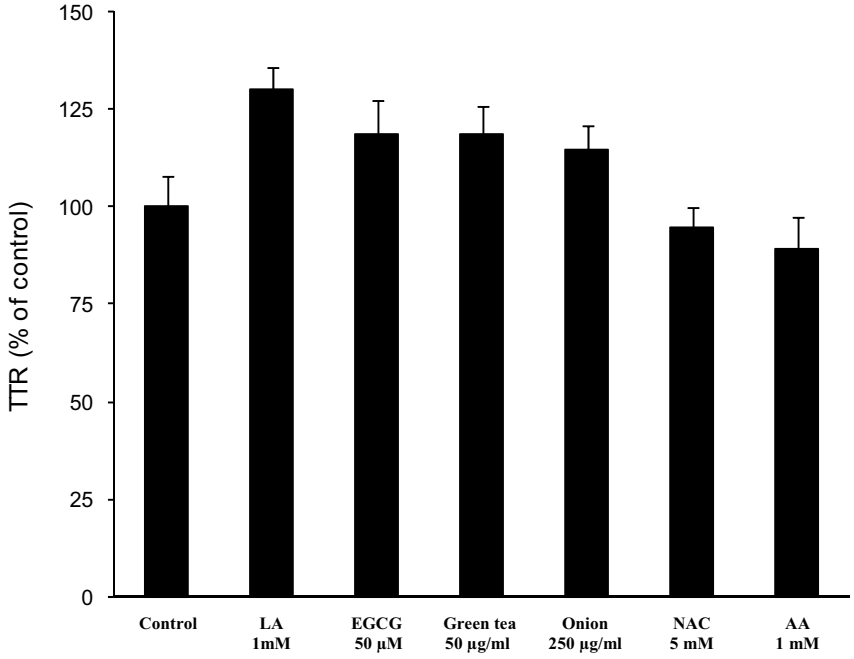


Figure 5.14: The effect of some antioxidants and plant extracts on TTR secretion of HepG2 cells after 24h of incubation. Data represent the mean of three replicates ± SD.

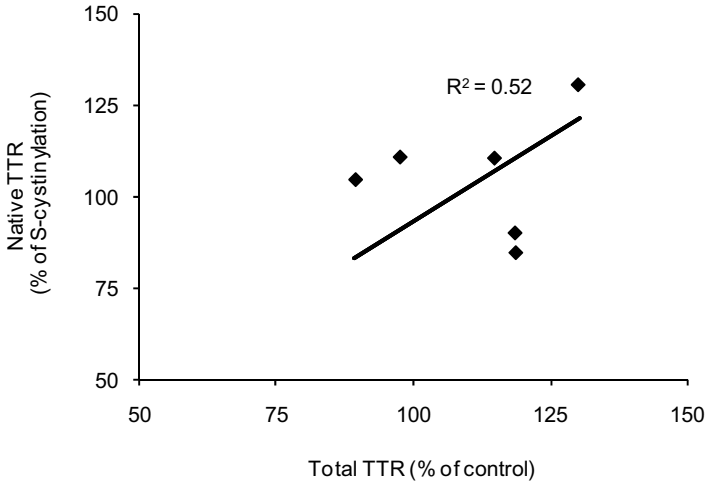


Figure 5.15: Relation between elevation in total TTR and attenuation to its native variant as resulting from lipoic acid, NAC, AA, EGCG, onion, and green tea treatments. Data represent the mean of three replicates.

5.3.2 Discussion

Transthyretin is a homotetrameric plasma protein that, in conditions not yet completely understood, may aggregate, forming the fibrillar material associated with TTR amyloidosis. This amyloid causes organ dysfunction and ultimately can lead to death (Bergen et al. 2004; Saraiva 2001). In plasma, TTR is extensively modified with thiol adducts. The unmodified TTR only accounts for ~5%–15% of the total TTR, where the other 85%–95% is posttranslationally modified in the form of S-sulfonation and S-thiolation (S-cysteinylation, S-cysteinylglycine, and S-glutathionylation) (Ando et al. 1997b; Kishikawa et al. 1996; Lim et al. 2003a; Terazaki et al. 1998).

A number of reported experiments indicate that the unmodified (native) TTR has very low capacity to form fibrils, whereas extensively S-conjugated of Cys10 of TTR might increase the fibril forming capacity and lead to more rapid progression of familial TTR amyloidosis. Interestingly also, a higher percentage of S-conjugated TTR to the unmodified TTR has been reported in patients with symptomatic amyloid disease (Lim et al. 2003c; Zhang and Kelly 2005).

More attention is also given to tetramer structure stability of TTR since it was reported that dissociation of the TTR tetramer occurs prior to fibril formation (Morais-de-Sa et al. 2004). A recent study of (Kingsbury et al. 2008) indicated that the unmodified TTR has a very high stable tetramer compared to the S-cysteinylated one, the later decreases the stability of both tetramer and dimer structure but to different extents. This study also hypothesizes that factors stabilizing the TTR structure would inhibit aggregation, thereby may giving protection against amyloid disease.

SELDI-TOF-MS technique applied in our study offers an accurate and reproducible method for detection of molecular variants of TTR (Schweigert et al. 2004). Whereby, the obtained results demonstrated that TTR secreted by HepG2 cells has a molecular structure quite similar to the purified standard and

serum TTR (Figure 5.13), in which all three main variants are contained representing, the native (13699.5 ± 9.7 Da), S-cystinylated (13821.9 ± 10.2 Da), and S-glutathionylated TTR (14007.2 ± 11.5 Da), with the S-cystinylated being the dominant variant. These results are in agreement and confirmed those of (Lim et al. 2003b; Schweigert et al. 2004; Terazaki et al. 1998). Other forms such as S-sulfonated and S-cysteinylglycinated TTR were not observed, may be due to dehydration or phosphorylation (Schweigert et al. 2004). Interestingly, a variant with molecular mass of 13453.8 ± 8.3 Da has been detected only in TTR secreted by HepG2 in all experiments. Since there is no comparable data available about TTR microheterogeneity from cell culture, it was difficult to attribute this variant, which needs further investigations.

Among the 16 substances tested including antioxidant agents and plant extract, six were able to modify the microheterogeneity of TTR to the native form. The potency of these substances arranged in a descending order is as following: LA, NAC, onion, AA, EGCG and green tea, whereas all other substances showed no significant effect. When these results are correlated to the total TTR secreted by HepG2 treated with such antioxidants or plant extracts as determined by ELISA assay Figure (5.14), data revealed a weak correlation (R^2=0.52) between total elevation in TTR due to the antioxidants or plant extract and its shifting to the native form (Figure 5.15).

Although it has been reported that free radical injury may result in amyloid deposits and could participate in the formation of amyloid fibrils (Ando et al. 1997a), the results suggest a protective role of administrating antioxidants either by reducing the toxic effects of free radicals or by reducing amyloid fibril formation in amyloidosis. Our results suggest a week relation between antioxidants capacity of the studied substances and its potency to elevate the native TTR since the reported antioxidants capacity was ordered as following green tea > EGCG > NAC > AA > α-LA (Ivekovic et al. 2005), whereby this order wasn't in consist with the order of attenuation of native TTR.

Since LA, NAC and onion extract exhibited the highest effect in increasing the native TTR compared to other antioxidants including AA, EGCG and green tea, and since LA, NAC and onion extracts are sulfur containing compounds, it can be assumed that the sulfur (thiol) content of these substances plays a vital role. With regard to the structure-function relationship among these sulfur containing antioxidants, LA has a strained cyclic disulfide in a 1,2-dithioline ring and in its reduced form, contains two thiol groups. Due to its unique strained cyclic disulfide structure, LA exerts significant antioxidant activities both *in vivo* and *in vitro*. It is more potent reducing agent than GSH itself and reacts immediately with plasma proteins and directly reduces disulfide groups and can reduce GSSG to GSH, and cystine to cysteine. Both LA and DHLA are more easily reduced/ oxidized compared to monothiols. Therefore, LA might be considered as the ideal and universal antioxidant (Atmaca 2004; Packer et al. 1995b; Roy and Packer 1998a). Furthermore it is unique among the antioxidant because it keeps its protective functions in both reduced and oxidized forms (Atmaca 2004; Navari-Izzo et al. 2002). Although the wide use of NAC as antioxidants, it exhibits lower effect than LA because it contain only one thiol group. Likewise, onion contains several cysteine sulfoxide derivatives (Dini et al. 2008), the results showed that they produce lower effect compared to LA. This may be due to the concentration applied in the study.

Since the mechanism of elevating the native TTR remains unknown, and since the antioxidants potency didn't completely fulfill explanation of this elevation or shifting to the native TTR. It can therefore be assumed that sulfur content of antioxidants may explain partially the elevation in native TTR, we also assume that it may play a role on stabilizing the tetramer structure of TTR and finally thereby play a vital role in amylogenetic diseases.

5.4 Protective effect of antioxidants and plant extracts on diethylnitrosamine induced hepatotoxicity in HepG2 cells

5.4.1 Results

5.4.1.1 Cytotoxicity of Diethylnitrosamine

Confluent HepG2 cells were treated with increasing concentrations of diethylnitrosamine (DEN) (0-100 mM) for 24, 48, and 72h. Following incubation, cell viability was determined using the neutral red assay (NR). As shown in Figure (5.16), DEN cytotoxicity is expressed as percentage of control cells viability. DEN significantly reduce cell viability in a concentration-dependent manner ($p<0.01$). The initial (IC90) and midpoint (IC50) values, the concentration resulting in 10 and 50% inhibition of cytotoxicity parameters, were calculated from the dose response curves. The IC90 value of DEN was 46.8, 35.1, and 23.3 mM after 24, 48, and 72h respectively, where the IC50 value was 64.2, 40.1 and 34.9 mM after 24, 48, and 72h respectively. DEN concentration above 100, 70, and 60 mM blocks completely cell viability after 24, 48, and 72h respectively. Cell viability of control cells was mostly greater than 95% for all experiments.

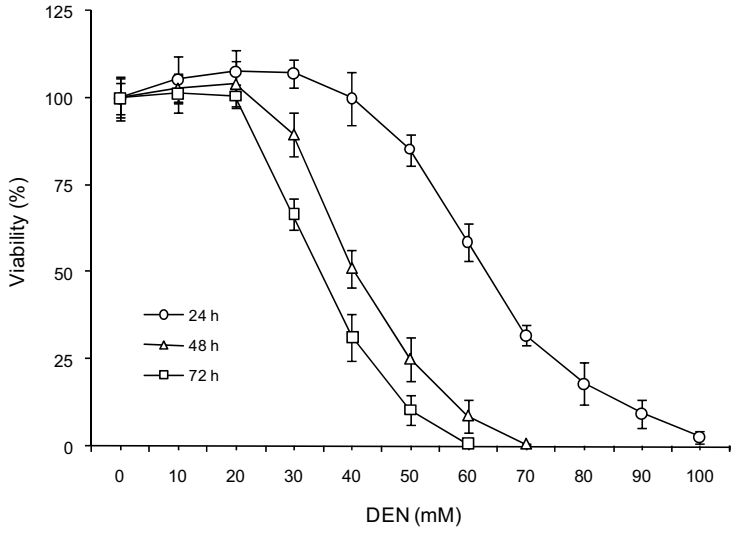


Figure 5.16: Cytotoxicity of DEN as determined by NR assay after 24, 48, and 72h of incubation with HepG2 cells. Data represent the mean of six replicates ±SD.

5.4.1.2 Cytotoxicity of antioxidants and plant solvent extracts

In order to define the non-toxic concentrations, (that has no initial cytotoxic effect), of the studied antioxidants and plant solvent extracts, confluent HepG2 cells were incubated for 24 and 48h with wide concentration range of each substance as following; EC, EGC (0-100 µM), ECG, EGCG (0-50 µM), AA, NAC, LA (0-10 mM), α-TOC (0-200 µM), green tea, artichoke, spinach, onion, broccoli, orange peel, potato peel and eggplant peel extracts (0-1 mg/ml). Cytotoxicity was then measured by NR assay. Figures (5.17 and 5.18) illustrate the cytotoxicity of these components as percent of control cell viability. Further, Table (5.4) summarizes the initial and midpoint concentration of each substance. At represented concentrations in Table (5.4), green tea and potato peel exhibit initial and midpoint cytotoxicity after 24 and 48h. They induce approximately full death at concentration $\leq$1 and 0.5 mg/ml respectively. Initial cytotoxicity was also observed by lipoic acid and broccoli where their midpoint appeared only after 48h. Artichoke, spinach and eggplant peel recorded only initial cytotoxicity after 48h. All other antioxidants and plant extracts didn't show detectable cytotoxicity at the applied concentration ranges. Furthermore EC, ECG, EGC and EGCG, especially after 24h induce higher viability (126%) compared to control cells.

5.4.1.3 Effect of antioxidants and plant extracts on diethylnitrosamine cytotoxicity

To study the protective effects of antioxidants and plant extracts on DEN cytotoxicity, a confluent HepG2 cells were pre-incubated for 8h with the non-toxic serial concentration of the antioxidants and plant extracts and then with a constant dose of 35 mM DEN, a concentration that induces $\approx$ 40% mortality in the HepG2 cells after 48h as calculated from concentration-response curve was then added for further 48h. Cells treated with medium containing 35 mM DEN served as negative control. Protective effects of these substances on DEN cytotoxicity are illustrated in Figures (5.19 and 5.20) as percent of non-treated control. As shown, significant ($p<0.05$) protective effect towards DEN

cytotoxicity were achieved by application of LA (5-50 µM), α-TOC and AA (5-25 µM), spinach and orange peel (5-125 µg/ml), egg plant peel (5-25 µg/ml), artichoke (1-10 µg/ml), and onion at (5 µg/ml). The protective effect ranged from 66.5 -112 as percent of the non-treated control. Such effects were ordered as following: spinach > LA> artichoke > orange peel > eggplant peel > α-TOC> onion > AA. Other concentration ranges as well as all other antioxidant and plant extracts induce slight to significant increase on DEN cytotoxicity. Green tea, broccoli and ECG in combination with DEN caused higher cytotoxicity.

Table 5.4: The initial and midpoint concentrations of antioxidants and plant solvent extracts.

Components	IC_{90}		IC_{50}	
	24h	48h	24h	48h
EC (µM)	-	-	-	-
ECG (µM)	-	-	-	-
EGC (µM)	-	-	-	-
EGCG (µM)	-	-	-	-
AA (mM)	7.60	3.12	-	-
NAC (mM)	-	-	-	-
α-LA (mM)	6.25	1.70	-	4.15
α-TOC (mM)	-	-	-	-
Green tea (mg/ml)	0.16	0.16	0.49	0.38
Artichoke (mg/ml)	-	0.12	-	-
Spinach (mg/ml)	-	0.10	-	-
Onion (mg/ml)	-	-	-	-
Broccoli (mg/ml)	0.37	0.66	-	0.95
Orange peel (mg/ml)	-	-	-	
Potato peel (mg/ml)	0.16	0.11	0.37	0.36
Eggplant peel (mg/ml)	-	0.17	-	-

Data represent the mean of six replicates.

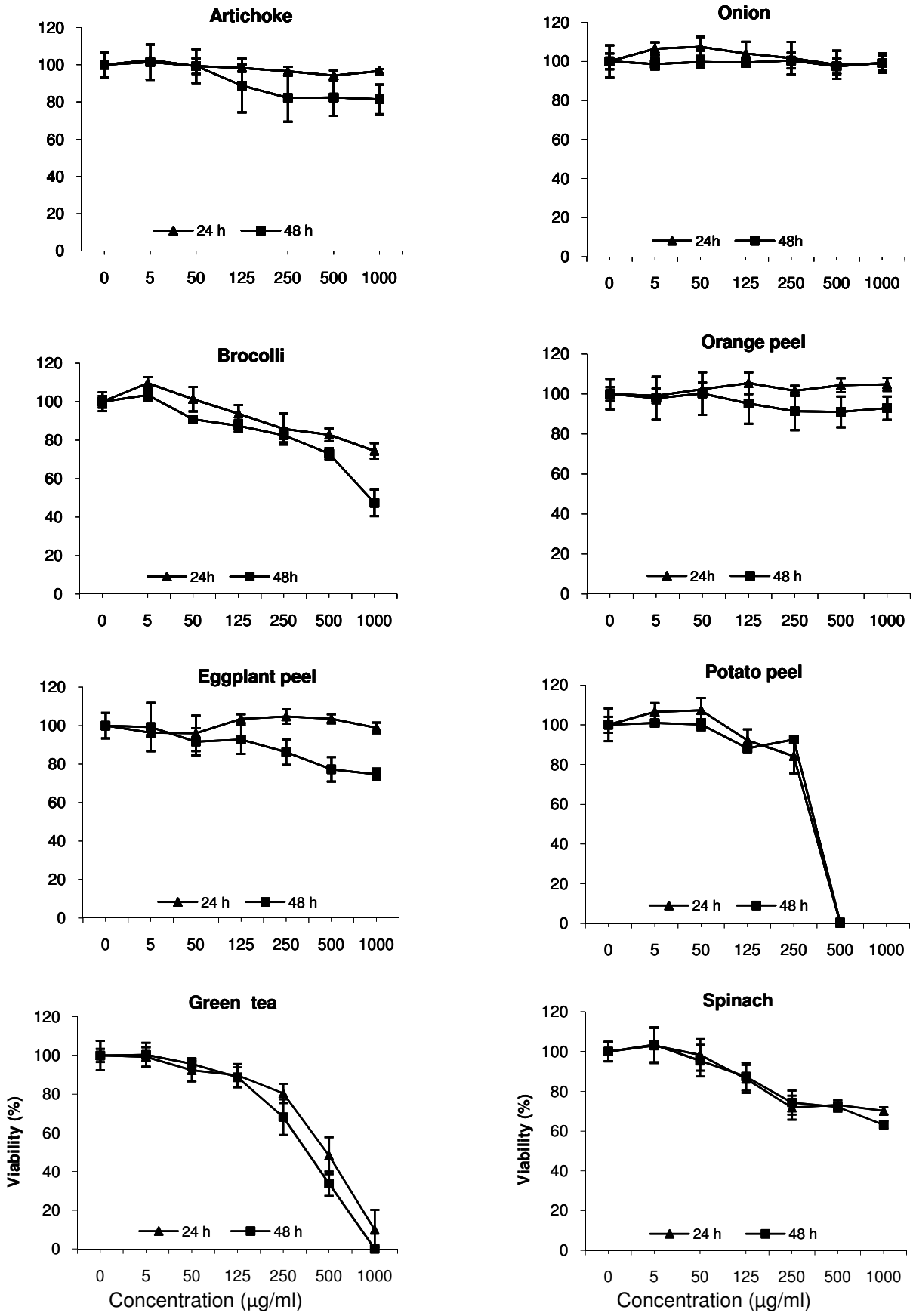


Figure 5.17: Cytotoxic effect of plants solvent extracts on HepG2 cells as determined by NR assay.*

* Confluent HepG2 cells were incubated with serial concentration of plant solvent extract for 24 and 48h. Cell viability was then measured by NR assay. Data represent the mean of six replicates ± SD.

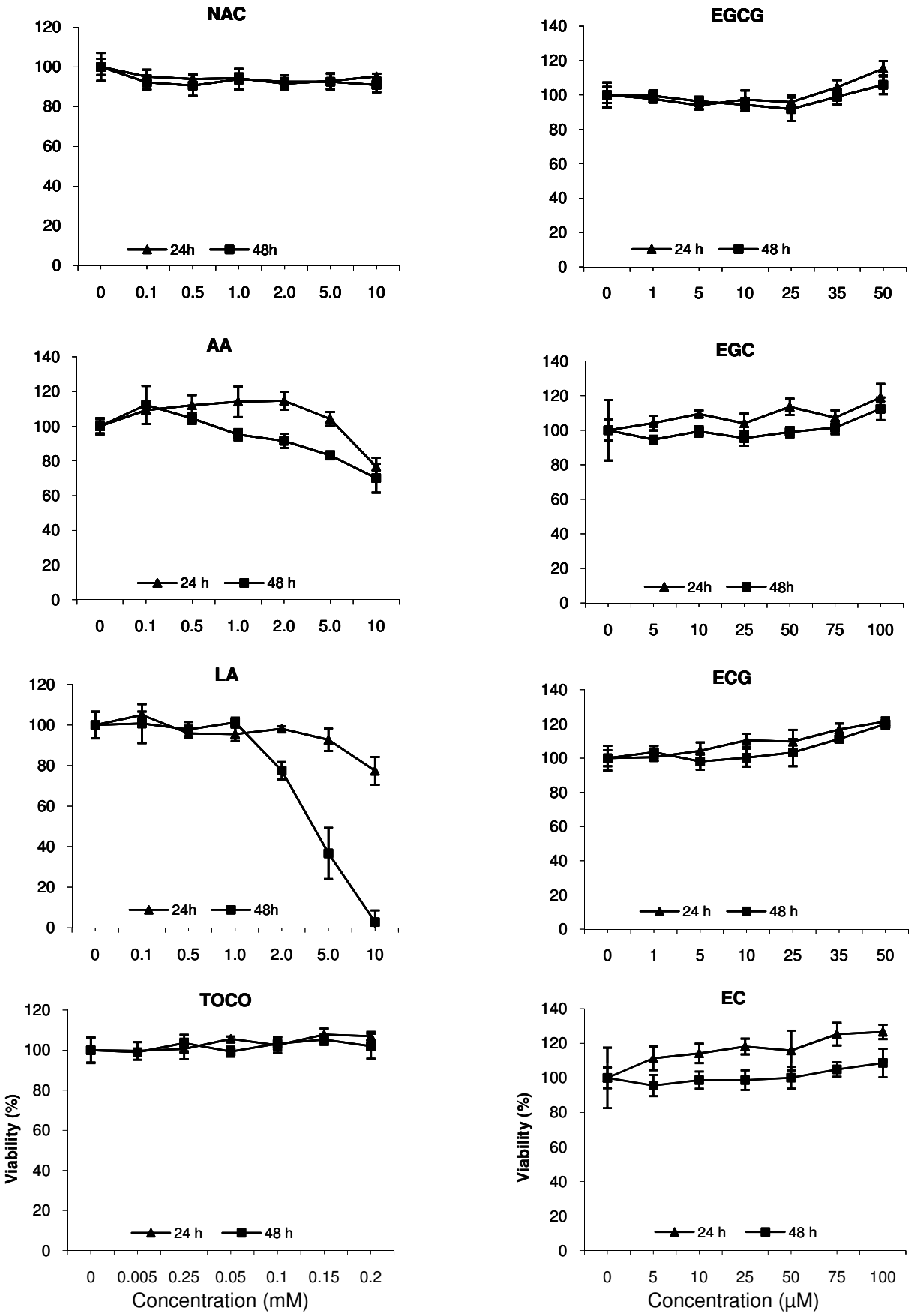


Figure 5.18: Cytotoxic effect of antioxidants on HepG2 cells as determined by NR assay.*

* Confluent HepG2 cells were incubated with serial concentration of antioxidants for 24 and 48h. Cell viability was then measured by NR assay. Data represent the mean of six replicates ± SD.

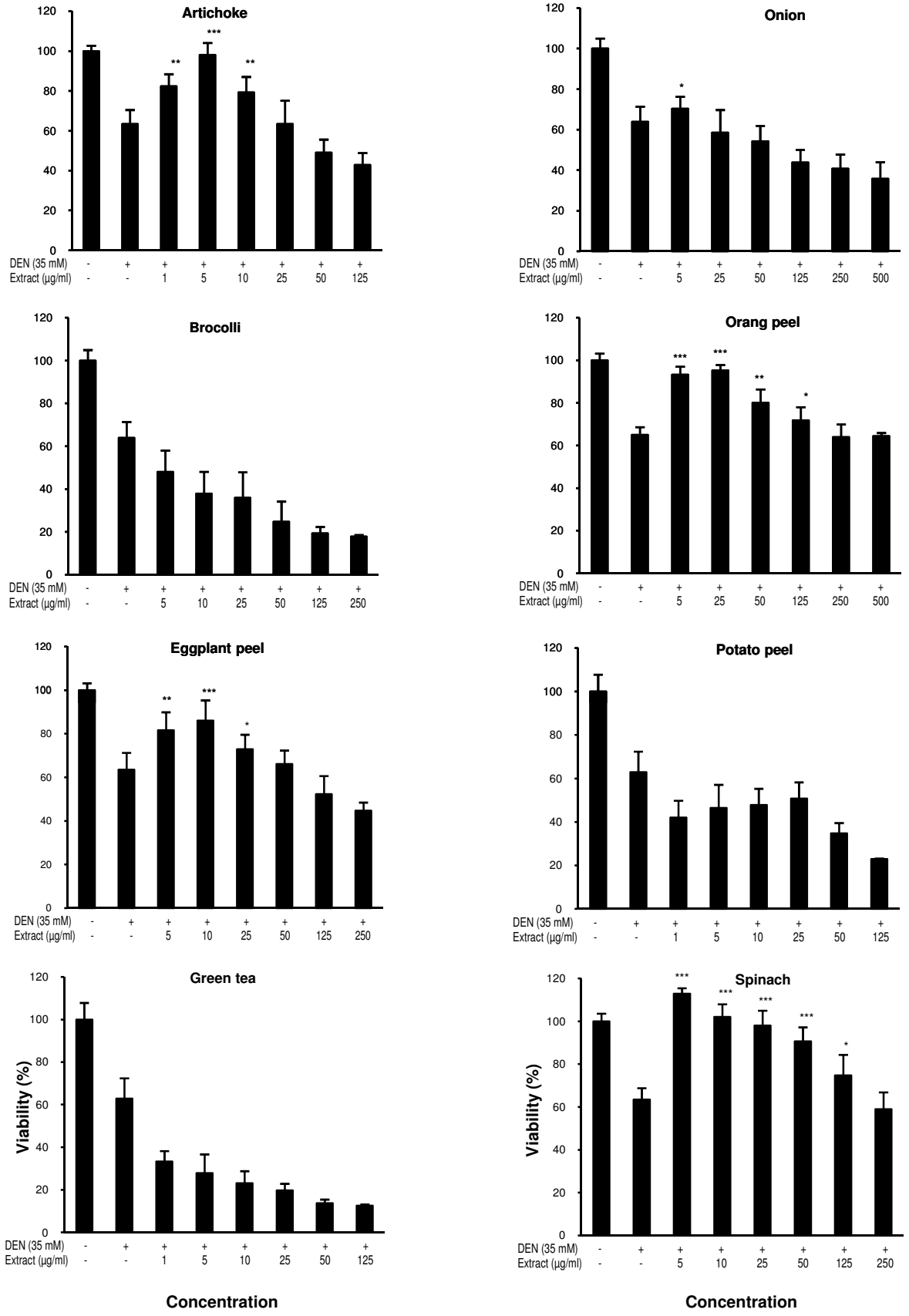


Figure 5.19: Effect of plant solvent extracts on DEN induced cytotoxicity on HepG2 cells as determined by NR assay*

* Confluent HepG2 cells were first incubated with serial concentrations of plants solvent extracts (which showed no cytotoxic effects) for 8h and then treated with fixed concentration of 35 mM DEN for further 48h. Cell viability was then measured by NR assay. Data represent the mean of six replicates ± SD.

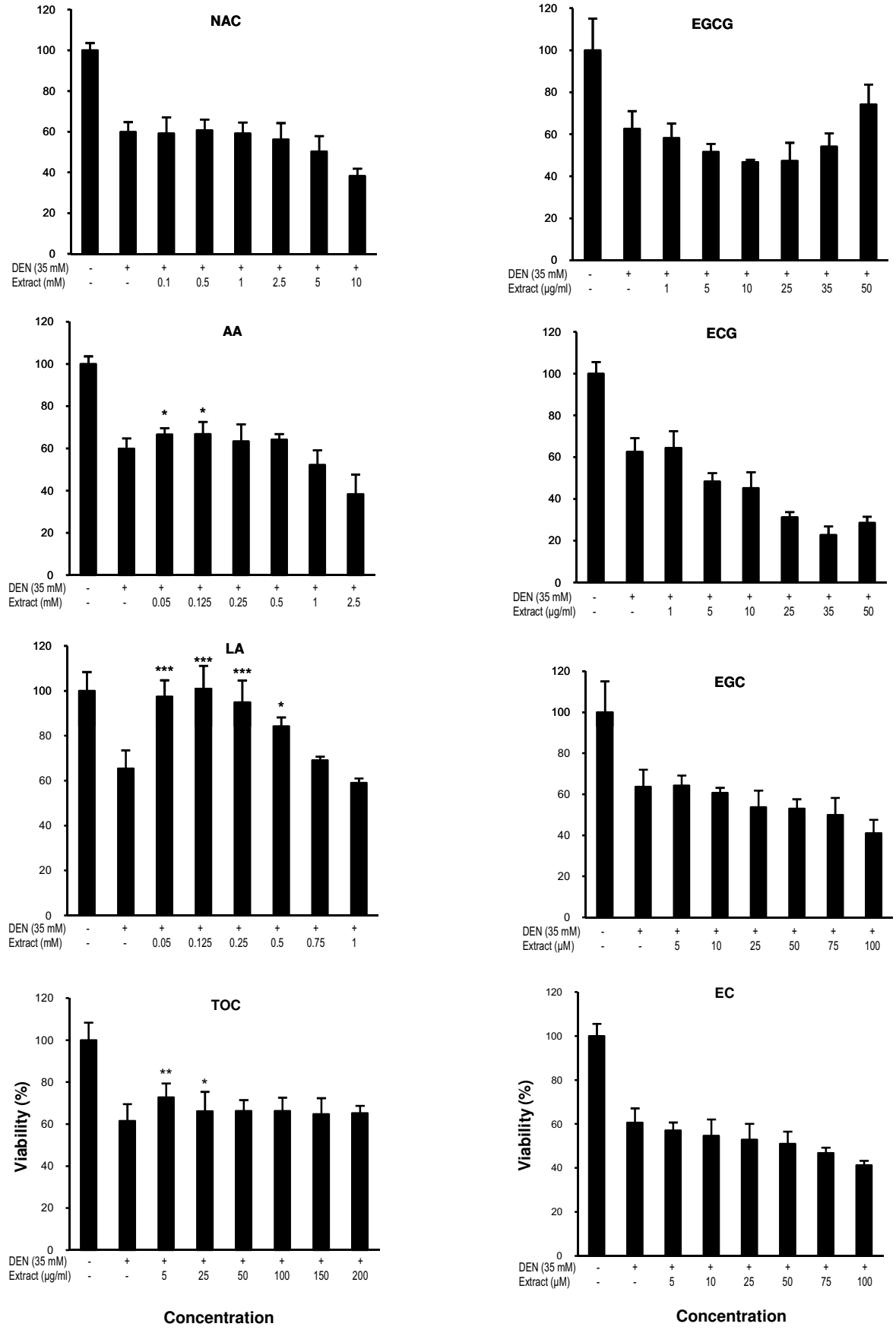


Figure 5.20: Effect of antioxidants on DEN induced cytotoxicity on HepG2 cells as determined by NR assay*

* Confluent HepG2 cells were first incubated with serial concentrations of antioxidants (which showed no cytotoxic effects) for 8h and then treated with fixed concentration of 35 mM DEN for further 48h. Cell viability was then measured by NR assay. Data represent the mean of six replicates ± SD.

5.4.2 Discussion

In vitro cytotoxicity assays provide a potentially useful tool in studying the acute toxicity of various xenobiotics. Numerous publications recommended the use of these *in vitro* assays to predict the susceptible risk resulting from agents or complex mixtures *in vivo*. Moreover, they offer major advantages in speed, simplicity, cost, safety and reproducibility compared to *in vivo* studies (Yamamoto et al. 2001). Despite the large number of alternative methods that have been proposed as cytotoxicity assays, such as trypan blue dye exclusion, lactic dehydrogenase, protein content and MTT, neutral red assay has proved to be sensitive, reproducible, non-radioactive, technically fast and simple especially when performed in microtiter trays and an automatic scanning spectrophotometer, and can be a valuable tool for determining the cytotoxicity of xenobiotics under controlled *in vitro* conditions. It is based on the *in vitro* incorporation of a supravital dye, neutral red (NR), into lysosomes of living uninjured cells (Borenfreund et al. 1990; Borenfreund and Puerner 1985; Zhang et al. 1990).

HepG2 is a highly differentiated human hepatoma cell line, which retains many of the cellular functions often lost by primary cells culture such as expression of hepatocyte-specific cell surface receptors and syntheses of plasma proteins. These cells retain cytochrome P450-dependent mixed function oxidases and glucuronic acid and sulfate conjugation activities involved in phase I and phase II metabolism of xenobiotics (Doostdar et al. 1988; Grant et al. 1988). It reflects metabolism of such compounds *in vitro* better than experimental models with metabolically incompetent cells and exogenous activation mixtures. Therefore, it is frequently used as an *in vitro* system to study the metabolism and toxicity of wide range of toxicants including nitrosamines (Nakama et al. 1995; Natarajan and Darroudi 1991)

Diethylnitrosamine (DEN) is one of the most important environmental carcinogens, present in a variety of foods, including cured meats, smoked and

salt-dried fish, grain products, cheese, soybeans and alcoholic beverages. It was also detected in tobacco smoke, in baby pacifiers and baby-bottle nipples as contaminant in the rubber, and it can be synthesized endogenously. (Lijinsky 1999; Tricker and Preussmann 1991; Yurchenko and Molder 2006) DEN is primarily used as a hepatocarcinogen to induce hepatocellular carcinoma. Moreover, it can induce carcinogenesis in other organs like kidney, trachea, lung, esophagus, forestomach, and nasal cavity. DEN is biologically and chemically inert therefore it needs activation by cytochrome P450 enzymes which are synthesized by liver cells to produce its cytotoxic, mutagenic and carcinogenic effects (Enzmann et al. 1995; Verna et al. 1996)

According to our results, DEN was able to induce cell death in a concentration dependent manner. It can produce its cytotoxic effect after 24, 48 and 72h. Although DEN was active at all three endpoints, it requires considerably higher concentrations for its effects especially after 24h. This may suggest that the amount of cytochrome P450 enzymes (P4502A6, P4502A1 and other forms of P450) produced by HepG2 cell do not significant activate DEN (Jover et al. 2001; Zhuge et al. 2003). Despite the fact that *in vitro* data concerning the DEN cytotoxicity are very limited. The data were in agreement with (Zhuge et al. 2003) with exception to that the midpoint cytotoxicity (IC50) was quite higher than in the present study. This may due to using another assay (MTT). It may also reflects the NR sensitivity compared with MTT assay especially after long incubation periods with the toxic agents (Yamamoto et al. 2001). Metabolic de-ethylation of DEN is the main activation pathway in the formation of genotoxic products. This activation leads to the formation of acetaldehyde and an ethylcarbonium ion, which is considered to be the main DNA-reactive species. In addition to enzymatic de-ethylation, DEN also undergoes de-nitrosation reaction leading to the formation of nitrite (Sierra et al. 2001).

This study was conducted to evaluate the protective effect of eight antioxidant agents, including EC, ECG, EGC, EGCG, AA, NAC, LA, α-TOC, and eight plant solvent extracts of green tea, artichoke, spinach, onion, broccoli, orange peel,

potato peel and eggplant peel on DEN cytotoxicity in HepG2 cells using neutral red assay. While evaluating the cytotoxic effects of such antioxidants and plant extracts, it was important to exclude those concentrations exhibiting cytotoxic effects especially when applied towards DEN. To our knowledge, the available data concerning the cytotoxic potency of antioxidant and plant extracts is very limited compared to those discussing their benefits. Results explored that green tea and potato peel extracts were more toxic than other substances. They initiated cytotoxic effect on HepG2 cell with approximately 160 µg/ml after 24h. Moreover both of them completely block cell viability at concentration above 1 and 0.5 mg/ml respectively. Data of green tea cytotoxicity were in consist with (Schmidt et al. 2005) where 1-3 mg/ml induced a significant decrease in primary rat hepatocytes viability. This cytotoxic effect may be due to the H_2O_2 generated by green tea in the medium and may account for all cytotoxic effects (Chai et al. 2003). Cytotoxic effect resulting from potato peel suggested to be due to their glycoalkaloids contents which tend to concentrate in the potato peel rather than the flesh (Charles et al. 2002). Broccoli and lipoic acid were also able to initiate cytotoxic effects to the HepG2 cells after 24h but the IC50 appeared only after 48h. Isothiocyanates and other breakdown products of glucosinates found in broccoli have been associated with its genotoxicity and cytotoxicity in bacterial and mammalian *in vitro* test systems (Charles et al. 2002; Kassie et al. 1996; Linscombe et al. 1998). The IC50 of lipoic acid was in agreement with (Casciari et al. 2001). Stimulating and generating of H_2O_2 as well as superoxide anion may be the proposed action of lipoic acid induced cytotoxicity (Moini et al. 2002a; Yamasaki et al. 2009).

Artichoke, spinach and eggplant peel induce only initial cytotoxicity after 48h and didn't elicit the IC50 at the tested concentration. Toxicity of these extracts might due to the pro-oxidants effects resulting from their flavonoids, carotenoids and other active components (Rietjens et al. 2002; Satoh et al. 2008). All other antioxidants and plant extracts including EC, ECG, EGC, EGCG, AA, NAC, α-TOC, onion and orange peel didn't exhibit detectable cytotoxicity in

the tested concentration ranges. These data are in agreement with (Li and Xie 2000; Rietjens et al. 2002; Yen et al. 2002).

The nontoxic concentrations of antioxidants and plant extracts were then studied as protective agents against DEN induced cytotoxicity. Data revealed that at lower concentrations of spinach, LA, artichoke, orange peel, eggplant peel, α-TOC, onion and AA in descendingly order, significantly reduce DEN cytotoxicity, while their higher concentrations as well as all other antioxidants and plant extracts, induce slight to significant increasing in DEN cytotoxicity. Since spinach, a rich source of lipoic acid (Packer et al. 1995b), and synthetic lipoic acid recorded the highest protective effects against DEN cytotoxicity, we therefore suggest that its protective effects may be attributed to lipoic acid. Many other active constituents may be also involved such as flavonoids (Cho et al. 2008; Lomnitski et al. 2003), coumaric acid derivatives and uridine (Bergman et al. 2001) carotenoids (β-carotene, lutein and zeaxanthin) and vitamin C (Ismail et al. 2004; Sisodia et al. 2008).

Lipoic acid serves through several mechanisms to act as protective agent, including scavenging of free radicals, chelating of metal ions, regeneration of endogenous and exogenous antioxidants, such as ubiquinon, vitamins C and E and glutathione and inhibition of NF-κB, a transcription factor controlling the expression of several genes engaged in the inflammatory response (Lee et al. 2008a; Roy and Packer 1998b). Therefore, it has been widely used as therapeutic agent in several diseases (El Midaoui and de Champlain 2002; Ha et al. 2006), including advanced cancer by increasing the glutathione peroxidase activity and by reducing oxidative stress (Bustamante et al. 1998; Mantovani et al. 2003). On the other hand, (Perra et al. 2008) presented results demonstrating that α-LA treatment in conditions associated with fatty liver aggravates liver damage and strongly accelerates the growth of preneoplastic lesions.

Artichoke extract significantly inhibit the DEN cytotoxicity. This can be explained through the phenolic contents and antoxidative potency. Artichoke extracts contains several active constituents, such as hydroxycinnamic acids, flavonoids, cinarin, cynarin, cynaropicrin, grosheimin, inulin, luteolin but cannot account for the full potency of the extract (Gebhardt 1997; Zapolska-Downar et al. 2002). Artichoke extract may also exert its protective effect through diminishing the loss of total GSH and cellular leakage of GSSG resulting in oxidative stress induced hepatotoxicity (Mehmetcik et al. 2008). Orange peel is a byproduct; contains several active constitutes including sinapic acid, p-coumaric acid, naringin, hesperidin, neohesperidin, kaempferol, sinensetin, diosmin and carotenoids including luteolin, zeaxanthin, β-cryptoxanthin and β-carotene (Anagnostopoulou et al. 2005a; Wang et al. 2008; Wang et al. 2007). Many of these constitutes showed beneficial effects on acute toxicity such as hesperidin (das Neves et al. 2004), kaempferol (Leung et al. 2007) and carotenoids (Gradelet et al. 1997; Morganti and Fabrizi 2002). Moreover, It was reported that orange peel extract inhibits tumor growth *in vivo* (Fan et al. 2007). These findings are in consistent with our data concerning inhibition of DEN cytotoxicity. On the same line eggplant peel as byproduct showed significantly inhibition on DEN cytotoxicity. It contains significant amount of phenolic compounds including chlorogenic acid and quercetin. Eggplant peel extracts may also contain several anthocyanins especially nasunin which are reported to act preventively against the oxidative stress (Kimura et al. 1999; Noda et al. 2000).

α-TOC, onion and AA also inhibits DEN cytotoxicity but their effects were lower compared to other substances. These results were in agreement with (Bansal et al. 2005b) who concludes that the pre-treatment with vitamin E prior to the administration of DEN to rats, reduced oxidative stress, although it produced only slight changes in the hepatic injury, in a time-dependent manner, where other studies reported no significant effects (Lii et al. 1999). Vitamin C is assumed to block the synthesis of endogenous DEN from its precursors (Melnikov 1998). It is also able to reduce the severity of chemical

hepatocarcinogenesis (Shamaan et al. 1998). Likewise onion extracts exhibit also slight inhibition on DEN cytotoxicity this may due to organosulfur compounds like cysteine sulfoxide derivatives (Kris-Etherton et al. 2002).

Other antioxidants and plant extracts including NAC, EC, ECG, EGC, EGCG, green tea, broccoli and potato peel as well as the higher concentrations of other substances, induce elevation on DEN cytotoxicity. Green tea and broccoli were the most prominent promoters to this cytotoxicity where cell viability was 12.5 and 17.8% of untreated control respectively. These negative effects might due to the pro-oxidants effects resulting from their flavonoids, carotenoids and other active constitutes involved in these substances (Rietjens et al. 2002; Satoh et al. 2008). Although green tea extract and its catechins including EC, ECG, EGC and EGCG exhibit broad spectrum of medicinal activities like reducing the incidence of chemically induced tumors (Naczk and Shahidi 2004; Wang et al. 1992), they can themselves act as cell damaging and pro-oxidants especially at high concentrations due to the spontaneous H_2O_2 generation. On the other hand it has been suggested that pro-oxidants activities may be also an important mechanism of the anticancer activities found for green tea (Malik et al. 2003; Nakagawa et al. 2002).

Our data showed no significant relation between the protective effects towards DEN induced cytotoxicity and antioxidants potential and/or phenolic contents of the tested substances. Therefore we suggest that the antioxidant potency is not the only mechanisms, many other mechanisms have been speculated such as inhibition of DEN metabolism of toxic metabolites including inhabitation of cytochrome P450 enzymes of the CYP1A family which play a major role in the activation of a number of suspected human carcinogens, inhibition of RNS/ROS production, increased intracellular GSH, and inhibition of NF-κB activation (Dey and Cederbaum 2006; Lee et al. 2008b). We can conclude that some antioxidants and plant extracts at selective concentration may exhibit inhibitory effects against DEN induced hepatotoxicity whereas others can promote its effect. Different mechanisms for such inhibition can be speculated

at this point, underlining the need of more detailed studies to specifically identify these mechanisms. Experiments with pure substances would simplify this issue giving more consistent and direct results. A combinatorial study with different pure components could then be applied to determine the synergetic effects as those that may be present in the plant extracts tested.

6 SUMMARY

Dietary antioxidants are believed to play an important role in the prevention and treatment of a variety of diseases associated with oxidative stress. Although there is a wide range of dietary antioxidants, the bulk of the research to date has been focused on the nutrient antioxidants vitamin C, E, and carotenoids. Certain relatively uncommon antioxidants such as lipoic acid (LA), and phenolic compounds such as (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG), have not been extensively investigated although they may exert greater antioxidant potency than that of carotenoids and vitamins. Extracts from selected plants and plant byproducts may represent rich sources for one or more of such antioxidants and therefore exhibit higher effects than a single antioxidant due to the synergistic effects produced between such antioxidants. However, in the last decade a number of epidemiological, animal and *in vitro* studies have suggested a protective and therapeutic potency of these antioxidants in a broad range of diseases such as cancer, diabetes, atherosclerosis, cataract and acute and chronic neurological disorders.

Inflammation, the response of the host toward any infection or injury, plays a central role in the development of many chronic diseases. Several evidences demonstrated the rise of different types of cancer from sites of inflammation. This suggests that active oxygen species and some cytokines generated in the inflamed tissues can cause injury to DNA and ultimately lead to carcinogenesis. Diethylnitrosamine (DEN) is one of the most important environmental carcinogens, present in a variety of foods, alcoholic beverages, tobacco smoke and it can be synthesized endogenously. In addition to the liver it can induce carcinogenesis in other organs like kidney, trachea, lung, esophagus, fore stomach, and nasal cavity. Several epidemiological and laboratory studies indicate that nitroso compounds including DEN may induce hyperplasia and chronic inflammation which is closely associated with the development of hepatocellular carcinoma.

Despite increasing evidence on the potential of antioxidants in modulating the etiology of chronic diseases, little is known about their role in inflammation and acute phase response (APR). Therefore the aim of the present work was to study the protective effect of water and solvent extracts of eight plant and plant byproducts including green tea, artichoke, spinach, broccoli, onion and eggplant, orange and potato peels as well as eight antioxidants agents including EC, EGC, ECG, EGCG, ascorbic acid (AA), acetylcysteine (NAC), α-LA, and alpha-tocopherol (α-TOC) toward acute inflammation induced by interleukin-6 (IL-6) and hepatotoxicity induced by DEN *in vitro*. The negative acute phase proteins (APP), transthyretin (TTR) and retinol-binding protein (RBP) were used as inflammatory biomarkers analyzed by ELISA, whereas neutral red assay was used for evaluating the cytotoxicity. All experiments were performed *in vitro* using human hepatocarcinoma cell line (HepG2). Additionally the antioxidant activities was measured by TEAC and FRAP assays, phenolic content was measured by Folin–Ciocalteu and characterized by HPLC. Moreover, the microheterogeneity of TTR was detected using immunoprecipitation assay combined with SELDI-TOF MS.

Results of present study showed that HepG2 cells provide a simple, sensitive *in vitro* system for studying the regulation of the negative APP, TTR and RBP under free and inflammatory condition. IL-6, a potent proinflammatory cytokine, in a concentration of 25 ng/ml was able to reduce TTR and RBP secretion by approximately 50-60% after 24h of incubation. With exception of broccoli and water extract of onion which showed pro-inflammatory effects in this study, all other plant extracts, at specific concentrations, were able to elevate TTR secretion in normal condition and even under treatment of IL-6 where the effect was quite lower. Green tea followed by artichoke and potato peel exhibited the highest elevation in TTR concentration which reached 1.1 and 2.5 folds of control in presence and absences of IL-6 respectively. In general Plant extracts were ordered according their anti-inflammatory potency as following: in water extracts; green tea > artichoke > potato peel > orange peel > spinach > eggplant peel, where in solvent extracts; green tea >

artichoke > potato peel > spinach > eggplant peel > onion > orange peel. The anti-inflammatory effect of water extracts of green tea, artichoke and orange peel were significantly higher than their corresponding solvent extracts whereas water extracts of eggplant-, potato peels and spinach showed lower effect than their solvent extracts. On the other hand α-LA followed by EGCG and ECG exhibited the highest elevation in TTR concentration compared to other antioxidants. The relation between the anti-inflammatory potential and antioxidants activity and phenolic content for the investigated substances was generally weak. This may suggest the involvement of other mechanisms than antioxidants properties for the observed effect.

TTR secreted by HepG2 cells has a molecular structure quite similar to the purified standard and serum TTR in which all the three main variants are contained including native, S-cystinylated and S-glutathionylated TTR. Interestingly, a variant with molecular mass of 13453.8 ± 8.3 Da has been detected only in TTR secreted by HepG2. Among all investigated antioxidants and plant extracts, six substances were able to elevate the native preferable TTR variant. The potency of these substances can be ordered as following α-LA > NAC > onion > AA > EGCG > green tea. A weak correlation between elevation on TTR and shifting to the native form was observed. Similar weak correlation has also been observed between antioxidants activity and elevation in native TTR.

Although DEN was able to induce cell death in a concentration dependent manner, it requires considerably higher concentrations for its effects especially after 24h. This may be attributed to a lack in cytochrome P450 enzymes produced by HepG2. At selected concentrations some antioxidants and plant extracts significantly attenuate DEN cytotoxicity as following: spinach > α-LA > artichoke > orange peel > eggplant peel > α-TOC > onion > AA. Contrary all other substances especially green tea, broccoli, potato peel, and ECG stimulate DEN toxicity.

In conclusion, this study demonstrated that selected antioxidants and plant extracts may attenuate the inflammatory process, not only by their antioxidants potency but also by other mechanisms which remain unclear. They may also play a vital role on stabilizing the tetramer structure of TTR and thereby prevent amyloidosis diseases. Lipoic acid represents in this study unique function against inflammation and hepatotoxicity. Despite the protective effect demonstrated by investigated substances, attention should also be given to the pro-oxidant and potential cytotoxic effects produced at higher concentrations.

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9 STATUTORY DECLARATION

I hereby declare that this thesis has been written by myself without any external unauthorized help, that it has been neither presented to any institution for evaluation nor previously published from its parts. Any data, words or ideas, of the thesis, however very limited, including tables, graphs, maps etc., which are quoted from or based on other sources, have been acknowledged without exception.

Potsdam, 16 April 2009

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The antioxidant acetylcysteine changes the microheterogeneity of plasma transthyretin in patients with chronic kidney disease into a less amyloidogenic form. Schweigert, FJ, Henze A, Gerike B, Raila J, Wittstock A, Scholze A, El-Saadany M.A, Zidek W, Martin Tepel M. (submitted).

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