EFFECTS OF ACRYLAMIDE AND RESVERATROL ON RABBIT LIVER AND KIDNEY ANTIOXIDANT ENZYMES

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EFFECTS OF ACRYLAMIDE AND RESVERATROL ON RABBIT LIVER AND KIDNEY ANTIOXIDANT ENZYMES

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ABSTRACT

EFFECTS OF ACRYLAMIDE AND RESVERATROL ON RABBIT ANTIOXIDANT ENZYMES

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Resveratrol is one of the promising naturally occurring polyphenolic compound found in red wine having antioxidant and anti-carcinogenic properties. However, *in vivo* studies investigating the effects of resveratrol on antioxidant enzymes are limited. In the present study, we investigated, for the first time, the influence of resveratrol on liver and kidney antioxidant enzymes and oxidative stress markers in acrylamide treated and control rabbits.

New Zealand male rabbits were treated with acrylamide and resveratrol, separately in two different doses and conditions. Their combined effects were also investigated. While, acrylamide treatment significantly decreased the glutathione peroxidase (GPx) activity in liver (1.24-fold), it was significantly increased (1.20 – 1.40-fold) by combined effect of resveratrol and acrylamide in liver and kidney. Furthermore, alone resveratrol administration increased (~1.37 – fold) GPx activity in kidney. Although, glutathione reductase (GR) was found to be significantly increased (~1.30-fold) in two different dose of resveratrol treated rabbit liver, it was not changed in acrylamide and their combined treatments. Despite, glutathione (GSH) content was decreased around 1.6 fold as a result of acrylamide treatment in rabbit liver and kidney cytosols, GSH level was returned to

normal levels by resveratrol tretment in rabbit liver and kidney. Furthermore, acrylamide treatment significantly increased the SDH activity in blood serum (1.68-fold) and in liver (1.27-fold) with respect to control. On the other hand, resveratrol treatment brought this activity nearly normal level in acrylamide treated rabbits.. Besides, sorbitol deydrogenase (SDH) was found to be decreased (3.13-fold) significantly in rabbit liver cytosol as a result of single dose of 100 mg/kg b.w. resveratrol treatment. Moreover, catalase activity and MDA level were not affected from either resveratrol or acrylamide and with their combination effect in investigated rabbit organs.

An important liver damage marker enzyme other than ALT and AST, SDH was characterized in terms of substrate, cofactor and enzyme concentration in rabbits which have been not investigated before and found to be 200 mM, 141 µM and 0.5 µL, respectively in rabbit liver. Furthermore, the Km value was first calculated in liver of New Zealand rabbits as 55,5 mM.

In addition to these, *in vitro* effects of resveratrol on GST activity was also studied throughout this study. Resveratrol was shown to be a noncompetitive inhibitor for liver cytosolic GST against substrate CDNB with K_i of 175 µM. On the other hand, resveratrol was shown to be a competitive inhibitor for liver cytosolic GST against substrate GSH with K_i of 55 µM.

The results of the present study have demonstrated for the first time that resveratrol induced some of the antioxidant enzyme activities and as well nonenzymatic antioxidants in rabbit liver and kidney. The results of GPx, GR, SDH activities and GSH level have also suggested that resveratrol may have protective effects on acrylamide induced hepatoxicity and renal toxicity. Therefore, it may be a therapeutic approach for the oxidative stress-related diseases such as cancer. However, further *in vivo* studies are required to clarify the effect of resveratrol on both acrylamide-induced toxicity and bioavailability in the body.

Key Words: Resveratrol, antioxidant enzymes, acrylamide, oxidative stress, SDH

ÖZ

RESVERATROL VE AKRİLAMİDIN TAVŞAN SİTOZOLİK ENZİMLERİ ÜZERİNE ETKİSİ

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Resveratrol kırmızı şarapta bulunan antioksidan ve antikarsinojen özellikleri olan ümit vadeden, doğal bir polifenolik maddelerden biridir. Ancak, resveratrolün antioksidan enzimler üzerine etkisini araştıran *in vivo* çalışmalar yeterli değildir. Bu çalışmada, biz ilk kez akrilamid uygulanmış ve kontrol tavşanların karaciğer ve böbrek antioksidan enzimleri ve oksidatif stress işaretçileri üzerine resveratrolün etkisini araştırdık.

Çalışmada, akrilamid ve resveratrol Yeni Zellanda türü erkek tavşanlara farklı doz ve durumlarda ayrı ayrı uygulanmıştır. Ayrıca, akrilamid ve resveratrolun ortak etkilerini araştırmak için her ikisi birlikte uygulanmıştır. GPx aktivitesini akrilamid karaciğerde anlamlı bir şekilde düşürürken (1.24-kat), resveratrol ve akrilamidin birlikte uygulanması bu aktiviteyi anlamlı şekilde artırmıştır (1.20 – 1.40-kat). Ayrıca, resveratrolün yalnız verilmesi GPx aktivitesini böbrekte anlamlı şekilde artırmıştır (~1.37-kat). Resveratrolün iki faklı dozu GR aktivitesini tavşan karaciğerinde anlamlı şekilde artırmasına karşın, akrilamid ve birlikte uygulama bunu değiştirmemiştir. GSH miktarı, tavşan karaciğer ve böbreğinde akrilamid uygulaması ile yaklaşık 1.6 kat düşerken, bu resveratrol uygulaması ile birlikte normal seviyeye geri döndürülmüştür. Ayrıca, akrilamid

uygulaması SDH aktivitesini tavşan kan serumunda (1.68-kat) ve karaciğerinde (1.27-kat) artırmıştır. Bunun aksine, resveratrol ve akrilamidin birlikte uygulaması bu aktiviteyi normal seviyesine getirmiştir. Bunun yanısıra, tek doz 100 mg/kg b.w. resveratrol uygulaması SDH aktivitesini (3.13-kat) düşürmüştür. Buna ek olarak, katalaz aktivitesi ve MDA seviyesi resveratrol, akrilamid ve her ikisinin ortak uygulaması araştırılan organlar üzerine etki göstermemiştir.

Bunun yanısıra, ALT ve AST dışında önemli bir karaciğer hasarı işaretçisi enzim olan sorbitol dehidrogenaz daha önce araştırılmamış olan substrat, kofaktör ve enzim konsantrasyonu açısından karakterize edilmiş ve tavşan karaciğerinde sırasıyla 200 mM, 141 μM ve 0.5 μL olarak bulunmuştur. Ayrıca, Yeni Zellanda tavşanlarında Km ilk kez 55,5 mM olarak hesaplanmıştır.

Bunlara ek olarak, resveratrolün GST aktivitesi üzerine etkisi *in vitro* olarak araştırılmıştır. Resveratrolün sitozolik GST enzimi için CDNB substratına gore 175 μM K_i değeri ile yarışmasız inhibitor olduğu gösterilmiştir. Öte yandan, resveratrolün sitozolik GST enzimi için GSH substratına göre 55 μM K_i değeri ile yarışmalı inhibitor olduğu gösterilmiştir.

Bu çalışma, resveratrolün tavşan karaciğer ve böbreğinde hem antioksidan enzim aktivitelerini hem de enzimatik olmayan antioksidanları artırdığını gösteren ilk çalışmadır. GPx, GR, SDH aktiviteleri ve GSH değeri sonuçları, resveratrolün akrilamidle indüklenen renal ve hepatotoksisitede koruyucu etkisi olduğunu göstermiştir. Dolayısıyla, kanser gibi oksidatif stres kaynaklı hastalıklarda resveratrol iyileştirici amaçlı kullanılabilir. Ancak, resveratrolün akrilamid toksisitesinde koruyucu etkisini ve vücutta biyoyararlanımını tanımlamak için ileri çalışmalar yapılmalıdır.

Anahtar Kelimler: Resveratrol, antioksidan enzimler, akrilamid, oksidatif stress, SDH

To My Family

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AA	Acrylamide
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BSA	Bovine serum albumin
CDNB	1-chloro-dinitrobenzene
CYP	Cytochrome P450
DMSO	Dimethylsulfoxide
DNB-SG	1-glutathione-2,4-dinitrobenzene
EDTA	Ethylene diamine tetra acetic acid
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
MDA	Malondialdehyde
NADH	Nicotinamideadenine dinucleotide, reduced form
$NADP^+$	Nicotinamideadenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form

NQO1	DT-Diaphorase
PAH	Polycyclic aromatic hydrocarbon
PUFA	Poly unsaturated fatty acid
SDH	Sorbitol dehydrogenase
TBA	2-thiobarbituric acid
TCA	Trichloroacetic acid
TRIS	Tris(hydroxymethyl) aminomethane

CHAPTER 1

INTRODUCTION

With the growth of civilization and industrialization, an increasing number of chemicals are being introduced to our environment as xenobiotics. These chemicals are dangerous to living organisms, to humans, and to our ecosystems (Arinç *et al*., 2000). These chemicals cause mutations in organisms leading to different kind of chronic diseases particularly cancer. Despite enormous efforts to search for a cure, cancer still remains as a formidable challenge for public health in the world (Kundu and Surh, 2008). It is also expected that in the next 50 years, the number of cancer-related deaths may double (Mann *et al*., 2005). Other than classical cancer chemoprevention methods, the plant kingdom nowadays attracts considerable attention for the discovery of many biologically active substances with great therapeutic values. Furthermore, accumulating evidence from population-based and laboratory studies suggest that regular consumption of fruits and vegetables is inversely associated with the risk of certain malignancies. However it is not yet clear which components or combination of components in fruits and vegetables are protective and what is their mechanism of action (Canistro *et al*., 2009).

One of the promising naturally occurring polyphenol exhibiting health beneficial effects including chemopreventive, antioxidant, cardioprotective and anti-inflammatory activities is resveratrol (3,5,4'- trihydroxystilbene) (Penumastha *et al.,* 2006; Ferrero *et al.,* 1998; Ray *et al.,* 1999; Sgambato *et al.,* 2001; Sılan *et al.,* 2007).

1.1 POLYPHENOLIC COMPOUNDS

The expression “phenolic compounds” represents a considerable range of substances that possess an aromatic ring having one or more hydroxyl and methoxyl group substituents (Harborne *et al*., 1980, Hodek *et al.,* 2002). Up to now, thousands of different polyphenolic compounds, with more than 8,000 phenolic structures currently known are produced by plants as secondary metabolites (Urquiaga *et al*. 2000). These polyphenolic compounds are essential to the physiology of plants, being involved in diverse functions such as lignifications and structure, pigmentation, pollination, pathogen resistance and growth (Haslam, 1998).

Flavonoids represent the most common and widely distributed group of plant phenolics. Flavanoids are part of a family of naturally occurring polyphenolic compounds having a common C_6-C_3-C_6 structure consisting of two aromatic rings linked through an oxygenated heterocycle (Harborne, 1980). Since flavanoids have radical scavenging property, complexation with metal ions (copper, iodine etc.) and complexation with other molecules including macromolecules like proteins and polysaccharides, they show biochemical and pharmacological properties particularly for the cancer prevention (Haslam *et al.*, 1996). Moreover, flavanoids, particularly those present in dietary and medicinal plants, have been reported to possess substantial anticarcinogenic and antimutagenic activities (Gusman *et al.,* 2001). This property of flavanoids attracts the most attention of the scientists and since then several different mechanisms were proposed particularly in alteration of enzyme expression and/or activities.

Since antioxidant enzymes have role in neutralizing the damaging effects of free radicals generated from the xenobiotics and environmental chemicals; flavanoids accepted as the important antioxidant defenses having the potential to induce these antioxidant enzymes to protect the cells against free radicals. Among the distinct flavanoids, over the past decade, resveratrol has

emerged as one of the most promising naturally occurring compound with immense therapeutic potential particularly in cancer chemoprevention.

1.2 RESVERATROL

Resveratrol is a natural and antifungal polyphenol phytoalexin (trans 3,5,4-trihydroxy stilbene) present in various food products particularly in grapes, mulberries, red wine, root extract of weed, rasperries, blueberries, Scots pine, Eastern white pine and knotweed (Figue 1.1) having antioxidant, antiplatelet, angiogenic, anti-inflammatory, estrogenic, cardioprotective, cell growth modulatory, anticarcinogenic, antihypersensitive and immunomodulatory properties (Sılan *et al.,* 2007; Şener *et al.,* 2006; Kırımlıoğlu *et al.,* 2008; Athar *et al.,* 2009; Penumastha *et al.,* 2006; Ferrero *et al.,* 1998; Ray *et al.,* 1999; Russo *et al.,* 2001; Sgamboto *et al.,* 2001; Surh *et al.,* 1999; Bertelli *et al.,*1999).

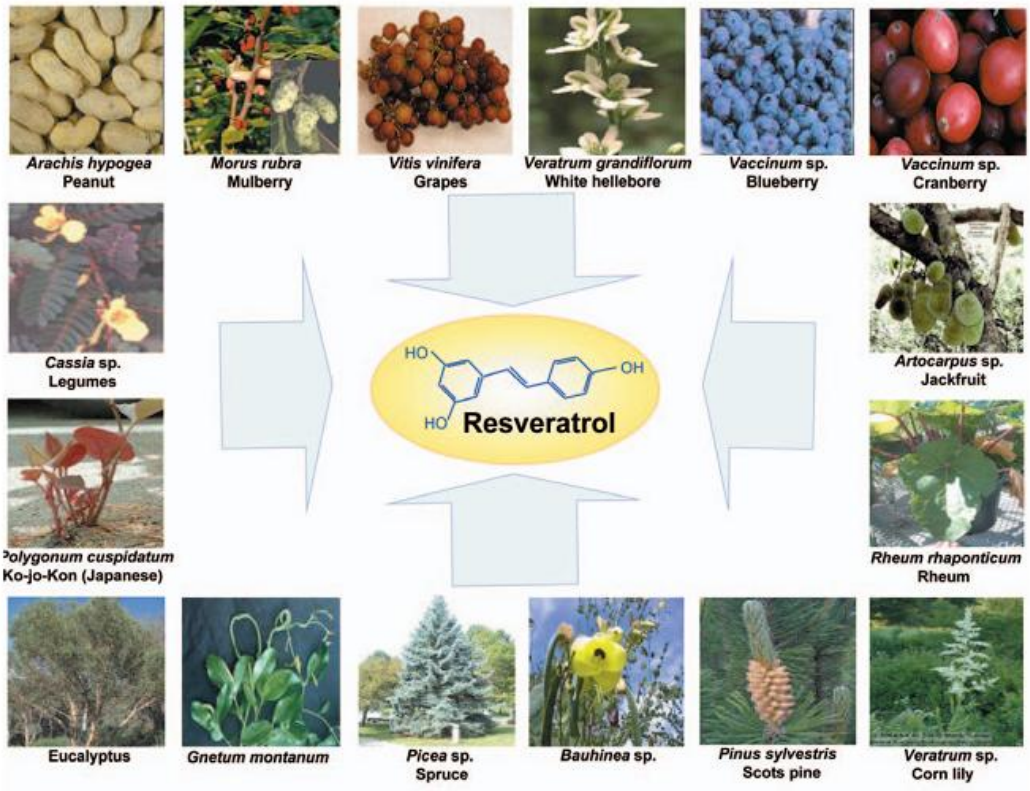


Figure 1. 1 Sources of Resveratrol. It is taken from Bharat *et al.,* 2004

Resveratrol is first identified in *Veratrum grandiflorum O. Loes* (White Hellebore) and then synthesized by *Polygonurn cuspidatum* (“Kojokon” in Japanese) roots, which have long been used as a traditional oriental medicine for therapeutic indications including heart diseases (Takaoka, 1939). In Figure 1.2., the structure of resveratrol is given.

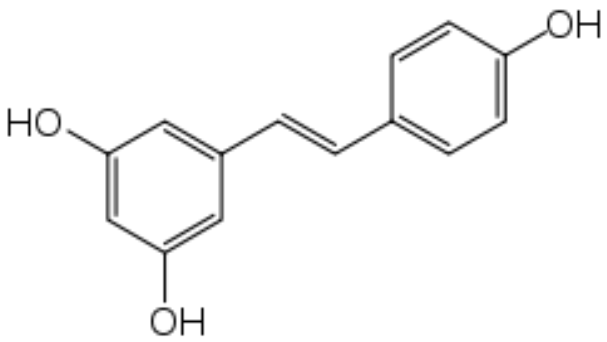


Figure 1. 2 Chemical structure of resveratrol

Resveratrol was first taken into consideration being as a therapeutic agent when its presence in wine was reported in 1992 by Siemann and Creasy (Siemann and Creasy, 1992). Since there is a high concentration of resveratrol exists in the skin of the grapes (~50 - 100 µg/g) depending on the grape cultivar, its geographic origin, and exposure to fungal infection, a significant amount of it is present in the red wine (~0.1-20 mg/L) (Fremont *et al.,* 2000; Savouret and Quesne, 2002). The amount of fermentation time a wine spends in contact with grape skins is an important determinant of its resveratrol content. In Table 1.1, the amount of resveratrol in distinct beverages can be observed.

Table 1. 1 Resveratrol Content in Beverages[a]

Beverage	Total resveratrol
Muscadine Wines	14.1 - 40 mg/L
Red Wines (Global)	1.98 - 7.13 mg/L
Red wine (Spanish)	1.92 - 12.59 mg/L
Red Grape Juice	1.14 - 8.69 mg/L
Rose Wine (Spanish)	0.43 - 3.52 mg/L
Pinot Noir (White wine)	0.40 - 2.0 mg/L
White Wine (Spanish)	0.05 - 1.80 mg/L
Peanut (boiled)	2.3 - 7.9 µg/g
Peanut Butter	0.2 - 0.4 µg/g

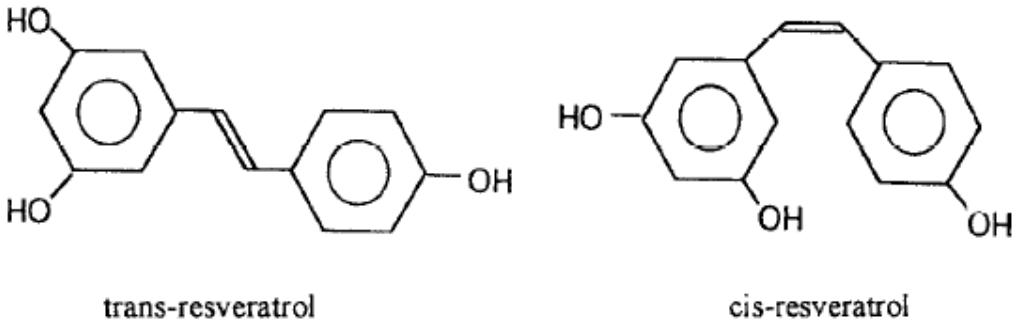
[a] Adapted from LeBlanc, 2005; Burns *et al.*, 2002

As it is also seen in the table 1.1, resveratrol concentration is much higher in red wines than white wines. This is due to the fact that after pressing of the grapes, the skins are removed in white wine production; on the contrary the grape skins are left for the red wine production for variable periods of time for obtaining the aromatic compounds (Elmali *et al.,* 2005; Soleas, 1997).

Resveratrol is produced naturally by several plants when under attack by pathogens such as bacteria or fungi. It is a stilbenoid, a derivate of stilbene, and is produced in plants with the help of the enzyme stilbene synthase. Its synthesis is induced by stress conditions such as infection by pathogenic bacteria or trauma, UV irradiation, or exposure to ozone, vicissitudes in climate, sunlight and heavy metals (Langcake *et al.,* 1976; Schubert *et al.,* 1997; Soleas *et al.,* 1997; Athar *et al.,* 2009). Particularly red wine contains high quantity of resveratrol because of the response of *Vitis vinifera* to fungal infection (Dercks *et al.,* 1989).

Resveratrol ($C_{14}H_{12}O_3$) is a white powder with slight yellow cast with a melting point of 253-255 °C and 228.24 $g.mol^{-1}$ molecular weight. Resveratrol is insoluble in water but dissolves in ethanol and

dimethylsulphoxide. The stilbene based structure of resveratrol consists of two phenolic rings linked by a styrene double bond to generate 3,4',5, -trihydroxystilbene (Aggarwal *et al*., 2004). Resveratrol exists as two stereoisomeric forms: *cis*- (*Z*) and *trans*- (€). The *trans*- form can undergo isomerisation to the *cis*- form when exposed to ultraviolet irradiation and heat. Moreover, the naturally existing form of resveratrol as being trans form was first detected in grapevines *(Vitis viniferu)* in 1976 (Langcake and Pryce, 1976; Athar *et al.,* 2009). Trans-resveratrol is the preferred steric form and is relatively stable if it is protected from high pH and light (Ignatowicz and Baer-Dubowska, 2001).



trans-resveratrol cis-resveratrol

Figure 1. 3 Chemical structure of trans and cis resveratrol

1.2.1 Therapeutic Effects of Resveratrol

Up to now, resveratrol is a frequently studied polyphenol not only in animal models but also in laboratory experiments demonstrating that this compound has numerous biological activities. The enormous beneficial effects can be summarized in Table 1.2.

Table 1. 2 Therapeutic activities of resveratrol[a]

Therapeutic Activities of Resveratrol	Reference
Antibacterial and antifungicidal activities	Creasy and Coffee, 1988
Antioxidant activity	Chanvitayapongs *et al.,* 1997
Free radical scavenging	Belguendouz *et al.,*1997; Fauconneau *et al.,* 1997
Inhibition of lipid peroxidation	Frankel *et al.,* 1993
Inhibition of eicosanoid synthesis	Pace-Asciak *et al.,* 1995; Kimura *et al.,* 1985
Inhibition of platelet aggregation	Pace-Asciak *et al.,* 1995; Bertelli *et al.,* 1995; Chung *et al.,* 1992
Anti-inflammatory activity	Jang *et al.,* 1997; Johnson *et al.,* 1998; Rotondo *et al.,* 1998
Vasorelaxing Activity	Chen and Pace-Asciak., 1996
Modulation of lipid and lipoprotein metabolism	Celotti *et al.,* 1996
Oestrogenic/anti-oestrogenic activity	Gehm *et al.,*1997, Mizutani *et al.,* 1998
Antitumoural activity	Jang *et al.,* 1997
Anti-aging activity	Jiang, 2008

[a] Adapted from *Gusman et al.,* 2001

1.2.1.1 Antioxidant Effect of Resveratrol

Resveratrol is both a free radical scavenger and a potent antioxidant due to its ability to promote activities of a variety of antioxidant enzymes as it is shown in Figure 1.4. The antioxidant effect of resveratrol is essentially depending on the redox properties of their phenolic hydroxy groups and the ability of the aromatic structure to support an unpaired electron due to electron delocalisation across the chemical structure (Duthie *et al.,* 2003; de la Lastra and Villegas, 2007).

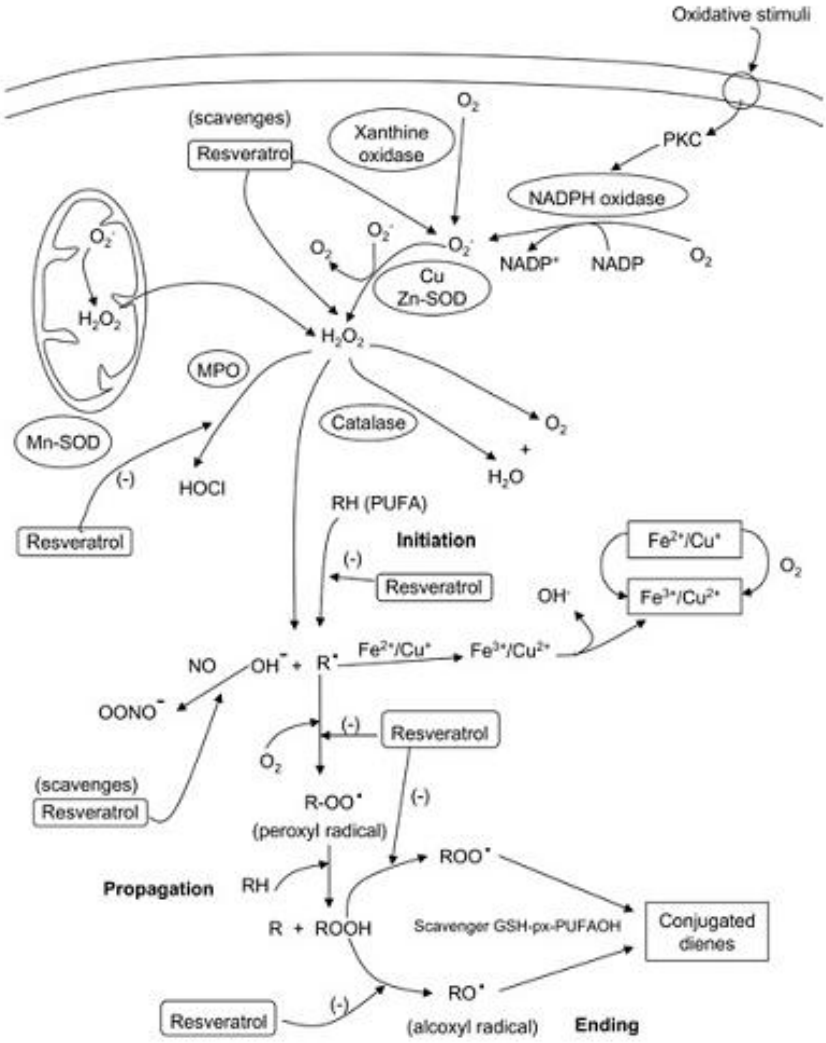


Figure 1. 4 Resveratrol Antioxidant Mechanism. It is taken from de la Lastra and Villegas, 2007.

Resveratrol shows potent antioxidant effect and can be a therapeutic approach for the oxidative stress-related diseases as cancer and arteriosclerosis (Jang *et al.,* 1997; Belguendouz *et al.,* 1998, Pace-Asciak *et al.,* 1995, Bravo *et al.,* 1998). Moreover, resveratrol is an excellent scavenger of hydroxyl, superoxide and other radicals. It also protects against lipid peroxidation in cell membranes and DNA damage caused by reactive oxygen species generation (Leonard *et al.,* 2003). All the protective role of resveratrol has been attributed to its antioxidant activity (Rice-Evans *et al.,* 1996).

In order to protect tissues against the deleterious effects of ROS, all cells have numerous defense mechanisms including antioxidant enzymes such as SOD (superoxide dismutase), catalase, glutathione reductase and glutathione peroxidase. Resveratrol can maintain the concentration of intracellular antioxidants found in biological systems. For instance, in one study, stilbene appeared to maintain the glutathione content in peripheral blood mononuclear cells isolated ex vivo from a healthy human from oxidative damage caused by 2-deoxy-D-ribose (Losa *et al*., 2003). In another study, in human blood platelets, resveratrol markedly decreased oxidation of thiol groups of proteins in these cells (Olas *et al*., 2004). Similarly, resveratrol induced an increase in glutathione levels in a concentration dependent manner in human lymphocytes activated with H_2O_2. Furthermore, resveratrol increased the amounts of several antioxidant enzymes in human lymphocytes, including glutathione peroxidase, glutathione S-transferase and glutathione reductase (Yen *et al*., 2003).

1.2.1.2 Cardioprotective Effect of Resveratrol

As a phenolic compound, since resveratrol contributes to the antioxidant potential of red wine, can play a role in the prevention of human cardiovascular diseases (Fremont *et al.,* 2000).

Epidemiological studies have revealed an inverse correlation between red wine consumption and the incidence of cardiovascular disease, a phenomenon commonly known as the ‘‘French Paradox’’, i.e. the fact that the incidence of heart infarction in France is about 40% lower than in the rest of Europe, despite smoking habits, lack of exercise and a diet being traditionally rich in saturated food particularly the cheese (Goldberg *et al.,* 1996, Hebbar *et al.,* 2005, de Lorgeril *et al.,* 2002).

Resveratrol has been found to prevent and improve the cure for the cardiovascular diseases in different ways as represented in Figure 1.5. One of the prevention is by reducing the cardiomyocyte apoptosis by upregulating nitric oxide particularly acting through the inducible nitric oxide synthase (iNOS) mRNA expression levels in the tissue, inhibiting membrane lipid peroxidation via a significant reduction in oxidative stress, protecting the vascular endothelium and inhibiting platelet aggregation (Fremont *et al.,* 2000; Shigematsu *et al.,* 2003; Wallerath *et al.,* 2002; Wu *et al.,* 2001; Imamura *et al.,* 2002).

Moreover, it is demonstrated that antioxidant enzymes and phase II enzymes including superoxide dismutase (SOD), catalase, GSH, glutathione reductase (GR), glutathione S-transferase (GST), and NAD(P)H:quinone oxidoreductase 1 (NQO1) can be induced by resveratrol in cultured cardiomyocytes and form resistance for cardiac cell injury when reactive oxygen species are present in the environment (Ross, 1997).

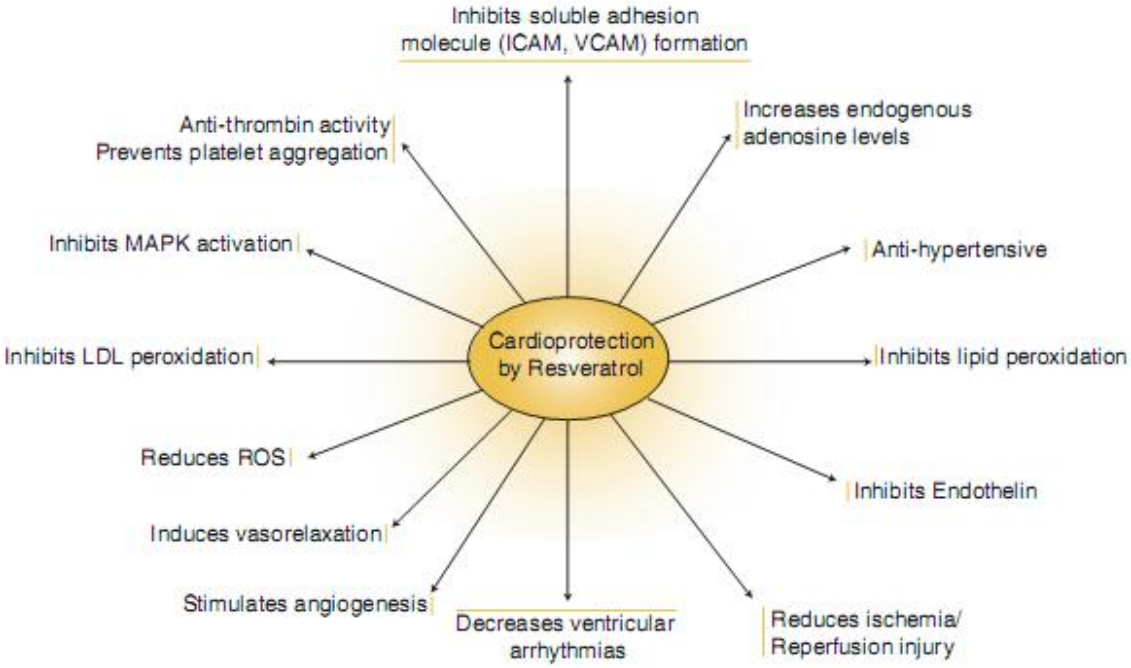


Figure 1. 5 Cardioprotection with resveratrol. It is taken from Das and Maulik, 2006.

1.2.1.3 Chemopreventive Effect of Resveratrol

The potent anti-carcinogenic property of resveratrol is first identified in 1997 when polynuclear aromatic hydrocarbon dimethylbenz(a)anthracene (DBMA) induced preneoplastic lesions in mouse mammary gland culture inhibits cellular events associated with tumor initiation, promotion, and progression in tumorigenesis (Jang *et al.,* 1997). Additionally, resveratrol has been shown to inhibit proliferation of a variety of cancer cells including breast, prostate, neck, ovary, muscle, colon, lung, esophagus, liver, non-melanoma skin cancer, neuroblastoma, leukemia, fibrosarcoma (Athar *et al.,* 2007; Roman *et al*., 2002; Dorrie *et al*., 2001; Wieder *et al*., 2001; Hayashibara *et al*., 2002; Tinhofer *et al*.; 2001; Clement *et al*., 1998).

The action mechanism of chemoprevention of resveratrol is still not so well defined however there exists such propositions: (1) inhibition of ribonucleotide reductase, DNA polymerase, protein kinase C, cycloxygenase-2 and hydroperoxidase activities; (2) inhibition of reactive oxygen species (ROS)-mediated carcinogenesis; (3) inhibition of cell division; (4) apoptotic cell death activation (Fontecave *et al.,* 1998; Sun *et al.,* 1998; Stewart *et al.,* 2000; Clément *et al.,* 1998; Subbaramaiah *et al.,* 1998; Jang *et al.,* 1997; Huang *et al.,* 1999, Ragione *et al.,* 1998; She *et al.,* 2001; Tinhofer *et al.,* 2001, Asensi *et al.,* 2002). Furthermore resveratrol has also been shown to suppress angiogenesis and metastasis. Other possible action mechanisms of resveratrol against cancer are represented in Figure 1.6.

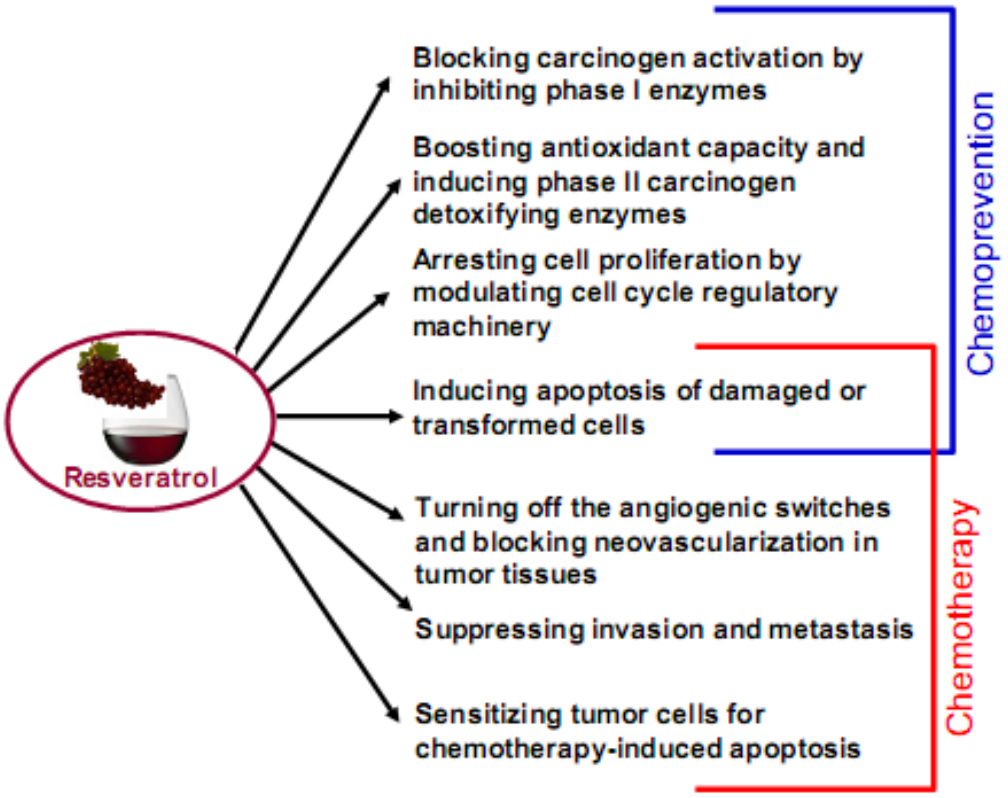


Figure 1. 6 Chemopreventive and chemotherapeutic action mechanisms of resveratrol. It is taken from Kundu and Surh, 2008.

The anti-carcinogenic effects of resveratrol appear to be closely associated with its antioxidant activity. The *in vivo* and *in vitro* studies suggests that use of resveratrol provides a new perspective in human cancer chemoprevention, in a combinatorial approach with either chemotherapeutic drugs or cytotoxic factors for the highly efficient treatment of drug refractory tumor cells (Seve *et al*., 2005).

1.3 CELLULAR DEFENSE SYSTEM AGAINST FREE RADICALS

Aerobic organisms possess antioxidant defense systems that protect themselves against reactive oxygen species (ROS) produced as a consequence of aerobic respiration and substrate oxidation (Figure 1.7). Small amounts of ROS, including hydroxyl radicals (•OH), superoxide anions ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), are constantly generated in aerobic organisms in response to both external and internal stimuli (Hurst *et al*., 1997; Jornot *et al*., 1998; Mills *et al*., 1998). Furthermore, reactive oxygen species (ROS) are involved in the cell growth, differentiation, progression, and death (Mates *et al*., 1999). Low levels of ROS may be beneficial and inevitable in many biochemical processes, including intracellular messaging in the cell, differentiation and cell progression or the arrest of growth, apoptosis, immunity, and defense against micro-organisms (Ghosh *et al*., 1998; Yin *et al*., 1995; Bae *et al*., 1997; Lee *et al*., 1998). Nevertheless, high doses and/or inadequate removal of ROS result in oxidative stress causing severe metabolic malfunctions including cancer, ischemia and damage to biological macromolecules including failures in immunity and endocrine functions (Chopra and Wallace, 1998; Czene *et al*., 1997; Wojtaszek *et al*., 1997). In order to avoid the negative aspects of ROS in the cell, several non-enzymatic and enzymatic antioxidant activities are found in organisms. In case of a ROS accumulation in the cell, the defense system promotes the regulation and expression of these enzymes.

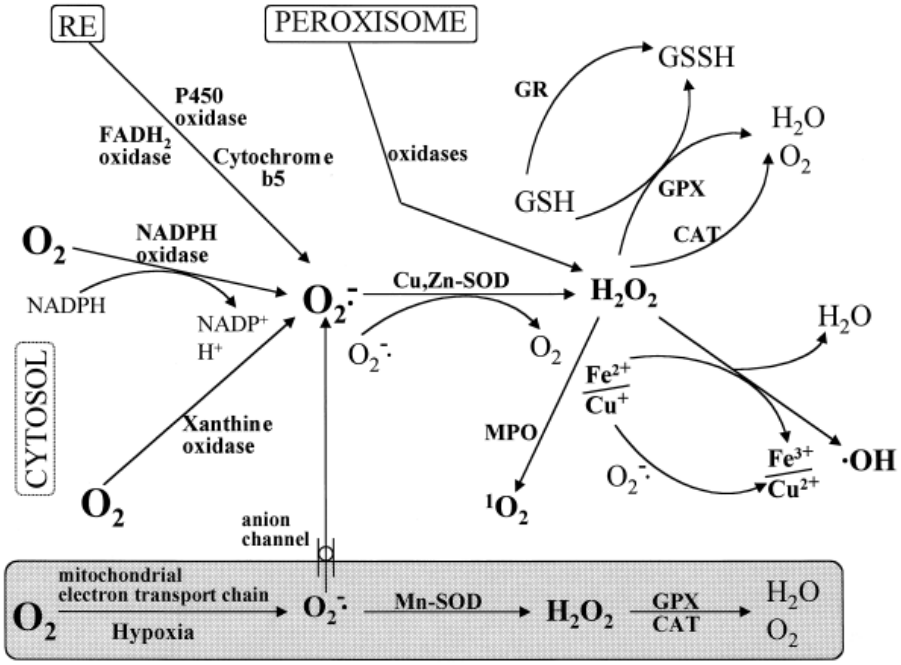


Figure 1. 7 Generation of reactive oxygen species and the defense mechanism against damage by active oxygen. It is taken from Mates *et al*., 1999.

During hypoxia superoxide generated may be degraded into the mitochondria by Mn-SOD or, if it reaches the cytosol, by Cu, Zn-SOD. In the endoplasmic reticulum, NADPH-CYP450 reductase can leak electrons onto O_2 generating $O_2^{\bullet -}$, $FADH_2$ and cytochrome b5 can also contribute to this system. Within peroxisomes, there are enzymes localized that produce H_2O_2 without intermediation of $O_2^{\bullet -}$. Contrarily to $O_2^{\bullet -}$, H_2O_2 is able to cross cell membranes and within the cells it can react with Fe^{2+} or Cu^{+} to form hydroxyl radicals via Fenton reaction (Mates *et al*., 1999).

As it is shown in Figure 1.7, CYP450s can form free radicals in the endoplasmic reticulum. Particularly, CYP2E1 has been identified as a source of reactive oxygen species in CYP2E1-dependent monooxygenation reactions (Ekstrom and Ingelman-Sundberg, 1989; Persson *et al*., 1990; Arinc *et al*., 2007). Moreover, CYP2E1 is accepted as the most efficient isozyme in the initiation of NADPH-dependent lipid peroxidation (Ekstrom and Ingelman-Sundberg 1989).

1.3.1 Antioxidant Enzymes

All cells in eukaryotic organisms contain powerful antioxidant enzymes to protect themselves against free radicals. The three major classes of antioxidant enzymes are the superoxide dismutases, catalases and glutathione (GSH) peroxidases. In addition, there are numerous specialized antioxidant enzymes reacting with detoxifying oxidant compounds. Indirect antioxidant enzymes can be glutathione reductase and glutathione S-transferases. Different subcellular sites and different cell types may contain varying amounts of the antioxidant enzymes (Soboll *et al*., 1995).

1.3.1.1 Superoxide Dismutases (E.C 1.15.1.1)

Superoxide dismutase (EC 1.15.1.1) is the metalloenzyme that catalyses the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2. Peroxide can be destroyed by CAT or GPX reactions in the following reactions (Fridovich, 1995; Sandalio *et al*., 1997; Teixeira *et al.*, 1998).

General reaction catalyzed by superoxide dismutase:

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

SOD has been shown to play key role in protecting cells and tissues against oxidative stress (Fridovich and Freeman, 1986; Tsan, 1997). Four different kind of SODs have been characterized in eukaryotes; a copper- and zinc-containing form (CuZnSOD) localized in the cytosol, a manganese-containing form (MnSOD) in the mitochondria, iron containing FeSOD in some prokaryotes and plants' outer mitochondrial membrane and a copper- and zinc- containing form in the extracellular matrix (ECSOD) (Marklund, 1982).

Copper-zinc superoxide dismutase is a homodimer with a molecular weight of 32 kD and mainly localized in the cytosol, but is also present in nucleus and peroxisomes. CuZnSOD is made up of two identical subunits containing one Cu^{2+} and one Zn^{2+} per subunit as its active site (Fridovich, 1975).

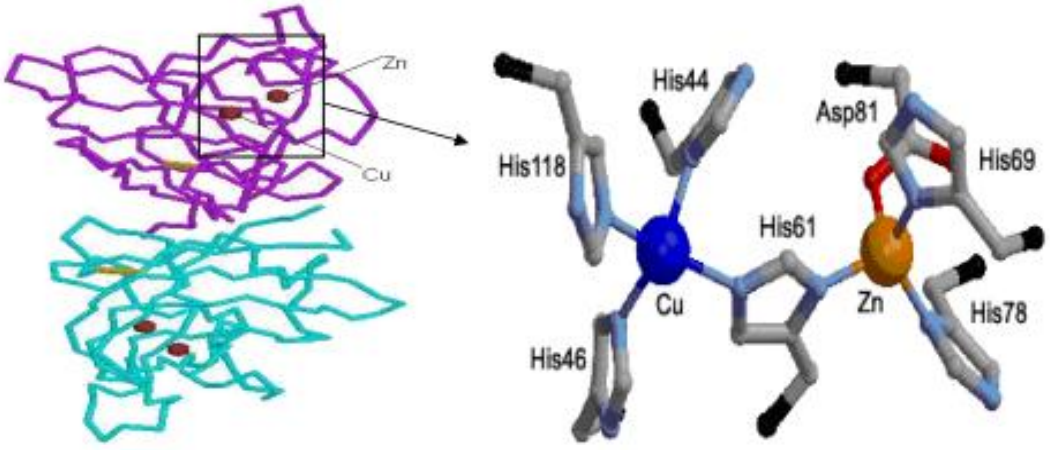


Figure 1. 8 Protein Structure and active site of CuZnSOD

Manganese superoxide dismutase is a homotetramer with a molecular weight of 23 kD. MnSOD is localized in the matrix of the mitochondria having manganese (III) at its active site (Oberley and Buettner, 1979). MnSOD has been shown to play a major role in promoting cellular differentiation and tumorgenesis and in protecting against hyperoxia-induced pulmonary toxicity (Buettner *et al*., 1978; Bozzi *et al*., 1976).

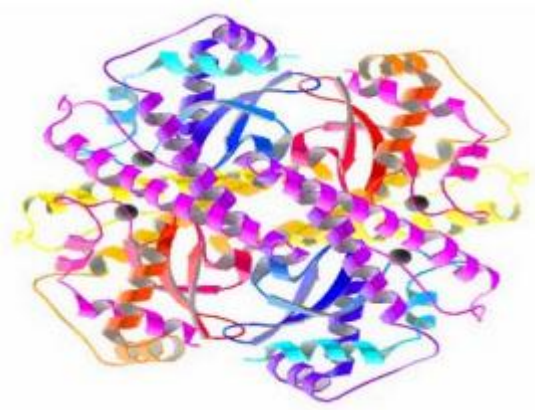


Figure 1. 9 Structure of MnSOD

The Mn- and Fe-SODs are very similar in their primary and tertiary structures. Fe-containing SOD appears to be located exclusively in chloroplasts (Salin & Bridges 1980; Kwiatowski *et al*. 1985).

Extracellular superoxide dismutase is a secretory tetrameric Cu/Zn containing glycoprotein, with a molecular weight of around 135 kD (Marklund, 1982). ECSOD is the least abundant of the SODs in tissues, but it is the major SOD in extracellular fluids such as plasma and extracellular matrix (Marklund, 1984; Sandstrom *et al.*, 1993 and Oury *et al*., 1994).

1.3.1.2 Catalase (E.C 1.11.1.6)

Catalase is a tetrameric hemoprotein with a molecular weight of 240 kD (Figure 1.11). A typical catalase contains four identical subunits, each with a heme in active site. There are many forms of catalase and most of them contain Fe-heme, but some contains Mn.

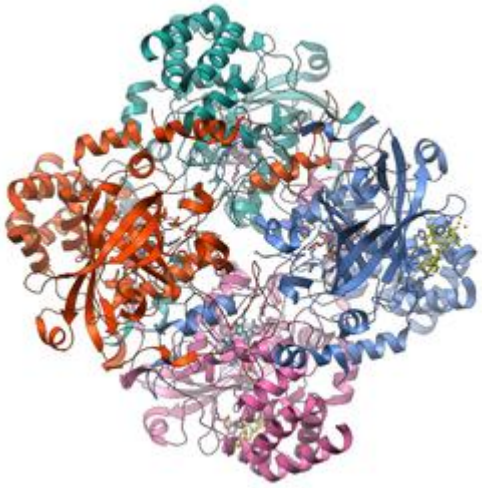


Figure 1. 10 Structure of catalase

Catalase, present in the peroxisomes of nearly all aerobic cells, serves to protect the cell from the toxic effects of hydrogen peroxide by catalyzing its decomposition into molecular oxygen and water without the production of free radicals (Davies *et al*.,1979). The major function of catalase within cells is to prevent the accumulation of toxic levels of hydrogen peroxide formed as a by-product of metabolic processes - primarily that of the electron transport pathway.

General reaction catalyzed by catalase:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

1.3.1.3 Glutathione Peroxidase (E.C 1.11.1.9)

In animal cells, glutathione peroxidases (GPx) are homotetrameric water soluble selenoenzymes wich catalyze the reduction of hydroperoxides at the expense of GSH (Flohe, 1989; Ursini *et al*., 1995). In this process, hydrogen peroxide is reduced to water, whereas organic hydroperoxides are reduced to alcohols. GPx active site contains selenium in the form of a selenocysteine residue, which is incorporated into the polypeptide backbone (Flohe *et al*., 1973; Rotruck *et al*., 1973; Stadtman, 1991).

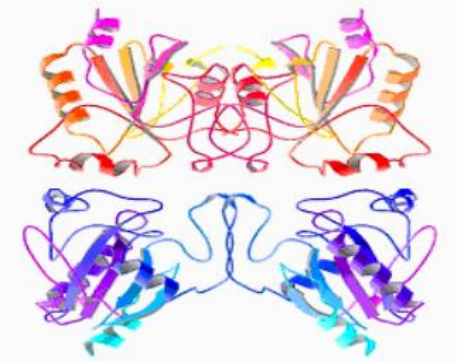


Figure 1. 11 Structure of GPx

There are several isozymes of GPx varying in celullar location and substrate specificity. GPx1 is the most abundant version, found in the cytoplasm of nearly all mammalian tissues, whose preferred substrate is hydrogen peroxide. GPx2 is an intestinal and extracellular enzyme, while GPx3 is extracellular, especially aboundant in plasma. GPx4 has a high preference for lipid hydroperoxides; it is expressed in nearly every mammalian cell, though at much lower levels. (Muller *et al*., 2007). So far, eight different isoforms of glutathione peroxidase (GPx1-8) have been identified in humans.

General reaction catalyzed by glutathione peroxidase:

$$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$$

1.3.1.4 Glutathione Reductase (E.C 1.6.4.2)

Glutathione reductase (GR) is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). The native enzyme of most GRs is a homodimer of 100-120 kD. This enzyme is essential for the GSH redox cycle wich maintains adequate levels of reduced cellular GSH. GR is known to play a key role in response to oxidative stress, heavy metals and herbicides in both plants and animals.

Furthemore, glutathione reductase has a crucial role in GPx and GST reactions as an adjunct in the control of peroxides and free radicals (Bompart *et al*., 1990). A high GSH/GSSG ratio is essential for protection against oxidative stress. Glutathione reductase has been mainly localized in chloroplasts, mitochondria, cytosol and peroxisomes.

General reaction catalyzed by glutathione reductase:

$$ROH + GSSG + NADPH + H^{+} \longrightarrow ROOH + 2GSH + NADP^{+}$$

GR is a homodimeric enzyme of which each subunit contains four well-defined domains (Williams *et al*., 1967; Dym and Eisenberg, 2001). The dimeric nature of the enzyme is critical for its function because both subunits contribute with essential residues to the constitution of the active site (Karplus and Schulz, 1989).

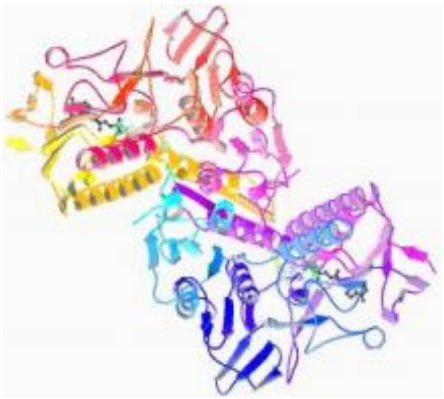


Figure 1. 12 Structure of GR

1.3.1.5 Glutatione S-Transferase (E.C 2.5.1.18)

Glutathione S-Transferases (GSTs, E.C 2.5.1.18) are a multi-gene family of dimeric ubiquitous enzymes involved in the removal of toxic metabolites from the cell, maintenance cellular sulfhydryl groups in their reduced form and, in a few instances, activation of a wide variety of chemicals. GSTs are soluble

proteins with typical molecular masses of around 50kDa (Eaton and Bammler, 1999; Bendich *et al*., 1988). Generally GSTs catalyze the conjugation of reduced glutathione (GSH) with a variety of nonpolar compounds that contain an electrophilic carbon, nitrogen or sulphur atom (Strange *et al*., 2000; Hayes *et al*., 2005). The general substrate of GST in cytosolic extracts of liver is CDNB (1,2-dichloro-4-nitrobenzene). GSTs are probably present in all life forms, in microbes, flies, plants and mammals. Furthermore, the GST proteins have evolved by gene duplication to perform a range of functional roles using the tripeptide glutathione (GSH) as a cosubstrate or coenzyme (Armstrong, 1997).

GSTs catalyze GSH dependent detoxification of reactive electrophile xenobiotics such as genotoxic chemicals, carcinogens, cytotoxic cancer chemotherapeutic agents; environmental carcinogens such as herbicides, insecticides and pollutants; endogenous compounds such as epoxides and quinones which are the by-products of oxidative stress (Hayes *et al*., 2005). Some of the substrates of GSTs are given in Table 1.3.

Some environmental xenobiotics and dietary carcinogens become toxic only after activation *in vivo*. Since Cytochrome P450s are the most efficient enzymes of carcinogen activation, this CYP-catalyzed oxidation are also frequently followed by conjugation with GSH via GST, and other detoxifying enzymes (Eaton and Bammler, 1999). GSTs are also involved in the metabolism of by-products of oxidative stress resulted from degradation of aromatic aminoacids (phenylalanine and tyrosine), synthesis of steroid hormones, synthesis of important metabolites of arachidonic acid, modulation of signaling pathways. It was also demonstrated that bioactivation of toxic metabolites by GST may cause organ damages (Bladeren van, 1988; Sherratt *et al*., 1997; Anders and Dekant, 1998).

Table 1. 3 Exogenous and endogenous substrates of GST[a]

Substrates for Glutathione Peroxidases			
Exogenous Substrates			**Endogenous Substrates**
Environmental Carcinogens	**Pesticides**	**Drugs**	(by-products of oxidative stress)
BDPE	Lindane	cis - platin	α-β-unsaturated aldehydes
AFB 8,9 epoxide	Alachlor	chlorambucil	hydroperoxides
5-hydroxy methyl - chrysene sulfate	Atrazine	cyclophosphamide	epoxides
Styrene oxide	DDT	BCNU	quinones
4 - Nitroquinoline	methyl parathion	thiotepa	dopaminochrome
Actonein	EPN	fosfomycin	aminochrome
Hexachlorobutadiene	malathion	ethacyric acid	catechol estrogens
Butadiene	muconaldehyde	nitroglycerine	maleylacetoacetate
Trichloroethylene	tridiphane	menadione	
Methylene chloride		acetominophen	
Ethylene oxide		adriamycin	
Inorganic arsenic		busulfan	
		carmustine	
		crotonyloxymethyl-2-cyclohexenone	
		melphalan	

[a] Adapted from Eaton et. al., 1999; Hayes *et al.,* 2005; Coles and Kaldbular, 2003

Glutathione transferases metabolize xenobiotics via catalyzing the first of four steps required for the synthesis of mercapturic acids (Keen, 1978). Subsequent reactions in this pathway entail sequential removal of the γ – glutamyl moiety and glycine from the glutathione conjugate, followed finally by N-acetylation of the resulting cysteine conjugate. It is crucial to recognize that GST enzymes are part of an integrated defense strategy. Thus, it is also demonstrated that the effectiveness depends on also glutamate cysteine ligase and glutathione synthase to supply GSH and the actions of transporters to remove glutathione conjugates from the cell.

General reaction catalyzed by glutathione S-transferase:

$$GSH + R - X \longrightarrow GSR + HX$$

There are three main families of GSTs which are cytosolic, mitochondrial and microsomal GSTs. Cytosolic and mitochondrial GST share some similarities in their three-dimensional structure but express no structural resemblance to the microsomal GST enzymes (Hayes *et al*., 2005). Additionally, GSTs are predominantly expressed in the cytosol, where their GSH dependent catalytic functions include the conjugation and resulting detoxification of herbicides, the reduction of organic hydroperoxides formed during oxidative stress and biotransformation of xenobiotics (Edwards *et al.*, 2000 and Marrs, 1996).

As in the concern of the present study, cytosolic GSTs are predominantly found in the cytoplasm of the cell. Mammalian cytosolic GSTs are all dimeric with subunits of 199-244 amino acids in length. There have been seven classes of cytosolic GSTs in mammalian species depending on the amino acid sequence similarities which are Alpha, Mu, Pi, Sigma, Theta, Omega and Zeta. In non-mammalian species other classes are designated as Beta, Delta, Epsilon, Lambda, Tau and 'U' class (Figure 1.14).

Cytosolic GSTs catalyze several reactions including conjugation, reduction and isomerization. They also bind covalently and non-covalently to hydrophobic nonsubstrate ligands including bilirubin, dyes, PAHs, heme and hormones which contribute to intracellular transport, disposition of xenobiotics and hormones (Litwack *et al.*, 1971; Habig *et. al*., 1974; Ketley *et. al*., 1975). Cytosolic GSTs exhibit genetic polymorphisms and this phenomenon has been associated with susceptibility to various diseases such as cancer and inflammatory diseases (Strange *et al*., 2000).

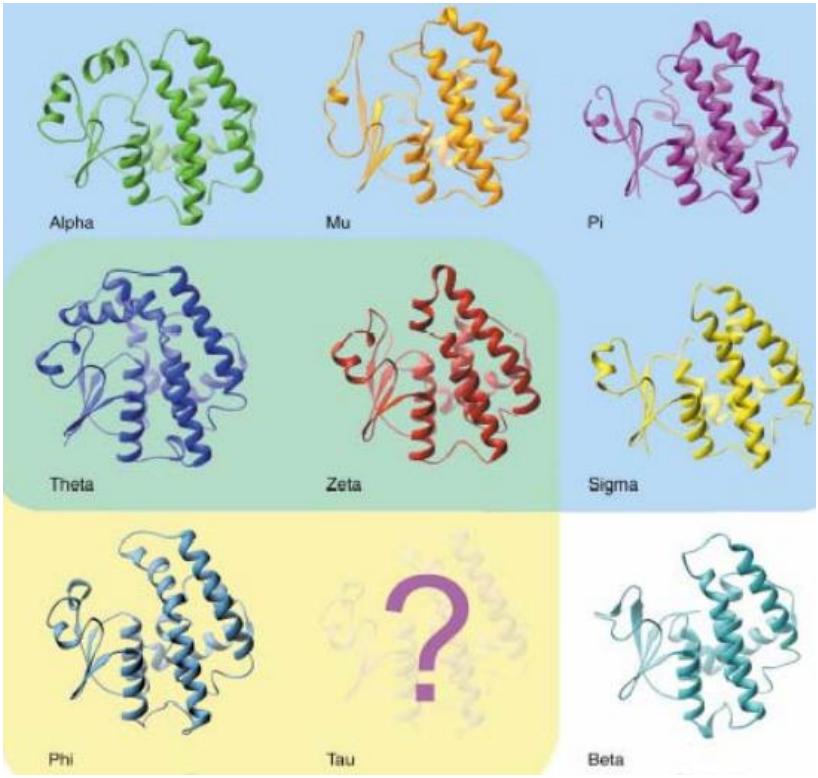


Figure 1. 13 Structure of GST subunits. The GST specific to mammals (alpha, mu, pi and sigma) have a blue background; the plant specific (phi) and bacteria specific (beta) GSTs have yellow and white backgrounds, respectively; GSTs (theta and zeta) that have counterparts in both animals and plants have green backgrounds. Although there is little sequence similarity between enzymes of different classes, there is significant conservation in overall structure (Dixon *et al*., 2002).

1.3.2 Non-Enzymatic Antioxidants

Antioxidants are the compounds that are functioning for the neutralization of the oxidizing effects of the free radicals. Other than antioxidant enzymes in the body for the protection against free radicals, a number of compounds act as non-enzymatic antioxidants. One of the important cellular non-enzymatic antioxidant is glutathione. Glutathione (GSH; $C_{10}H_{17}N_3O_6S$), shown in Figure 1.15 is a tripeptide (L-glutamyl-L-cysteinyl-glycine) and first reported in 1988 as a phitothion, distributed in the intracellular space of plants, animals, and

microorganisms has two general functions: to remove toxic metabolites from the cell and to maintain cellular sulfhydryl groups in their reduced form (Bendich, 1988). The cysteine provides an exposed free sulphydryl group (SH) that is very reactive, providing an abundant target for radical attack via disulfide bond formation. Reaction with radicals oxidizes glutathione, but the reduced form is regenerated in a redox cycle involving glutathione reductase and the electron acceptor NADPH. Other than glutathione, there exists several non-enzymatic antioxidants in the body such as ascorbic acid and α-tocopherol.

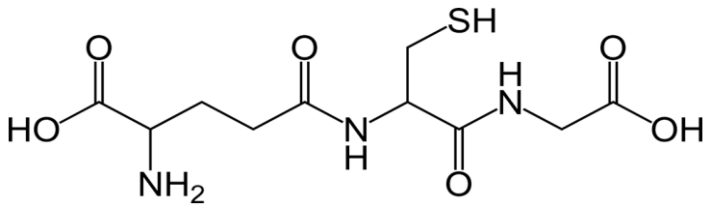


Figure 1. 14 Chemical structure of glutathione. –SH is the most biochemically active thiol group that can react with nucleophiles.

1.4 BIOMARKERS OF OXIDATIVE STRESS

Oxidative stress in the body can be easily recognized via alteration in the antioxidant enzyme activities and measurement of non-enzymatic antioxidant content. Other than these, there exist some biomarkers to detect the *in vivo* oxidative stress. These include DNA oxidation, glycoxidation, aminoacid oxidation and most importantly lipid peroxidation.

1.4.1 Lipid Peroxidation

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress. Lipid peroxidation is probably the most extensively investigated process which is induced by free radicals. Particularly, polyunsaturated fatty acids (PUFAs) are prone to free radical attack because the presence of a double bond

weakens the carbon-hydrogen bond at the adjacent carbon atom (Betteridge, 2000). Both isolated PUFAs (especially arachidonate) and those incorporated into lipids are readily attacked by free radicals, becoming oxidized into lipid peroxides (Halliwell and Chirico, 1993). These lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds. This process proceeds by a free radical chain reaction including initiation, propagation and termination (Figure 1.16). Thus, it is an important marker of early and reversible tissue damage.

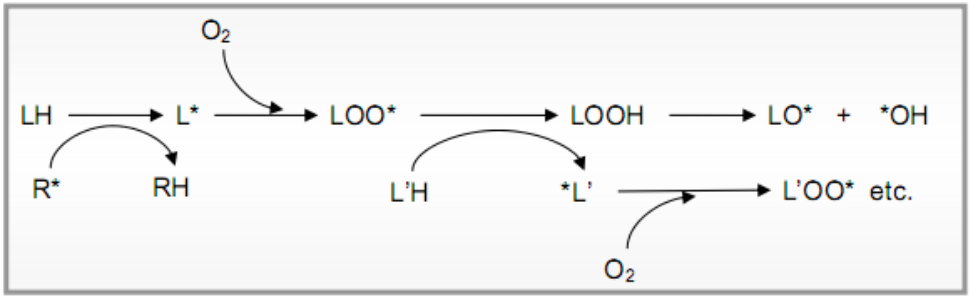


Figure 1. 15 Lipid peroxidation chain reaction

Lipid peroxidation can have profound effects on cellular function. Extensive peroxidation in cell membranes will result in changes in fluidity, increased permeability, a decrease in membrane potential, and eventually membrane rupture (Betteridge, 2000).

1.4.2 Sorbitol Dehydrogenase (E.C 1.1.1.14)

Sorbitol dehydrogenase, the second enzyme in the polyol pathway, uses NAD^+ as a cofactor and catalyzes the reversible oxidation-reduction of sorbitol and fructose. SDH has been identified in several human and animal tissues (King *et al*., 1958; Williams *et al*., 1957; Williams *et al*., 1954; Arcus *et al*., 1956; Holzer *et al*., 1955). It is located primarily in the cytoplasm and mitochondria of the liver, kidney, and seminal vesicles (Gerlach, 1957; Gerlach, 1959).

The use of the SDH assay is based on the finding that SDH activity in the serum is normally low but increases during acute episodes of liver damage due to exposure to environmental toxic chemicals leading to oxidative damage (King, 1965; Gerlach and Hiby, 1965). Unlike the transaminases, which may be of limited value as a biomarker for liver damage due to concentration variations across species, measurement of SDH has become a preferred marker of hepatic damage due to its high degree of organ specificity. Therefore, SDH has been considered as a very specific indicator of acute liver damage (Rose and Henderson, 1975; Schön and Wüst, 1960; Asada and Galambos, 1963). The catalytic sites contain both a serine and a histidine residue, which are hydrophilic side chains. The residues require NAD^+ and a zinc ion to be present for catalytic activity (Pauly *et al*., 2003).

General reaction catalyzed by sorbitol dehydrogenase:

$$\text{Sorbitol} + NAD^+ + H^+ \longrightarrow \text{Fructose} + NADH$$

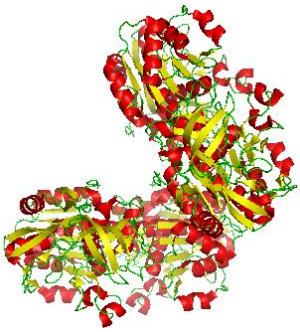


Figure 1. 16 Structure of SDH

1.5 ACRYLAMIDE

Acrylamide (2-propenamide) is an α-β unsaturated (conjugated) reactive low molecular weight vinyl compound produced industrially and is known as an animal neurotoxin, a reproductive toxin and a carcinogen formed in baked or fried carbohydrate rich food like chips and crisps (McCollisster, 1964; Wallace and Wallace, 1986; Friedman, 2003; Rice, 2005; Rosen and Hellenas, 2002; Tareke *et al*., 2002; Weiss, 2002). In the present study,

acrylamide is in concern as the oxidative damage causing reagent in rabbits and acrylamide induced toxicity was investigated.

Acrylamide is is an important chemical for waste water and drinking water purification, cosmetics, sugar manufacturing, petroleum and paper industry. Acrylamide is also extensively used in laboratories for the preparation of polyacrylamide gels for electrophoresis (Bergmark, 1997; Friedman, 2003; Exon, 2006). Acrylamide is also found in cigarette-smoke (Smith, 2000).

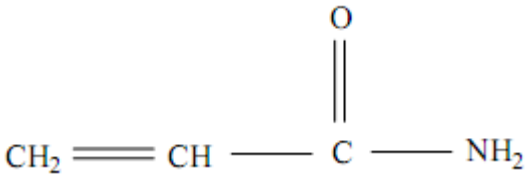


Figure 1. 17 Structure of acrylamide

Acrylamide can be reactive in three different ways. Firstly, it can undergo radical-mediated polymerization which is best attained anaerobically. Secondly, it was demonstrated that acrylamide might be formed through the Maillard reaction from amino acids (e.g.asparagine) and reducing sugars (e.g. glucose) (Mottram *et al*., 2002). Glycidamide, a metabolite of acrylamide formed via CYP2E1 metabolism, binds to DNA and can cause genetic damage. Thirdly, acrylamide can undergo addition to thiol, hydroxyl, or amino groups. A nice example is the thiol addition representing a detoxification pathway yielding primarily AA-GSH conjugates as urinary excreteable end products (Catalgol *et al*., 2009). Prolonged exposure has induced tumours in rats, but cancer in man has not been convincingly shown. The International Agency for Research on Cancer (IARC) and European Union has classified acrylamide as “probably carcinogenic to humans”(IARC, 1994).

In vivo effects of acrylamide and/or resveratrol, alone or in combination, in different doses and conditions on rabbit liver, kidney, and lung cytochrome P450 (CYPs) enzymes and NQO1 were investigated in our laboratory

(Nuyan, 2008). Microsomal CYP2E1-dependent *p*-nitrophenol hydroxylase, NDMA N-demethylase and aniline 4-hydroxylase activities were found to be significantly increased in acrylamide-treated rabbit liver (1.80-3.0 fold) and kidney (1.6-fold). Rabbit liver and kidney CYP2E1 protein levels (determined by western blot analyisis) also increased approximately 2-fold due to acrylamide treatment. In rabbit liver, resveratrol was found significantly effective in decreasing both acrylamide-induced CYP2E1-dependent enzyme activities (approximately 1.5-1.80 fold) and CYP2E1 protein levels (approximately 1.5-1.70 fold). Additionally, resveratrol significantly decreased acrylamide-induced CYP2E1 protein level (2-2.5 fold) in rabbit kidney. However, no significant change was observed in rabbit lung CYP2E1-dependent enzyme activities and CYP2E1 protein levels due to acrylamide, resveratrol or their combined treatments.

The results of the above study (Nuyan, 2008) have demonstrated for the first time that acrylamide induces rabbit liver and kidney CYP2E1-dependent enzyme activities and CYP2E1 protein levels. The induction of CYP2E1 enzyme activity and protein level by acrylamide treatment can stimulate formation of other toxic compounds and procarcinogens metabolized by CYP2E1 which in turn further potentiates the risk of hepatotoxicity, mutagenicity and carcinogenicity. The results of this study have also suggested that resveratrol may have protective effects on acrylamide induced CYP2E1 related toxicity.

Metabolism of AA in the body may result in generation of reactive oxygen species (ROS) which play a role in the oxidative stress of AA (Bergmark *et al*., 1991; Patel *et al*.,2003; Shuming *et al*.,2009). On the basis of results obtained by Yousef and El-Demerdash, 2006, acrylamide causes disturbances in the oxidative status and enzyme activities and indicates a risk of organ damage during exposure to acrylamide.

1.6 AIM OF THE PRESENT STUDY

Cancer becomes one of the leading death causing disease in today's world and incidence rates of cancer continue to rise with a significant ratio in the last century. Even more, cancer is the second leading cause of death after cardiovascular diseases in the world. There is a link between certain cancers and human exposure to toxic chemicals in the environment. Acrylamide is one of these toxic chemicals and known as an animal neurotoxin, a reproductive toxin and a carcinogen. Specifically, the carcinogenic effects of acrylamide has received great attention in recent years due to detection of acrylamide and its residues in human diet and also detection of acrylamide adducts (hemoglobin and DNA) in blood of both animals and humans (Tareke *et al*., 2000; Rosen and Helenas, 2002; Bergmark 1997; Hagmar, 2001).

Flavanoids is accepted as the important antioxidant defenses having potential to induce antioxidant enzymes to protect the cells against free radicals. In this study, acrylamide induced oxidative stress was studied against the active polypenolic compound resveratrol. Resveratrol is a recently important flavonoid with its well-known chemoprevention effects on different types of cancer (Jang *et al*., 1997; Dong, 2003). Resveratrol is found in grapes, red wine, mulberries and peanuts that can be easily consumed by humans in their diets like other phenolic compounds. Among several mechanisms, these flavonoids implement their chemoprevention effect by modulation of enzyme expression and/or activities such as inhibition of CYPs (mainly metabolize procarcinogens to more toxic compounds) or activation of anti-oxidant enzymes such as GPXs. Since acrylamide is a toxic compound, it is aimed that resveratrol exerts its free radical scavenging effect and antioxidant property on acrylamide-induced toxicity (Belguendouz *et al.,*1997; Chanvitayapongs *et al.,* 1997; Hebbar *et al*., 2005). Thus in the present study the protective effects of resveratrol on acrylamide-induced toxicity investigated in terms of antioxidant enzymes.

In this regards, the aims of the present study were:

1. to investigate the *in vivo* protective effects of resveratrol against carcinogenic acrylamide on rabbit liver, lung and kidney antioxidant enzyme activities including catalase, glutathione peroxidase and glutathione reductase which have not been investigated before

2. to determine the *in vivo* carcinogenic effect of acrylamide and polyphenolic compound resveratrol and their combined effects in non-enzymatic antioxidant, reduced glutathione content

3. to examine the *in vivo* carcinogenic acrylamide induced lipid peroxidation and the protective/preventive effect of resveratrol

4. to characterize one of the important hepatic damage marker enzymes –sorbitol dehydrogenase- in terms of substrate, cofactor and enzyme concentration in rabbits for the first time

5. to determine the *in vivo* hepatotoxic effect of acrylamide and polyphenolic compound resveratrol and their combined effects on rabbit liver and serum sorbitol dehydrogenase activities which have not been investigated before

6. to elucidate the possible *in vitro* effect of plant polyphenolic compound resveratrol for its ability to modulate glutathione-S transferase enzyme activities for the first time

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

Resveratrol was a kind gift from Mikrogen Pharma, İstanbul, Turkey.

Acrylamide (A8887), Acetic acid (glacial) (27225), Ammonium sulphate (A4418), Bovine serum albumin (BSA; A7511), Butylated hydroxytoulene (B1378), Carboxymethyl cellulose (C4146), 1-chloro-dinitrobenzene (CDNB; C6396), Ethanol (32221), ϵ-amino caproic acid (ϵ-ACA; A2504), Fructose (F0127), Glutathione reduced form (G4251), Glutathione oxidized form (G4251), Glutathione reductase (G3664), Hydrochloric acid (HCl; H1758), Phenylmethanesulfonyl fluoride (PMSF, P7626) , Sodium azide (S8032), Sodium potassium tartarate (Rochell salt; S2377), 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris; T1378), 2-Thiobarbituric acid (TBA, T5500) and Triton X-100 (T9284) were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

Copper (II) sulfate (Cu_2SO_4), Dimethyl sulfoxide (DMSO; 2951), Ethylene diamine tetra sodium salt (EDTA; 08421), Hydrogen peroxide 35% (H_2O_2; 304191), Perchloric acid (109065), Potassium chloride (KCl; 104935), Potassium dihydrogen phosphate (KH_2PO_4; 04871), Di-potassium hydrogen phosphate (K_2HPO_4; 05101), Sodium hydroxide (06462) and Trichloroacetic acid (TCA; 00256) were the products of E. Merck, Darmstadt, Germany.

β-nicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH; A1395), β-nicotinamide adenine dinucleotide reduced form (NADH; N1829) were purchased from Applichem Biochemica, Chemica Synthesis Services, Darmstadt, Germany.

All the other chemicals used in this study were of analytical grade and were obtained from commercial sources at the highest grade of purity.

This study was done in cooperation with Mine Nuyan's thesis work which constitutes the "*In vivo* Interaction of Carcinogenic Acrylamide with Cytochrome P450 Isozymes and Phase I Enzymes In Rabbit Liver, Kidney and Lung". In this study, the protective and preventive effects of resveratrol against carcinogenic chemical acrylamide were analyzed in terms of oxidative stress. Additionally, a crucial hepatic damage marker enzyme sorbitol dehydrogenase was characterized and *in vitro* inhibition of glutathione S-transferase enzyme by resveratrol was analyzed.

2.2 ANIMALS AND TREATMENTS

2.5 month old Adult male New Zealand white rabbits, initially weighing 2.0-2.9 kg, were purchased from Lameli Husbandry, Ankara. The rabbits were caged separately and housed for 7 days before the beginning of the treatments for adaptation to their new environment at 23-25 °C on 12 h light/12h dark cycle with free access to water and commercial chow. The procedures involving animals and their care were carried out in accordance with the Declaration of Helsinki.

After one week period, the rabbits were randomly selected and divided into 5 groups consisting of 4-5 animals. The animals were treated with two different compounds in two different doses and conditions:

1) Acrylamide dissolved in distilled water was injected subcutaneously to the animals

2) Resveratrol dissolved in carboxymethyl cellulose was administered to the animals, intragastrically.

First group consisted of control animals administered by physiological saline. Second group, acrylamide ("AA" group) was injected 3 dose of 100mg/kg body weight (b.w.) acrylamide at day 1, day 5 and day 8. Third group,

resveratrol group (“25 mg RESV” group) was administered 3 dose of 25mg/kg b.w. resveratrol at day 1, day 5 and day 8. Fourth group (“RESV+AA” group) was treated with 25mg/kg b.w. resveratrol at day 1, day 5 and day 8; 6 hours following resveratrol treatment, 100mg/kg b.w. acrylamide was administered to the animals at day 1, day 5 and day 8. In the fifth group (“AA+RESV” group), the animals were injected with 100mg/kg b.w. acrylamide at day 1, day 5 and day 8, then, at day 8, 6 hours following the last acrylamide treatment, a single dose of 100mg/kg b.w. resveratrol was administered to the animals. Finally, in the sixth group (“100 mg RESV”) a single dose of 100 mg/kg b.w. resveratrol was administered to the animals at day 8. Schematic representation of the treatments was represented in Figure 2.1. All of the animals were sacrificed 20h after last treatment by decapitation.

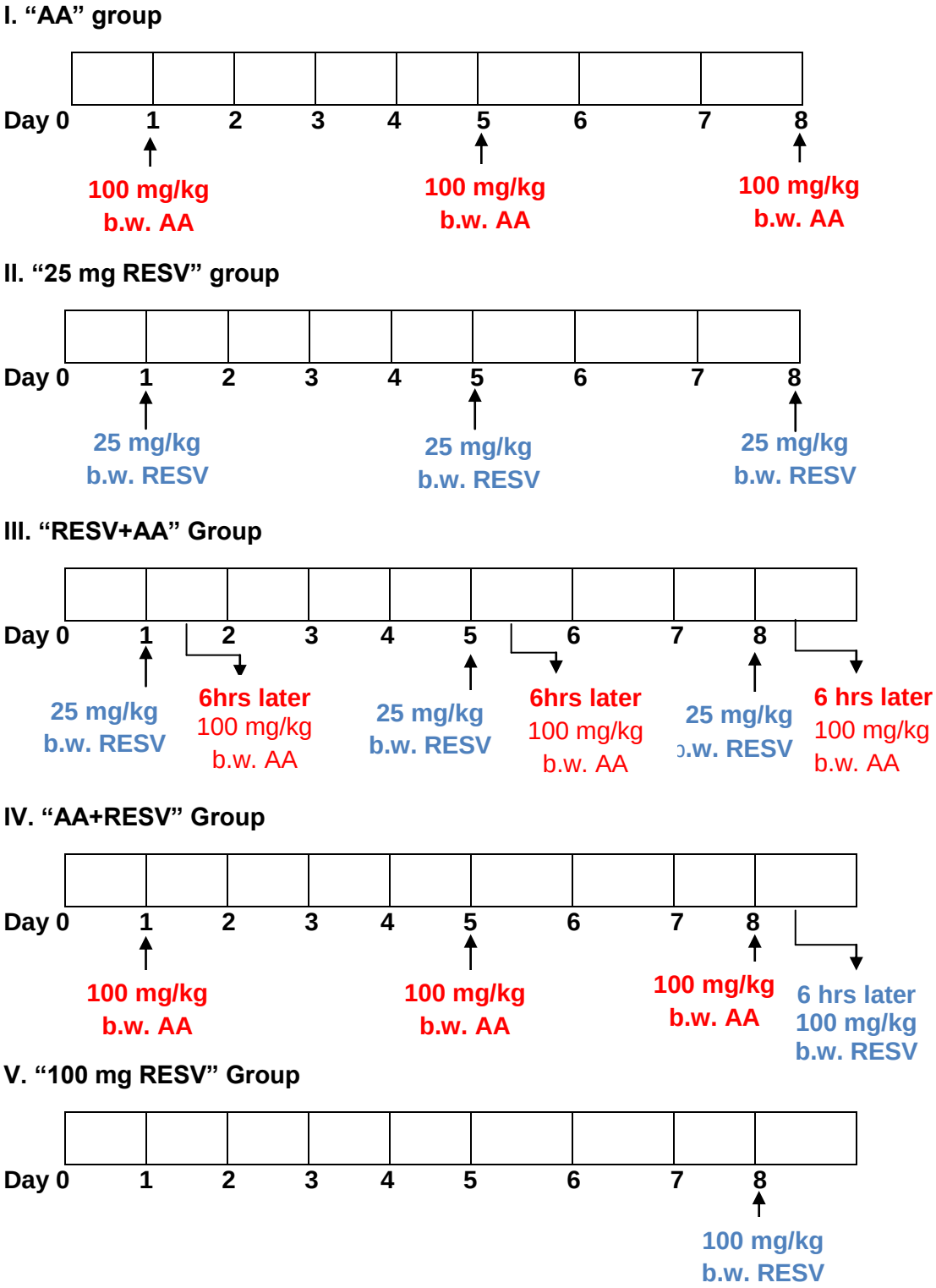


Figure 2. 1 Schematic representation of the experimental design consisting acrylamide and resveratrol treatment

2.3 METHOD

2.3.1 Preparation of Rabbit Liver Cytosols

Rabbit liver microsomes were prepared according to the method of Arınç and Adalı (1990) with slight modifications. Adult male New Zealand rabbits purchased from Lameli Husbandry, Ankara, Turkey, were killed by decapitation. The livers each weighing 40-80 g was removed immediately after killing the animals. Gall bladders were removed from the livers to prevent inhibitory effect of the gall bladders' content on monoxygenase activity. Then, the livers were placed in crushed ice. The connective and fatty tissues removed from the liver and followingly the livers were washed several times with cold distilled water and then with 1.15% KCl to remove the excess blood. All subsequent steps were carried out at 0-4 ° C. After draining and blotting on a filter paper, the livers were weighed for each rabbit tissue and minced by scissors. Then, the minced liver tissues were homogenized in 1.15% KCl solution containing 2 mM EDTA, 0.25 mM ϵ – ACA and 0.1 mM PMSF at a volume equal to 3 times the weight of liver by using Potter-Elvehjem glass homogenizer coupled with a motor (Black and Decker, V850, multispeed drill) driven Teflon pestle at 2 400 rpm. Fifteen passes were made for the homogenization of liver tissue.

The resulting homogenate was centrifuged at 10 031 rpm (10 800xg)(Sigma 3K30 Centrifuge, Osterode am Harz, Germany) with 12156 rotor for 25 minutes to remove cell debris, nuclei and mitochondria. Then, the supernatant fraction containing endoplasmic reticulum and soluble fraction of the cells was filtered through double layers of cheese-cloth by the aid of Buchner funnel. The microsomes were sedimented from supernatant by centrifugation at 45 000 rpm(145 215xg) for 50 minutes using a T 1270 type rotor in Sorvall Combi Plus Ultracentrifuge (Sorvall, Du Pont Company, Newton, Connecticut, USA). The supernatant fraction (cytosol) was obtained and the pellet was processed for microsomal separation. The soluble fraction containing cytosol was centrifuged at 13 100 rpm (16 000xg) (Sigma 3K30 Centrifuge, Osterode am Harz, Germany) with 12156 rotor for 25 minutes to

obtain a clear solution and to remove fatty interferences. The supernatant fraction was taken and shocked with liquid nitrogen and then stored at -80 ° C in order to use in the relevant cytosolic enzyme studies. Liver cytosolic fraction contained approximately 20 - 35 mg of protein per mL.

2.3.2 Preparation of Rabbit Kidney and Lung Cytosols

Rabbit kidney and microsomes were prepared essentially with the same procedure used for the preparation of rabbit liver cytosols with some modifications (Arınç *et al*, 2000a,b).

After decapitation of the rabbits, the kidney and lungs were removed and immediately placed in crushed ice. All subsequent steps were carried out at 0-4 ° C. After removal of fatty and connective tissues, organs were washed several with cold distilled water and then with 1.15% KCl. After draining and blotting on a filter paper and weighed, the lung and kidney tissues are weighed for each rabbit. After mincing, kidney and lung tissues were homogenized in 1.15% KCl solution containing 2 mM EDTA, 0.25 mM ϵ–ACA and 0.1 mM PMSF by using Potter-Elvehjem glass homogenizer coupled with a motor (Black and Decker, V850, multispeed drill) driven Teflon pestle at 2 400 rpm. The volume of the 1.15% KCl solution containing 2 mM EDTA, 0.25 mM ϵ–ACA and 0.1 mM PMSF was equal to 2.2 times the weight of kidney and 2.5 times the weight of lung tissues. Fifteen passes were made for the homogenization of kidney and lung tissues.

The resulting kidney or lung homogenate was centrifuged at 10 031 rpm (10 800xg) (Sigma 3K30 Centrifuge, Osterode am Harz, Germany) with 12156 rotor for 25 minutes to remove cell debris, nuclei and mitochondria. Then, the supernatant fraction containing endoplasmic reticulum and soluble fraction of the cells was filtered through double layers of cheese-cloth by the aid of Buchner funnel. The microsomes were sedimented from supernatant by centrifugation at 45 000 rpm (145 215xg) for 50 minutes using a T 1270 type rotor in Sorvall Combi Plus Ultracentifuge (Sorvall, Du Pont Company, Newton, Connecticut, USA). The supernatant fraction (cytosol) was obtained

and the pellet was processed for microsomal separation. The soluble fraction containing cytosol was centrifuged at 13 100 rpm (16 000xg) (Sigma 3K30 Centrifuge, Osterode am Harz, Germany) with 12156 rotor for 25 minutes to obtain a clear solution and to remove fatty interferences. The supernatant fraction was taken and shocked with liquid nitrogen and then stored -80 ° C in order to use in the relevant cytosolic enzyme studies. Lung cytosolic fraction contained approximately 20-32 mg of protein per mL. Kidney cytosolic fraction contained approximately 21-35 mg of protein per mL.

2.3.3 Preparation of Serum

Blood samples were collected into tubes immediately after killing the animals. The tubes kept for 15 minutes without shaking in a vertical position in order to avoid hemolysis. Then blood samples were centrifuged at 1 000 x g for 15 minutes. After centrifugation the supernatant was taken gently and stored at -20 ºC.

2.3.4 Protein Determination

The protein concentration of cytosols was measured according to the method of Lowry *et al.* (1951). As a standard, crystalline bovine serum albumin was used. Before protein determination, cytosols were centrifuged at 13 100 rpm (16 000xg) (Sigma 3K30 Centrifuge, Osterode am Harz, Germany) with 12156 rotor for 25 minutes to obtain a clear solution and to remove fat and protein interferences, otherwise there can exist a precipitation among time which affect the experimental results. Initial dilution (1:200) was performed for cytosols before the preparation of the reaction tubes. Moreover, dilution within tube was carried out by taking 0.10, 0.25, 0.50 mL of initially diluted samples into reaction tubes and completed it to final volume of 0.5 mL with distilled water. Then, 2.5 mL of Lowry alkaline copper reagent (prepared as 2 % copper sulphate, 2 % sodium potassium tartarate and 0.1 N NaOH containing 20% sodium carbonate in a ratio of 1:1:100 in the written order) was added to each tube. All tube contents were mixed and let stand for 10 minutes at room temperature for copper reaction in alkaline medium. After

that, 2 N folin reagent was diluted 1:1 ratio by distilled water and 0.25 mL of diluted reagent was added to each tube and mixed within 8 seconds by vortex. The tubes were incubated for 30 minutes at room temperature. The intensity of resulting color was measured at 660nm. Standard tubes with five different protein concentrations (0.02, 0.05, 0.10, 0.15 and 0.20 mg/mL of BSA) were prepared from crystalline bovine serum albumin, no dilutions were performed and same steps were carried out for standard tubes. A standard curve was plotted according to the readings of intensity of standards and by using the slope of the standard curve the protein amounts of the samples were calculated. Protein concentration was calculated by the following formula.

$$[\text{Protein}]_{(\text{mg/mL})} = \frac{OD_{660}}{\text{slope of standard curve}} \times \text{Tube dilution} \times \text{Dilution factor (200)}$$

2.3.5 Characterization and Determination of Sorbitol Dehydrogenase Activity

Since SDH is an important biomarker of hepatic damage, firstly we need to characterize the enzyme in terms of substrate, cofactor and enzyme content concentration in rabbit liver cytosolic fraction.

Firstly, the effect of substrate concentration on SDH activity for liver cytosolic fraction was determined by using different substrate concentrations in the reaction cuvette.

In 1 mL quartz cuvettes 0.700 mL 0.2 M potassium phosphate buffer pH 7.2, required volume of dH_2O, 5 µL 10x diluted liver cytosol, 70 µL 1 mg / mL NADH were added. Then it was mixed by inversion and measured the absorbance at 340 nm against blank (distilled water) for approximately 2 minutes. Each time, there should be blank reading (reaction with no

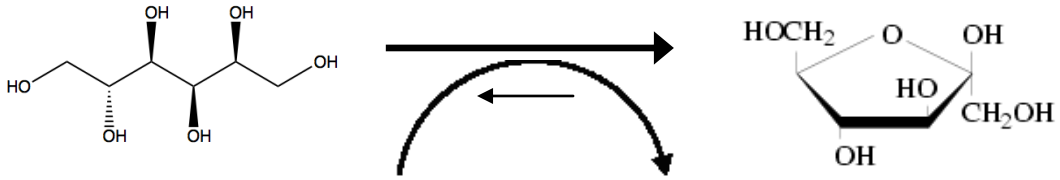
substrate) for making substraction of non-enzymatic product formation from the SDH assay. Then enzymatic reaction was initiated with the addition of required volume with different D-Fructose concentrations was followed at 340 nm ($Є_{340}$= 6.22 $mM^{-1}.cm^{-1}$) for 3 minutes at 32ºC at Hitachi U-2800 Spectrophotometer (Hitachi Technologies Corporation, Tokyo, Japan). The final reaction volume should be 1 mL and the decrease in absorbance was recorded against a blank tube. Used D-fructose concentrations were in the range between 50 – 800 mM as the final concentration in the cuvette. All different D-fructose concentrations were done by diluting 5 M D-fructose stock solution and final concentrations were adjusted by changing the volumes of dH_2O and fructose in the reaction mixture. At these fructose final concentrations enzyme reached saturation and afterwards substrate inhibition took place.

Another important parameter in SDH characterization is the cofactor concentration on the enzyme. The effect of cofactor concentration on SDH activity for liver cytosolic fraction was determined by using different NADH concentrations in the reaction cuvette.

In 1 mL quartz cuvettes 0.700 mL 0.2 M potassium phosphate buffer pH=7.2, required volume of dH_2O, 5 µL 10x diluted liver cytosol, required volume of NADH were added. Then it was mixed by inversion and measured the absorbance at 340 nm against blank (distilled water) for approximately 2 minutes. Each time, there should be blank reading (reaction with no substrate) for making substraction of non-enzymatic product formation from the SDH assay. Then enzymatic reaction was initiated with the addition of required volume with 50 µL 5 M D-Fructose concentrations was followed at 340 nm ($Є_{340}$= 6.22 $mM^{-1}.cm^{-1}$) for 3 minutes at 32ºC at Hitachi U-2800 Spectrophotometer (Hitachi Technologies Corporation, Tokyo, Japan). The final reaction volume should be 1 mL and the decrease in absorbance was recorded against a blank tube. Used NADH concentrations were in the range between 24 – 470 µM as the final concentration in the cuvette. All different NADH concentrations were done by diluting 1 mg / mL, 5mg / mL and

10 mg / mL stock solution which is appropriate and final concentrations were adjusted by changing the volumes of dH_2O and NADH in the reaction mixture. At these NADH final concentrations enzyme reached saturation and afterwards cofactor inhibition took place.

Sorbitol dehydrogenase activity was determined essentially according to the method of Rose and Henderson (1975). Assay conditions for liver cytosolic enzymes were optimized as described in “Results”. Sorbitol dehydrogenase uses NAD^+ as a cofactor and catalyzes the reversible oxidation-reduction of sorbitol and fructose. The rate of oxidation of NADH is directly proportional to the rate of conversion of D-Fructose to D-Sorbitol by SDH. The rate of decrease in absorbance at 340 nm allows measurement of SDH activity.



D-Sorbitol NAD^+ NADH D-Fructose

Figure 2. 2 Conversion of Sorbitol to Fructose by Sorbitol Dehydrogenase

In 1 mL quartz cuvettes 0.700 mL 0.2 M potassium phosphate buffer pH 7.2, 0.175 mL dH_2O, 5 µL 10x diluted liver cytosol, 70 µL 1 mg / mL NADH were added as it is illustrated in Table 2.1. Then it was mixed by inversion and measured the absorbance at 340 nm against blank (distilled water) for approximately 2 minutes. Each time, there should be blank reading (reaction with no substrate) for making substraction of non-enzymatic product formation from the SDH assay. Then enzymatic reaction was initiated with the addition of 50 µL 5 M D-Fructose at 340 nm ($Є_{340}$= 6.22 $mM^{-1}.cm^{-1}$) was followed for 3 minutes at 32ºC at Hitachi U-2800 Spectrophotometer (Hitachi Technologies Corporation, Tokyo, Japan). The decrease in absorbance was recorded against a blank tube.

Table 2. 1 The constituents of the incubation mixture used for the measurement of sorbitol dehydrogenase activity in rabbit liver cytosol

Constituents	Stock solutions	Volume to be added (mL) to 1 mL quartz cuvette	Final concentration in 1 mL quartz cuvette
Potassium Phosphate Buffer pH=7.2	0.2 M	0.700	0.14 M
NADH	1 mg / mL	0.070	0.070 mg / mL
D-Fructose	5 M	0.050	0.250 M

One unit of SDH activity is equal to the conversion of 1.0 µmole D-fructose to D-sorbitol per liter per minute at pH 7.2 at 32ºC. SDH activity was then described as the amount of NADH consumed in one minute by 1 mg protein containing cytosolic fraction. The enzyme activity was calculated using 6.22 (mM^{-1} cm^{-1}) as an extinction coefficient ($Є_{340}$). The following formula was used for the calculation of enzyme specific activity.

$$\frac{E}{U/g} = \frac{OD_{test} - OD_{blank}}{Є_{340} \times b \text{ light path}} \times \text{Tube Dilution } 1000/5 \times \text{Dilution Factor } 10 \times \frac{1}{\text{mg protein}}$$

Serum SDH activity measurement was done according to the same method as in liver cytosol activity measurement with some modifications. Firstly we spent some time for the amount of serum to be added to the reaction mixture. We started with 5x and 10x enzyme dilution however we could not able to obtain any enzymatic activity. Then we found out that serum should be used without dilution. In 1 mL quartz cuvettes 0.700 mL 0.2 M potassium phosphate buffer pH 7.2, 0.130 mL dH_2O, 50 µL serum with no dilution,70 µL 1 mg / mL NADH were added. Then it was mixed by inversion and measured the absorbance at 340 nm against blank (distilled water) for

approximately 2 minutes. Each time, there should be blank reading (reaction with no substrate) for making substraction of non-enzymatic product formation from the SDH assay. Then enzymatic reaction was initiated with the addition of 50 µL 5 M D-Fructose at 340 nm ($Є_{340}$= 6.22 $mM^{-1}.cm^{-1}$) was followed for 3 minutes at 32ºC at Hitachi U-2800 Spectrophotometer (Hitachi Technologies Corporation, Tokyo, Japan). The decrease in absorbance was recorded against a blank tube. The serum sample was 100 times more than the liver cytosol sample. Consequently, the specific activity calculation of sorbitol dehydrogenase activity for serum was given below.

$$\frac{E}{(\mu mol\, min\, mg)} = \frac{OD_{test} - OD_{blank}}{Є_{340} \times b\,(light\, path)} \times \text{Tube Dilution (1000/50)} \times \frac{1}{\text{mg protein}}$$

2.3.6 Determination of Catalase Activity

Catalase activities of rabbit cytosols were determined according to the method of Aebi (1964). In the UV range, H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240nm (ε_{240}=0.0364 μM^{-1} cm^{-1}). Catalase activity can be calculated from the difference in the absorbance at 240 nm per unit time. The reaction taking place in the reaction tube is given in Figure 2.3.

$$2H_20_2 \longrightarrow 2H_2O + O_2$$

Figure 2.3 Degradation of H_2O_2 by catalase

Before mixing the assay medium, enzyme solution (Cytosol) was pretreated 1% Triton X-100 for 10 minutes (ten fold dilution of cytosol 1 sample 9 triton

x-100), and then the mixture diluted further 200 or 300 fold to make a total dilution of 2000 or 3000 with 50mM phosphate buffer. After that 2 ml of diluted sample is added into quartz cuvettes. 1mL 30mM H_2O_2 was further added as it is in Table 2.2. The reaction was started by the addition of H_2O_2 and followed by the decrease in absorbance at 240 nm for about 1 min at Hitachi U-2800 Spectrophotometer (Hitachi Technologies Corporation, Tokyo, Japan). The decrease in absorbance was recorded against a blank tube containing 1 mL of 50mM phosphate buffer in place of substrate H_2O_2.

Table 2.2 The constituents of the incubation mixture used for the measurement of catalase activity in rabbit liver, kidney and lung cytosols

Constituents	**Stock solutions**	**Volume to be added (mL) to 3mL quartz cuvette**	**Final concentration in 3mL quartz cuvette**
200X diluted Potassium Phosphate Buffer pH=7.0	50 mM	2 mL	0.166 mM
Hydrogen Peroxide (H_2O_2)	30 mM	1 mL	10 mM

The enzyme activity was calculated as the amount of hydrogen peroxide decomposed by 1 mg total protein containing cytosol in one minute by using 0.0364 μM^{-1} cm^{-1} as an extinction coeffient (ε_{240}). Specific enzyme activity was calculated from the difference in the absorbance at 240 nm per unit time using the following formula.

$$\frac{E}{\mu mol/min/mg} = \frac{OD_{test} - OD_{blank}}{\epsilon_{340} \times b\ (light\ path)} \times \text{Tube Dilution } 3/2 \times \text{Dilution Factor } 2000 \text{ or } 3000 \times \frac{1}{mg\ protein}$$

2.3.7 Determination of Glutathione Reductase Activity

Glutathione reductase (GR) activity was measured according to the method of Carlberg and Mannervik (1975) with some modifications including the type of buffer used and reaction volume. Glutathione reductase catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to glutathione (GSH) as it is iluustrated in Figure 2.4. One GR activity unit is defined as the amount of enzyme catalyzing the reduction of one µmole of GSSG per minute at pH 7.6 and 25°C. As shown in the reaction, one molecule of NADPH is consumed for each molecule of GSSG reduced. Therefore, the reduction of GSSG is determined indirectly by the measurement of the consumption of NADPH. The rate of decrease at 340 nm is directly proportional to the GR activity in the sample.

$$GSSG + NADPH + H \xrightarrow{GR} 2GSH + NADP^{+}$$

Figure 2. 4 Conversion of GSSG to GSH by GR

In 3 mL quartz cuvettes 1.390 mL dH_2O, 400 µL 0.5 M phosphate buffer pH 7.6, 20 µL 50mM EDTA, 100 µL 2 mM NADPH were mixed. 70 µL 30 mM GSSG was added to the sample cuvette whereas the blank tube contained 70 µL dH_2O instead of GSSG. Then enzymatic reaction was initiated with the addition of 20 µL cytosol without dilution at 340nm ($Є_{340}$= 6.22 $mM^{-1}.cm^{-1}$) was followed for 5 minutes at Hitachi U-2800 Spectrophotometer (Hitachi Technologies Corporation, Tokyo, Japan). The decrease in absorbance was recorded against a blank tube (Table 2.3 and Figure 2.5).

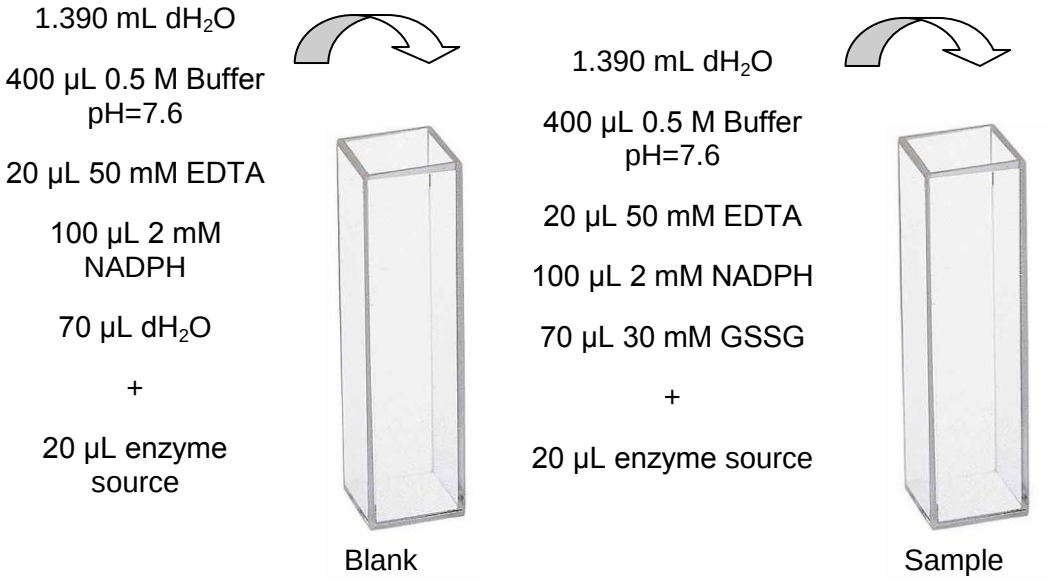


Figure 2. 5 Reaction constituents for the measurement of glutathione reductase activity in rabbit liver and kidney cytosol

Table 2. 3 The constituents of the incubation mixture used for the measurement of GR activity in rabbit liver and kidney cytosols

Constituents	**Stock solutions**	**Volume to be added (mL) to 1 mL quartz cuvette**	**Final concentration in 1 mL quartz cuvette**
Potassium Phosphate Buffer pH=7.6	500 mM	0.4	10 mM
EDTA	50 mM	0.020	0.5 mM
GSSG	30 mM	0.070	1.05 mM
NADPH	2 mM	0.100	0.1 mM

The oxidation of 1 pmol of NADPH/min under these conditions was used as a unit of glutathione reductase activity. The specific activity was expressed as units per mg of protein. GR activity was then described as the amount of NADPH consumed in one minute by 1 mg protein containing cytosolic fraction. The enzyme activity was calculated using 6.22 (mM^{-1} cm^{-1}) as an extinction coefficient (ϵ_{340}). Specific enzyme activity was calculated from the difference in the absorbance at 340 nm per unit time using the following formula.

$$\frac{E}{U/mg} = \frac{OD_{test} - OD_{blank}}{\epsilon_{340} \times b\ (light\ path)} \times \text{Tube Dilution } 2000/20 \times \text{Dilution Factor no DF} \times \frac{1}{mg\ protein}$$

2.3.8 Determination of Glutathione Peroxidase Activity

Glutathione peroxidase (GPx) activity was measured according to the method of Paglia and Valentine (1967). GPx activity measurement was based on the measurement of degree of NADPH oxidation at 340nm with glutathione reductase which use oxidized glutathione and NADPH as a substrate. Since oxidized glutathione is produced by GPx, the degree of NADPH is directly proportional to GPx activity. The reaction taking place in the reaction tube is given in Figure 2.8.

$$2GSH + H_2O_2 \longrightarrow GS{-}SG + 2H_2O$$

Figure 2. 6 Conversion of GSH to GSSG by GPx

In 3 mL quartz cuvettes, 2.425 mL 0.1 M Tris - HCl Buffer pH= 8.0, 75 µL 80 mM reduced glutathione (GSH freshly dissolved), 100 µL 0.24 U glutathione reductase (appropriate dilution must be done with freshly prepared 3,6M ammonium sulphate), 100 µL 40 fold diluted cytosolic fraction (app. 0.075mg protein), 100 µL 30mM sodium azide (NaN_3) (inhibit catalase) were mixed

and incubated for 5 min at RT. Then 100 µL 1.5 mM hydrogen peroxide was added both sample and blank cuvettes and the enzymatic reaction was initiated with the addition of 100 µL 2 mM NADPH as it is shown in Figure 2.7 and Table 2.4. Afterwards the rate of disappearance of NADPH (freshly dissolved) at 340nm ($Є_{340}$= 6.22 $mM^{-1}.cm^{-1}$) was followed for 3 minutes at Hitachi U-2800 Spectrophotometer (Hitachi Technologies Corporation, Tokyo, Japan). The decrease in absorbance was recorded against a blank tube containing 100 µL of 0.1 M Tris-HCl buffer instead of cofactor NADPH.

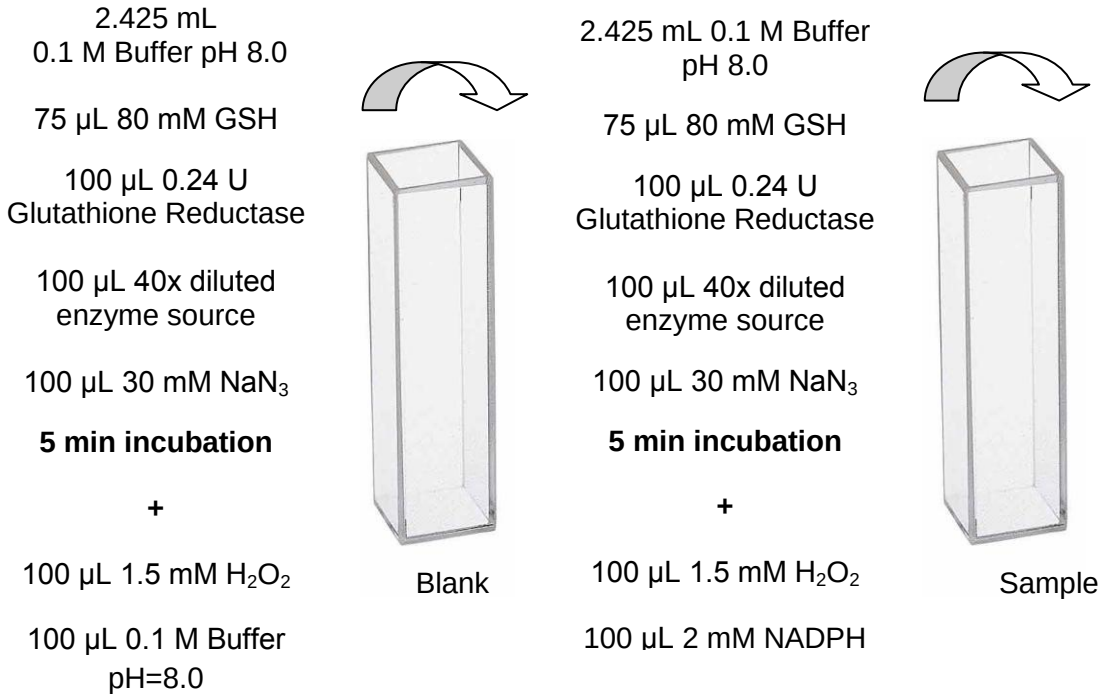


Figure 2. 7 Reaction constituents for the measurement of glutathione peroxidase activity in rabbit liver and kidney cytosol

This assay performed for the glutathione peroxidase enzyme activity measurement is a two enzyme coupled reaction. Glutathione reductase enzyme was also used to determine the rate of disappearance of NADPH in the reaction mixture. The reaction taking place in the cuvette can be represented in Figure 2.8.

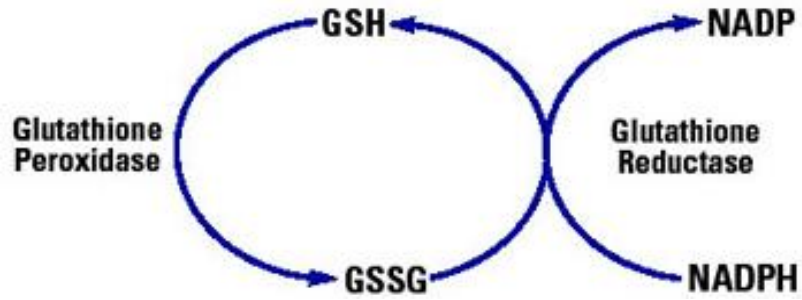


Figure 2. 8 Two enzyme coupled reaction for the measurement of GPx

Table 2. 4 The constituents of the incubation mixture used for the measurement of glutathione peroxidase activity in rabbit liver cytosols

Constituents	**Stock solutions**	**Volume to be added (mL) to 3mL quartz cuvette**	**Final concentration in 3mL quartz cuvette**
Tris-HCl Buffer pH=8.0	100 mM	2.425	80.83 mM
Reduced Glutathione (GSH)	80 mM	0.075	2 mM
Glutathione Reductase	0.24 U	0.100	0.008 U
Sodium azide (NaN_3)	30 mM	0.100	1 mM
Hydrogen Peroxide (H_2O_2)	1.5 mM	0.100	0.050 mM
NADPH	2.0 mM	0.100	0.067 mM

GPx activity was then described as the amount of NADPH consumed in one minute by 1mg protein containing cytosolic fraction. The enzyme activity was calculated using 6.22 (mM^{-1} cm^{-1}) as an extinction coefficient ($Є_{340}$). The following formula was used for the calculation of enzyme specific activity.

$$\frac{E}{\mu mol/min/mg} = \frac{OD_{test} - OD_{blank}}{Є_{340} \times b\ (light\ path)} \times \text{Tube Dilution } 3000/100 \times \text{Dilution Factor } 40 \times \frac{1}{mg\ protein}$$

2.3.9 Determination of Total Glutathione S-Transferase (GST) Activity

Total Glutathione S-Transferase (GST) activity was measured according to the method of Habig *et al.* (1974) with some modifications. 1-chloro-2,4-dinitrobenzene (CDNB) is a common substrate for all isozymes of GST. The principle of the method depends on the formation of 1-glutathione-2,4-dinitrobenzene (DNB-SG) from 1-chloro-2,4-dinitrobenzene by conjugation reaction catalyzed by GSTs (Figure 2.9). In GST catalyzed reaction, 1-glutathione-2,4- dinitrobenzene (DNB_SG) has absorbance at 340 nm. Glutathione S-transferase catalyzed conjugation reaction of 1-chloro-2,4-dinitrobenzene is monitored by the increase in the absorbance at 340 nm due to the 1-glutathione-2,4-dinitrobenzene (DNB-SG) formation.

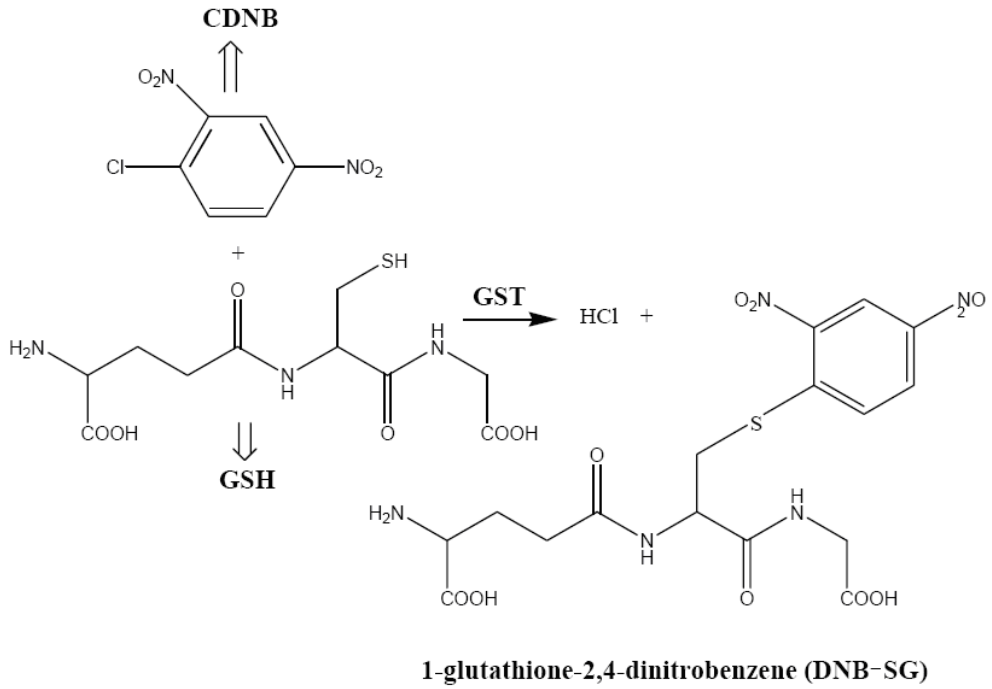


Figure 2. 9 1-glutathione-2,4-dinitrobenzene formation by GST catalyzed reaction

Into the 3 mL quartz cuvette, 2.5 mL 50 mM potassium phosphate buffer pH 7.0, 200 µL 20 mM GSH, 150 µL 20mM CDNB were added and the reaction was started with the addition of 150 µL 1000x diluted liver cytosol. 150 µL 1000x diluted liver cytosol was added to the sample cuvette whereas the blank tube contained 150 µL 50 mM Potassium phosphate buffer pH=7.0 instead of sample as it is illustrated in Table 2.5 and Figure 2.10. Then, thioether formation was followed at 340 nm for 2 minutes at Hitachi U-2800 Spectrophotometer (Hitachi Technologies Corporation, Tokyo, Japan). Each time, there should be blank readings (reaction with no enzyme) in order to subtract non-enzymatic product formation from the GST assay.

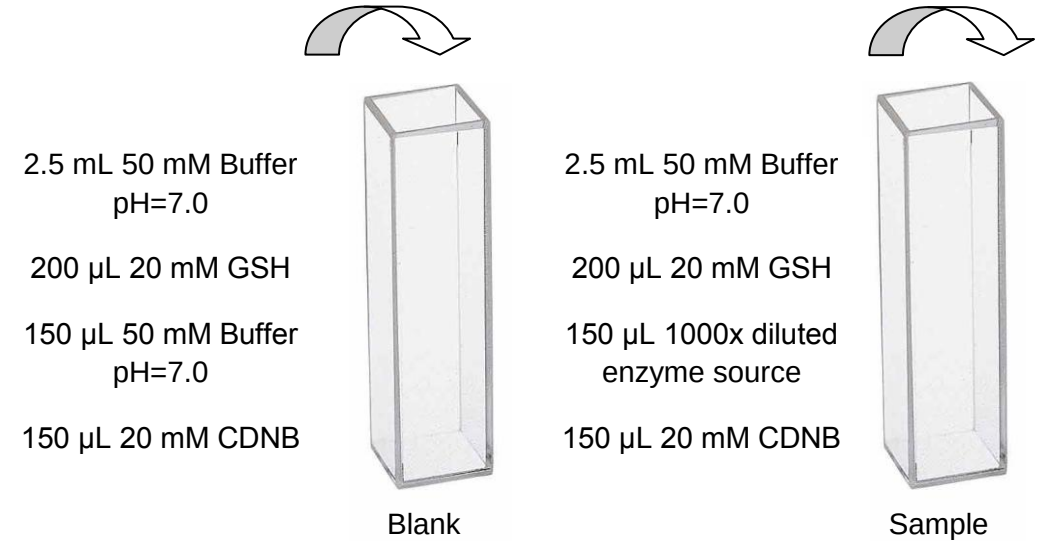


Figure 2. 10 Reaction constituents for the measurement of glutathione S-transferase activity in rabbit liver cytosol

Table 2. 5 The constituents of the incubation mixture used for the measurement of glutathione S-transferase activity in rabbit liver cytosol

Constituents	**Stock solutions**	**Volume to be added (mL) to 3mL quartz cuvette**	**Final concentration in 3 mL quartz cuvette**
Potassium Phosphate Buffer pH=7.0	50 mM	2.5	41.67 mM
CDNB* (in ethanol:dH_20, 3:2)	20 mM	0.150	1 mM
GSH	20 mM	0.200	1.3 mM

* CDNB should be freshly prepared and after weighing the proper amount of CDNB. It should be dissolved first in ethanol then the proper amount of dH_20 should be added (ethanol/ dH_20:3/2). Stored in dark.

Then, the enzyme activity was calculated as the amount of thioether (nmol) formed by 1 mg total protein containing cytosol in one minute by using 0.0096 μM^{-1} x cm^{-1} as an extinction coefficient of thioether formed by GST. The following formula was used for the calculation of enzyme specific activity.

$$\frac{E}{\mu mol/min/mg} = \frac{OD_{test} - OD_{blank}}{\epsilon_{340} \times b\ (light path)} \times \text{Tube Dilution } 3000/150 \times \text{Dilution Factor } 1000 \times \frac{1}{mg\ protein}$$

2.3.10 Determination of Reduced Glutathione (GSH) Amount in Soluble Fraction of Rabbit Liver and Kidney

Glutathione is a tripeptide formed from glutamic acid, cysteine and glycine. It is the major antioxidant produced by the cell, protecting it from free radicals such as reactive oxygen species. Also, glutathione is very important detoxifying agent, enabling the body to get rid of undesirable toxins and pollutants. Besides these important properties, glutathione plays a crucial role in maintaining a normal balance between oxidation and anti-oxidation.

In this study, glutathione amount of soluble fractions of liver and kidney was determined by the method of Akerboom and Sies (1981). The method involves the direct measurement of 5-thio-2-nitrobenzene (TNB) spectrophotometrically at 412 nm. DTNB method measures the ability of SH groups to reduce DTNB to form 1 mole of TNB (yellow) per mole of SH group. The reaction is given in Figure 2.13.

2 GSH + DTNB ⟶ GSSG + 2 TNB

Figure 2. 11 Formation of GSSG and TNB from GSH and DTNB

The reaction mixture contained 880 µL of 0.1 M potassium phosphate buffer containing 0.001 M EDTA at pH 7.0 and 25 µL of acid extract of soluble fraction of liver, 20 µL of 5-5'–Dithiobis 2-nitrobenzoic acid) (DTNB) from 1.5 mg/mL freshly prepared stock (dissolved in 0,1M Kpi Buffer at pH 7.0) and distilled water at final volume of 1 mL. Acid extracts of soluble fractions were prepared by mixing equal volumes of 2 M perchloric acid containing 4 mM EDTA and liver cytosols. Then, acid extracts were centrifuged at 5 000 x g for 5 minutes to remove proteins. Supernatant fractions contained soluble GSH. After mixing these constituents well, intensity of yellow color developed was measured at 412 nm by using Schimadzu UV-1201 spectrophotometer.

A 0.1 and 1 mM freshly prepared glutathione solution was used as standard. The tubes containing standards as six concentrations (1, 5, 10, 20, 50 and 100 nmoles) were mixed with other constituents and absorbances were recorded at 412 nm. A standard glutathione calibration curve was used for the calculation of reduced glutathione amount. GSH content was determined according to the following formula.

$$\text{GSH nmol/g} = \frac{OD_{412}}{\text{slope of the standard curve}} \times \text{Tube Dilution } 2/1 \times \text{Dilution Factor } 1/0{\cdot}025 \times \frac{1}{\text{mg protein}}$$

2.3.11 Determination of Lipid Peroxidation

The lipid peroxidation products were measured according to the method of Ahn *et al*. (1998) as described below. Malondialdehyde (MDA) which is an end product of lipid peroxidation reacts with thiobarbituric acid (TBA) and forms a colored complex having a maximum absorbance at 532 nm. This method determines the total thiobarbituric acid reactive substances and called TBARS method.

Minced liver sample (5g) was placed in a 50 ml test tube and homogenized with 15mL of distilled water. The homogenate (1mL) was transferred to a tube and 50µL 7,2 % butylated hydroxytoluene, 2mL thiobarbituric acid / trichloroacetic acid (20mM TBA and 15 % (w/v) TCA) solution were added. The sample was vortex mixed and then incubated in a 90˚C water bath for 15 minutes to develop red color. After cooling for 10 min in cold crushed ice, the samples were mixed and centrifuged at 3000 x g for 15 min at 5 ˚C by using NF centrifuge (Nuve Instruments Ltd., 06640 Ankara/Turkey). The absorbance of the resulting upper layer was read at 532 nm against a blank prepared with 1 mL of dH_2O and 2 mL of TBA / TCA solution. The amounts of TBARS were expressed as nmol of MDA per gram. Finally, TBARS concentrations were determined by using extinction coefficient of colored complex as $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and this test determines any compound that reacts with thiobarbituric acid, but the most abundant product is malondialdehyde. MDA content was determined according to the following formula.

$$\text{MDA nmol/g} = \frac{OD_{test} - OD_{blank}}{Є532 \times b\ (\text{light path})} \times \text{Tube Dilution } 3 \times 15\text{mL}/5\text{g}$$

2.3.12 Statistical Analysis

Statistical analysis was performed by using Minitab statistical software package for Windows. All results were expressed as means with their Standard Error of Means (SEM). Student's t-test and $p<0.05$ was chosen as the level for significance.

CHAPTER 3

RESULTS

In vivo effects of acrylamide and/or resveratrol, alone or in combination treatment on hepatic and renal antioxidant enzyme activities were studied by measuring catalase, glutathione peroxidase and glutathione reductase activities. In addition, the presence of oxidative stress – malondialdehyde analysis - was shown by thiobarbituric acid reactive substances (TBARS) test. Also, nonenzymatic antioxidant, reduced glutathione levels were compared in liver and kidney cytosols of both control and treated rabbits. Moreover, the conditions of the activity treatments of one of the major hepatic damage marker enzyme – sorbitol dehydrogenase (SDH) - was first optimized by using liver cytosolic fraction of control animals in terms of optimum substrate, cofactor and enzyme concentration conditions for the assay. Furthermore, SDH activities were measured in liver cytosol and blood serum of both control and treated rabbits. Moreover, *in vitro* effects of resveratrol on GST activity was studied. In addition type of inhibition of resveratrol was determined. Values given in tables for enzyme activities are the average duplicate of determinations.

3.1 DETERMINATION OF ANTIOXIDANT ENZYME ACTIVITIES

3.1.1 Catalase

Determination of catalase activity of liver, lung and kidney cytosols was carried out described as under the ‘Materials and Method’ section. Catalase activities of rabbit cytosols were determined according to the method of Aebi (1964).

Catalase activities of each rabbit liver, lung and kidney cytosol values for both control and treated rabbits were shown in Table 3.1. According to the results; although acrylamide is considered as a carcinogenic chemical, no statistically significant change was observed in all rabbits including the samples of liver, lung and kidney cytosols administered subcutaneously with 100 mg/kg b.w. acrylamide on days 1, 5 and 8 (presented as “AA” group) compared to controls. No statistically significant change was observed in all rabbits including the samples of liver, lung and kidney cytosols intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8 (presented as “25 mg RESV”) and rabbits intragastrically administered with 100 mg/kg b.w. resveratrol at day 8 (presented as “100 mg RESV” group) compared to controls. In addition, no statistically significant difference were observed in all rabbits including the samples of liver, lung and kidney cytosols administered 25mg/kg b.w. resveratrol and with 100 mg/kg b.w. acrylamide subcutaneously 6 hours following administration of resveratrol on days 1, 5 and 8 a.m. (presented as “RESV+AA” group) when compared to “AA” group. Furthermore, no statistically significant difference were observed in all rabbits including the samples of liver, lung and kidney cytosols administered 100mg/kg b.w. acrylamide subcutaneously on days 1,5 and 8 and with a single dose resveratrol intragastrically 6 hours following last administration of acrylamide on day 8 (presented as “AA+RESV” group) when compared to “AA” group. The preventive and protective activity of resveratrol against acrylamide can not be expressed in catalase enzyme assay. All measured catalase activities of each rabbit liver, lung and kidney cytosol values for both control and treated rabbits are given in Table 3.1.

Rabbit liver, lung and kidney cytosol catalase activities resulted in no statistically significant change in “AA”, “25 mg RESV” and “100 mg RESV” groups with respect to controls. In addition, “RESV+AA” and “AA+RESV” groups did not make any statistically significant change compared to “AA” group in all organs. In Figure 3.1, the effects of acrylamide and resveratrol in catalase activities of rabbit liver, lung and kidney cytosols are given.

Table 3. 1 Catalase activities of liver, lung and kidney cytosol

	Catalase Activities (µmol/min/mg protein) in Liver Cytosols					
Treatments	**Rabbits**					
	1	2	3	4	5	Average (Mean±SEM)
Control[1]	140.9	103.9	109.9	105.6		115.08 ± 17.4 (N=4)
AA[2]	102.1	134.4	114.2	125.6		119.08 ± 14.02 (N=4)[a]
25 mg RESV[3]	84.1	90.4	85.2	89,3		87.25 ± 3.07 (N=4)[a]
RESV+AA[4]	96.1	136.5	111.8	93.8		109.55 ± 9.83 (N=4)[b]
AA+RESV[5]	100.0	97.7	112.3			103.3 ± 4.53 (N=3)[b]
100 mg RESV[6]	72.4	117.9	130.5	126.3		111.8 ± 13.4 (N=4)[a]
	Catalase Activities (µmol/min/mg protein) in Lung Cytosols					
Treatments	**Rabbits**					
	1	2	3	4	5	Average (Mean±SEM)
Control[1]	50.4	63.3	58.9	61.1		58.42 ± 5.64 (N=4)
AA[2]	65.7	70.8	63.9	51.9	60.4	62.54 ± 3.15 (N=5)[a]
25 mg RESV[3]	58.8	55.1	63.2	57.2		58.58 ± 3.44 (N=4)[a]
RESV+AA[4]	59.8	75.6	69.5	60.3		66.30 ± 3.83 (N=4)[b]
AA+RESV[5]	52.4	56.4	45.5	65.8		55.02 ± 8.48 (N=4)[b]
100 mg RESV[6]	59.7	51.4	49.6	52.5		53.30 ± 2.22 (N=4)[a]
	Catalase Activities (µmol/min/mg protein) in Kidney Cytosols					
Treatments	**Rabbits**					
	1	2	3	4	5	Average (Mean±SEM)
Control[1]	220.0	172.0	218.5	178.5		197.3 ± 12.8 (N=4)
AA[2]	215.5	183.5	158.5	232.0	150.5	188.0 ± 35.3 (N=5)[a]
25 mg RESV[3]	264.5	227.5	218.5	188.5		224.8 ± 31.3 (N=4)[a]
RESV+AA[4]	187.5	198.5	221.0	192.5		199.9 ± 14.8 (N=4)[b]
AA+RESV[5]	196.5	238.0	150.0			194.8 ± 44.0 (N=3)[b]
100 mg RESV[6]	192.5	213.5	169.0	151.5		181.6 ± 27.1 (N=4)[a]

[a] no change with respect to controls [b] no change wit respect to “AA” group

[1] Control rabbits were injected with physiological saline

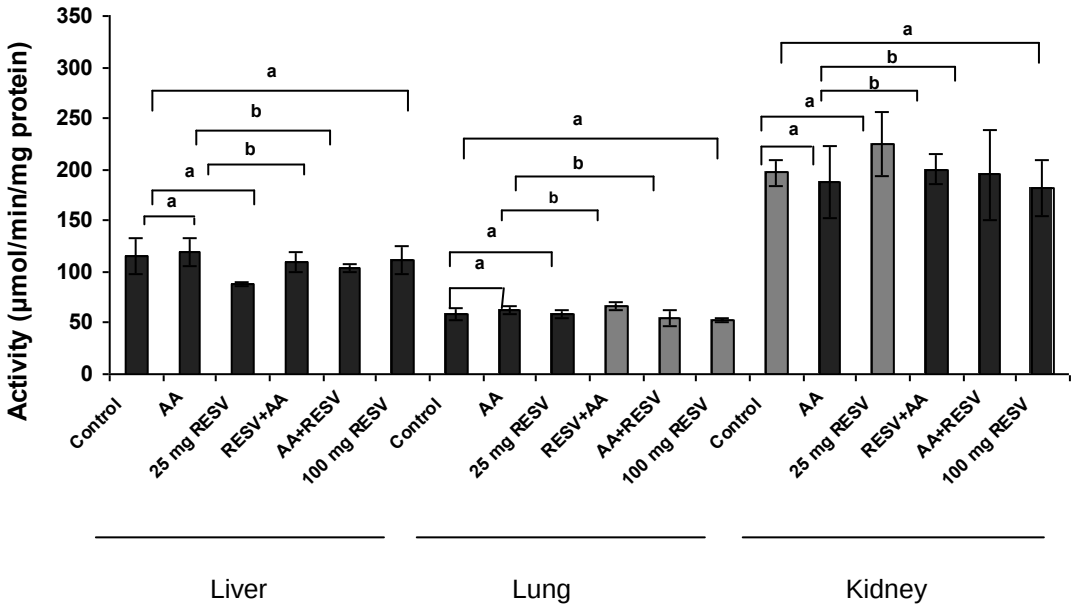
[2] “AA”: Rabbits were administered with acrylamide subcutaneously (s.c.) at a dose of 100 mg/kg b.w. on days 1,5 and 8

[3] “25 mg RESV”: Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8

[4] “RESV+AA”: Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8 a.m.; 6 hours following administration of resveratrol, rabbits were administered with acrylamide subcutaneously at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[5] “AA+RESV”: Rabbits were subcutaneously administered with acrylamide at a dose of 100 mg/kg b.w. on days 1,5 and 8 a.m., 6 hours following administration of acrylamide, rabbits were administered with resveratrol at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[6] “100 mg RESV”: Rabbits were intragastrically administered resveratrol at a dose of 100 mg/kg b.w. resveratrol at day 8.



[a] no change respective to controls [b] no change respective to "AA" group

Figure 3. 1 Catalase activities of rabbit liver, lung and kidney cytosols

Figure 3.1 is constructed from the data given in Table 3.1.

In addition, as it can be seen in Figure 3.1, catalase activity of rabbit kidney is 1.71 fold higher than rabbit liver and 3.38 fold higher than rabbit lung catalase activity. This shows catalase activity variance in distinct organs. It is observed that kidney catalase activity has the highest activity in all examined organs in this study.

3.1.2 Glutathione Peroxidase

Determination of glutathione peroxidase activity of liver cytosols was carried out described as under the 'Materials and Method' section by the method of Paglia and Valentine (1967). All measured glutathione peroxidase activities of each rabbit liver cytosol values for both control and treated rabbits were shown in Table 3.2. As told above, since acrylamide is a carcinogenic chemical, there was found a significant 1,24-fold ($p<0.01$) fall in GPx activity in all rabbits including the samples of liver cytosol in "AA" group compared to controls. On the other hand, although resveratrol is a red wine constituent having antioxidant properties, no statistically significant change was

observed in “25 mg RESV” and “100 mg RESV” groups compared with control. In addition, the results demonstrated the protective effect of resveratrol that GPx activity of rabbit liver cytosols resulted in statistically significant increase 1.20-fold ($p<0.05$) and 1.40-fold ($p<0.05$) in “RESV+AA” and “AA+RESV” group with respect to “AA” group, respectively. The activity was returned to normal level by resveratrol in acrylamide treated animals.

When kidney is taken into consideration, no statistically significant change was observed in “AA” treated animals with respect to controls. There was a significant increase 1.36-fold ($p<0.001$) and 1.34-fold ($p<0.01$) in “25 mg RESV” and “100 mg RESV” group, respectively. In addition, the results demonstrated the protective effect of resveratrol that GPx activity of rabbit kidney cytosols resulted in statistically significant increase 1.29-fold ($p<0.001$) and 1.28-fold ($p<0.01$) in “RESV+AA” and “AA+RESV” group with respect to “AA” group, respectively. The activity was returned to normal level by resveratrol in acrylamide treated animals. GPx activities of each rabbit liver cytosol values for both control and treated rabbits are given in Table 3.2.

Table 3. 2 Glutathione peroxidase activities of liver and kidney cytosol

	Glutathione Peroxidase Activities (µmol/min/mg protein) in Liver Cytosols					
Treatments	**Rabbits**					
	1	2	3	4	5	Average (Mean±SEM)
Control[1]	0.215	0.215	0.225	0.225		0.220 ± 0.006 (N=4)
AA[2]	0.180	0.170	0.165	0.195		0.177 ± 0.013 (N=4)[a**]
25 mg RESV[3]	0.195	0.180	0.250	0.220		0.211 ± 0.031 (N=4)[a]
RESV+AA[4]	0.220	0.200	0.220			0.213 ± 0.011 (N=3)[b*]
AA+RESV[5]	0.270	0.220	0.250			0.247 ± 0.025 (N=3)[b*]
100 mg RESV[6]	0.185	0.205	0.190			0.193 ± 0.010 (N=3)[a]
	Glutathione Peroxidase Activities (µmol/min/mg protein) in Kidney Cytosols					
Treatments	**Rabbits**					
	1	2	3	4	5	Average (Mean±SEM)
Control[1]	0.238	0.205	0.223	0.218		0.221 ±0.013 (N=4)
AA[2]	0.261	0.244	0.200	0.241	0.239	0.237 ± 0.007 (N=5) [a]
25 mg RESV[3]	0.325	0.306	0.286	0.289		0.301 ± 0.006 (N=4) [a***]
RESV+AA[4]	0.295	0.301	0.290	0.344		0.306 ± 0.007 (N=4) [b***]
AA+RESV[5]	0.288	0.357	0.337	0.239		0.305 ± 0.017 (N=4) [b**]
100 mg RESV[6]	0.325	0.333	0.313	0.210		0.295 ± 0.019 (N=4) [a**]

[a] no change with respect to controls [a**] p<0.01 with respect to controls

[a***] p<0.001 with respect to controls [b*] p<0.05 with respect to "AA" group

[b**] p<0.01 with respect to "AA" group [b***] p<0.001 with respect to "AA" group

[1] Control rabbits were injected with physiological saline

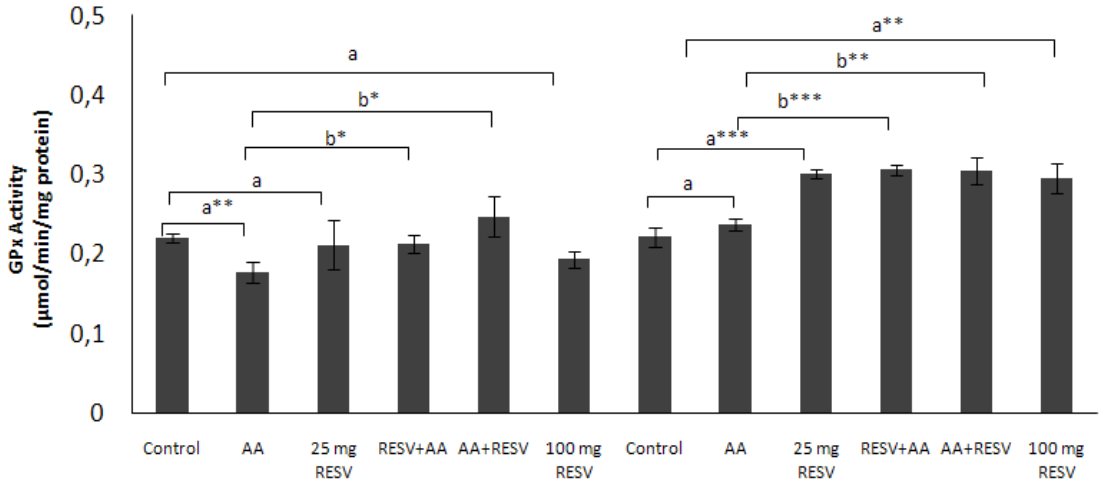
[2] "AA": Rabbits were administered with acrylamide subcutaneously (s.c.) at a dose of 100 mg/kg b.w. on days 1,5 and 8

[3] "25 mg RESV": Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8

[4] "RESV+AA": Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8 a.m.; 6 hours following administration of resveratrol, rabbits were administered with acrylamide subcutaneously at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[5] "AA+RESV": Rabbits were subcutaneously administered with acrylamide at a dose of 100 mg/kg b.w. on days 1,5 and 8 a.m., 6 hours following administration of acrylamide, rabbits were administered with resveratrol at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[6] "100 mg RESV": Rabbits were intragastrically administered resveratrol at a dose of 100 mg/kg b.w. resveratrol at day 8



a no change respective to controls

a** p<0.01 with respect to controls

a*** p<0.001 with respect to controls

b* p<0.05 with respect to "AA" group

b** p<0.01 with respect to "AA" group

b*** p<0.001 with respect to "AA" group

Figure 3. 2 GPx activities of rabbit liver and kidney cytosols

Figure 3.2 is constructed from the data given in Table 3.2.

3.1.3 Glutathione Reductase

Glutathione reductase (GR) activity was measured according to the method of Carlberg and Mannervik (1975). GR activity was determined by using liver and kidney cytosol for both control and treated rabbits. All measured GR activities of each rabbit liver and kidney values are given in Table 3.3 for both control and treated rabbits. The results demonstrated that GR activity of rabbit liver cytosols resulted in statistically significant increase 1.26-fold ($p<0.01$) and 1.31-fold ($p<0.05$) in "25mg RESV" and "100 mg RESV" group with respect to controls due to the antioxidant property of resveratrol, respectively. Additionally, though acrylamide a carcinogenic chemical, no statistically significant change was observed in "AA" group with respect to control. On the other hand, no statistically significant change was observed in "AA+RESV" group and "RESV+AA" group with respect to "AA" group in liver samples. Furthermore, no statistically significant difference was observed in "AA" group, "25 mg RESV" group and "100mg RESV" group with respect to

controls and no statistically significant change was observed in "RESV+AA" group and "AA+RESV" group when compared to "AA" group in kidney.

Table 3. 3 Glutathione Reductase Activities of Liver and Kidney Cytosol

	Glutathione Reductase Activities (U/mg) in Liver Cytosols						
Treatments	Rabbits						
	1	2	3	4	5	6	Average (Mean±SEM)
Control[1]	0.068	0.061	0.064	0.070	0.057	0.050	0.062 ± 0.007 (N=6)
AA[2]	0.055	0.064	0.071	0.069	0.058	0.047	0.061 ± 0.009 (N=6)[a]
25 mg RESV[3]	0.077	0.075	0.076	0.083			0.078 ± 0.004 (N=4)[a**]
RESV+AA[4]	0.065	0.064	0.042	0.042			0.053 ± 0.013 (N=4)[b]
AA+RESV[5]	0.052	0.072	0.079	0.053			0.064 ± 0.014 (N=4)[b]
100 mg RESV[6]	0.073	0.096	0.070	0.084			0.081 ± 0.012 (N=4)[a*]
	Glutathione Reductase Activities (U/mg) in Kidney Cytosols						
Treatments	Rabbits						
	1	2	3	4	5	6	Average (Mean±SEM)
Control[1]	0.091	0,077	0.088	0.088			0.086 ± 0.006 (N=4)
AA[2]	0.104	0.089	0.065	0.070	0.091	0.098	0.086 ± 0.015 (N=6)[a]
25 mg RESV[3]	0.109	0.099	0.099	0.082			0.097 ± 0.011 (N=4)[a]
RESV+AA[4]	0.098	0.099	0.096	0.101			0.098 ± 0.002 (N=4)[b]
AA+RESV[5]	0.098	0.112	0.110	0.072			0.098 ± 0.018 (N=4)[b]
100 mg RESV[6]	0.110	0.108	0.104	0.072			0.098 ± 0.018 (N=4)[a]

[a] no change wit respect to controls [a*] $p<0.05$ with respect to controls

[a**] $p<0.01$ with respect to controls [b] no change with respect to "AA" group

[1] Control rabbits were injected with physiological saline

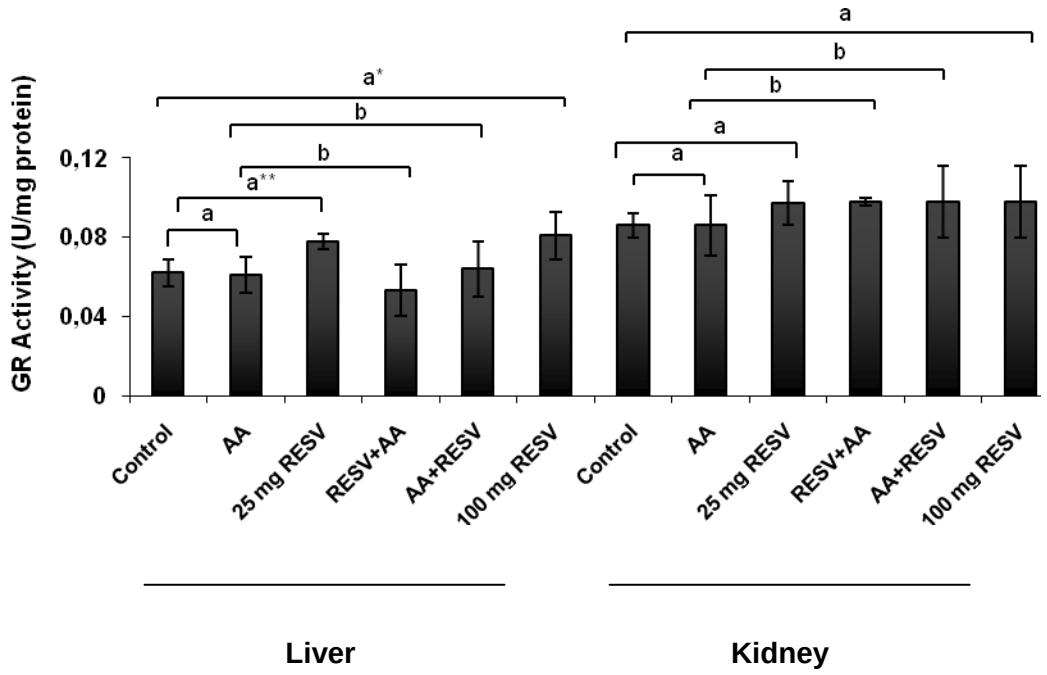
[2] "AA": Rabbits were administered with acrylamide subcutaneously (s.c.) at a dose of 100 mg/kg b.w. on days 1,5 and 8

[3] "25 mg RESV": Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8

[4] "RESV+AA": Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8 a.m.; 6 hours following administration of resveratrol, rabbits were administered with acrylamide subcutaneously at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[5] "AA+RESV": Rabbits were subcutaneously administered with acrylamide at a dose of 100 mg/kg b.w. on days 1,5 and 8 a.m., 6 hours following administration of acrylamide, rabbits were administered with resveratrol at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[6] "100 mg RESV": Rabbits were intragastrically administered resveratrol at a dose of 100 mg/kg b.w. resveratrol at day 8



[a] no change respective to controls [a*] p<0.05 with respect to controls

[a**] p<0.01 with respect to controls [b] no change respective to "AA" group

Figure 3. 3 GR activities of rabbit liver and kidney cytosols

Figure 3.3 is constructed from the data given in Table 3.3.

3.2 REDUCED GLUTATHIONE LEVELS

Reduced glutathione (GSH) which is the most important biomolecule against chemically induced toxicity can participate in the elimination of the reactive intermediates by reduction of hydroperoxides in the presence of GPx and GST. GSH also functions as free radical scavenger in the repair of radical caused biological damage.

Determination of reduced glutathione amount of soluble fractions of liver and kidney cytosols was determined by the method of Akerboom and Sies (1981) as described under the 'Materials and Method' section.

Reduced glutathione (GSH) amount was determined by using liver and kidney cytosol for both control and treated rabbits. All measured reduced

glutathione content of each rabbit liver and kidney values are given in Table 3.4 for both control and treated rabbits. The results demonstrated that GSH content of rabbit liver cytosol resulted in statistically significant 1.61-fold ($p<0.05$) decrease in “AA” group with respect to control. No statistically significant change was observed in the “25 mg RESV” group and “100 mg RESV” group with respect to controls and no statistically significant difference was observed in “AA+RESV” and “RESV+AA” group with respect to “AA” group in liver samples. Moreover, the result demonstrated that GSH content of rabbit kidney cytosol resulted in statistically significant 1.69-fold ($p<0.05$) decrease in “AA” group with respect to controls due to the carcinogenic property of acrylamide. Also, the results indicated a significant increase 1.30-fold ($p<0.05$) and 1.41-fold $p<0.05$) in “RESV+AA” and “AA+RESV” groups, respectively in comparison with “AA” group proofing the protective effect of resveratrol. In addition, although resveratrol has been considered as a potent antioxidant, no statistically significant difference was observed in alone “25 mg RESV” group and “100mg RESV” group with respect to controls. The effects of *in vivo* acrylamide and resveratrol treatment on liver and kidney reduced glutathione amount are given in Figure 3.4.

Also it should be noted that, reduced glutathione (GSH) levels of rabbit kidney cytosolic fraction is 2.11 fold higher than rabbit liver GSH content as it can be seen in Figure 3.4.

Table 3. 4 Reduced glutathione content of liver and kidney cytosol

	Reduced Glutathione Content (nmol/mg) in Liver Cytosol					
Treatments	Rabbits					
	1	2	3	4	5	Average (Mean±SEM)
Control[1]	37.2	59.3	42.2	52.6	58.6	49.48 ± 9.90 (N=5)
AA[2]	35.3	20.3	37.7	29.8		30.77 ± 7.73 (N=4)[a*]
25 mg RESV[3]	30.2	19.0	41.8	62.6		38.40 ± 18.63 (N=4)[a]
RESV+AA[4]	36.1	39.4	60.2	29.2		41.23 ± 13.34 (N=4)[b]
AA+RESV[5]	30.0	17.7	24.8	18.8		22.83 ± 5.71 (N=4)[b]
100 mg RESV[6]	36.9	30.8	38.4			37.37 ± 2.24 (N=3)[a]
	Reduced Glutathione Content (nmol/mg) in Kidney Cytosol					
Treatments	Rabbits					
	1	2	3	4	5	Average (Mean±SEM)
Control[1]	27.0	25.9	24.3	16.6		23.47 ± 4.71 (N=4)
AA[2]	13.6	14.0	12.9	15.1		13.90 ± 0.89 (N=4)[a*]
25 mg RESV[3]	20.4	17.2	20.0	13.5		17.77 ± 3.21 (N=4)[a]
RESV+AA[4]	16.9	16.1	21.4	18.9		18.31 ± 2.39 (N=4)[b*]
AA+RESV[5]	23.7	18.3	20.1	16.1		19.55 ± 3.21 (N=4)[b*]
100 mg RESV[6]	24.7	21.1	14.5			20.14 ± 5.18 (N=3)[a]

[a] no change wit respect to controls [a*] $p<0.05$ with respect to controls

[b] no change wit respect to "AA" group [b*] $p<0.05$ with respect to "AA" group

[1] Control rabbits were injected with physiological saline

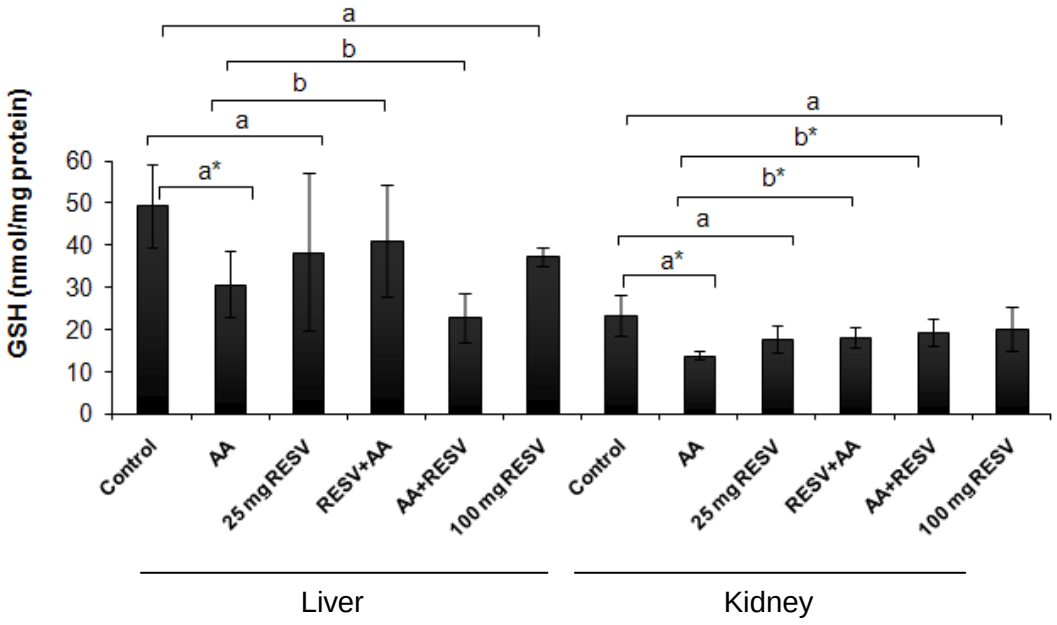
[2] "AA": Rabbits were administered with acrylamide subcutaneously (s.c.) at a dose of 100 mg/kg b.w. on days 1,5 and 8

[3] "25 mg RESV": Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8

[4] "RESV+AA": Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8 a.m.; 6 hours following administration of resveratrol, rabbits were administered with acrylamide subcutaneously at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[5] "AA+RESV": Rabbits were subcutaneously administered with acrylamide at a dose of 100 mg/kg b.w. on days 1,5 and 8 a.m., 6 hours following administration of acrylamide, rabbits were administered with resveratrol at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[6] "100 mg RESV": Rabbits were intragastrically administered resveratrol at a dose of 100 mg/kg b.w. resveratrol at day 8



[a] no change respective to controls [a*] $p<0.05$ with respect to controls

[b] no change respective to "AA" group [b*] $p<0.05$ with respect to "AA" group

Figure 3. 4 Reduced glutathione content of rabbit liver and kidney cytosols

Figure 3.4 is constructed from the data given in Table 3.4.

3.3 LIPID PEROXIDATION

Lipid peroxidation is a free radical induced process leading to oxidative damage of unsaturated lipids. Under normal physiological conditions, low levels of lipid peroxides are found in tissues, however, free radicals which react with lipids can cause peroxidative changes that result in enhanced lipid peroxidation. So, it is an important marker of early and reversible tissue damage and of decrease in antioxidant defense capacity of tissue.

The lipid peroxidation products were measured according to the method of Ahn *et al*. (1998) as described under the 'Materials and Method' section.

All measured malondialdeyhde (MDA) levels of each rabbit liver cytosol values for both control and treated rabbits were shown in Table 3.5. According to the results, although we expect an elevation due to toxic metabolite acrylamide, no statistically significant change was observed in all

rabbits including the samples of liver cytosol in “AA” group compared to controls. On the other hand, in spite of the potential antioxidant resveratrol, no statistically significant fall was observed in “25 mg RESV” group and “100 mg RESV” group compared to controls. In addition, the results demonstrated that no statistically significant difference were observed in all rabbits including the samples of liver cytosols in “RESV+AA” group and “AA+RESV” group when compared to “AA” group. The effects of acrylamide and resveratrol cannot be expressed in MDA analysis of rabbit liver cytosolic fractions. All measured MDA levels of each rabbit liver cytosol values for both control and treated rabbits are given in Table 3.5.

Table 3. 5 Malondialdehyde content of liver cytosol

	MDA Levels (nmol/g) in Liver Cytosol					
Treatments	Rabbits					
	1	2	3	4	5	Average (Mean±SEM)
Control[1]	4.47	5.31	4.00	4.15		4.48 ± 0.58 (N=4)
AA[2]	3.80	3.95	4.30	4.06	3.81	3.98 ± 0.21 (N=5)[a]
25 mg RESV[3]	4.18	4.01	5.08	5.76		4.76 ± 0.82 (N=4)[a]
RESV+AA[4]	5.50	4.58	5.85	3.52		4.86 ± 1.04 (N=4)[b]
AA+RESV[5]	3.95	4.35	3.43	4.10		3.96 ± 0.39 (N=4)[b]
100 mg RESV[6]	4.84	4.55	3.43	3.86		4.17 ± 0.64 (N=4)[a]

[a] no change with respect to controls [b] no change with respect to “AA” group

[1] Control rabbits were injected with physiological saline

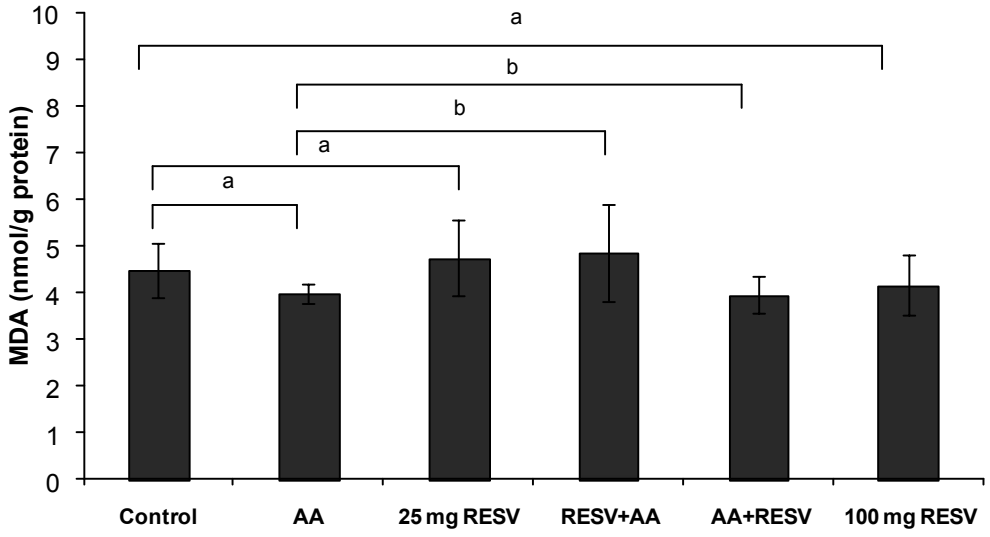
[2] “AA”: Rabbits were administered with acrylamide subcutaneously (s.c.) at a dose of 100 mg/kg b.w. on days 1,5 and 8

[3] “25 mg RESV”: Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8

[4] “RESV+AA”: Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8 a.m.; 6 hours following administration of resveratrol, rabbits were administered with acrylamide subcutaneously at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[5] “AA+RESV”: Rabbits were subcutaneously administered with acrylamide at a dose of 100 mg/kg b.w. on days 1,5 and 8 a.m., 6 hours following administration of acrylamide, rabbits were administered with resveratrol at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[6] “100 mg RESV”: Rabbits were intragastrically administered resveratrol at a dose of 100 mg/kg b.w. resveratrol at day 8



[a] no change with respect to controls [b] no change with respect to "AA" group

Figure 3. 5 Malondialdehyde levels of rabbit liver cytosols

Figure 3.5 is constructed from the data given in Table 3.5.

3.4 EFFECT OF ACRYLAMIDE AND RESVERATROL ON SORBITOL DEHYDROGENASE OF RABBIT BLOOD SERUM AND LIVER CYTOSOLIC FRACTION

Sorbitol Dehydrogenase (SDH) has been identified in both human and animal tissues and found primarily in the cytoplasm and mitochondria of liver and kidney cells and in the seminal vesicles. Unlike the transaminases, which may be of limited value as a biomarker for liver damage due to concentration variations across species, measurement of SDH has become a preferred marker of hepatic damage in both clinical veterinary medicine and drug safety studies, due to its high degree of organ specificity. Therefore, SDH has been considered as a very specific indicator of acute liver damage and disease (Rose and Henderson, 1975). SDH uses NAD^+ as a cofactor and catalyzes the reversible oxidation-reduction of sorbitol and fructose. The rate of oxidation of NADH is directly proportional to the rate of conversion of D-Fructose to D-Sorbitol by SDH. The rate of decrease in absorbance at 340 nm allows measurement of SDH activity.

The varying fructose concentration on SDH activity can be observed in Figure 3.6. The optimum fructose concentration was found as 200 mM. K_M was calculated for sorbitol dehydrogenase as 55,5 mM.

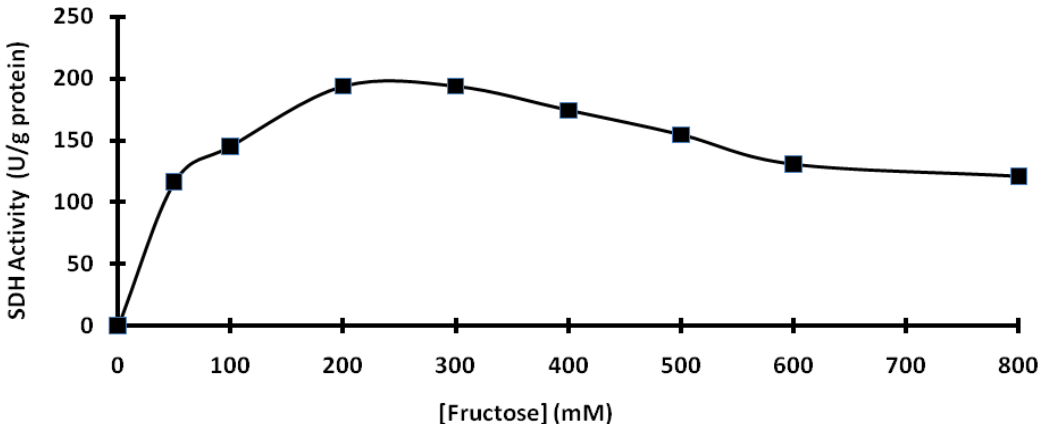


Figure 3. 6 Effect of fructose concentration on SDH activity

The varying NADH concentration on SDH activity can be observed in Figure 3.7. The optimum NADH concentration was found as 141 µM.

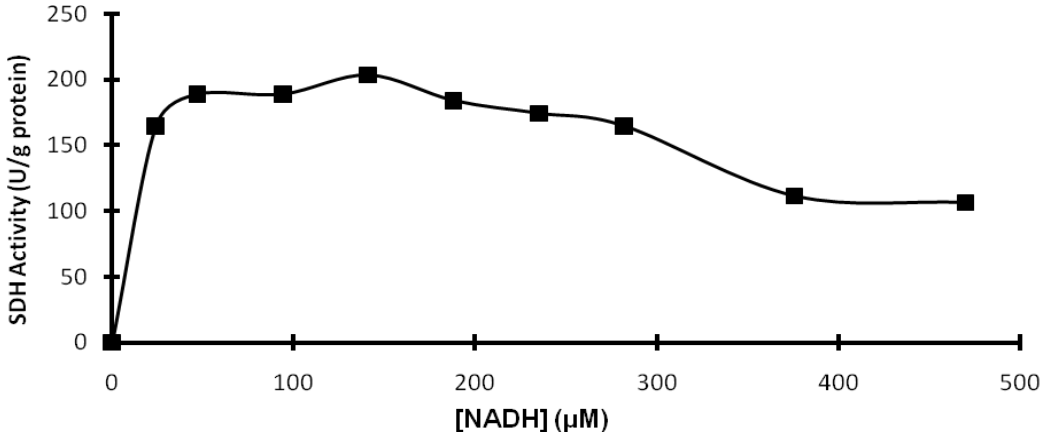


Figure 3. 7 Effect of NADH Concentration on SDH Activity

We spent some time for the amount of serum and liver cytosol to be added to the reaction mixture. For serum, we started with 5x and 10x enzyme dilution however we could not able to obtain any enzymatic activity. Then we found out that serum should be used without dilution. For liver cytosolic fraction, we obtained enzymatic activity with 10x diluted enzyme concentration.

Our SDH characterization data and other authors' findings are given in Table 3.6

Table 3. 6 Reaction rate assays for sorbitol dehydrogenase

Authors	Temp (ºC)	Fructose (mM)	NADH (µM)	Buffer (mM)	Enzyme vol.
Gerlich and Hiby	25	400	400	110 TEA (pH 7.4)	1.0 mL (Serum)
Schön and Wüst	37	150	250	75 Tris/HCl (pH 7.6)	0.5 mL (Serum)
Asada and Galambos	32	130	1000	65 phosphate (pH 7.4)	0.5 mL (Serum)
King	25	220	240	110 TEA (pH 7.4)	1.0mL (Serum)
Rose and Henderson	37	500	247	90 Tris/HCl (pH 6.6)	0.1 m (Serum)
Kalın (this thesis)	32	200	141	140 phosphate (pH 7.2)	0.5 µL (Liver cytosol) 0.05 mL (Serum)

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) assays are common laboratory tests that are used for screening of liver diseases. On the contrary, as told above, transaminases cannot always be used as a marker of liver damage. This was also seen in the results of Master thesis of Mine Nuyan, 2008.

According to the AST results, no statistically significant change was observed in "AA" group compared to controls. However, acrylamide is defined as reasonably anticipated to be a human carcinogen based on sufficient

evidence of carcinogenicity in experimental animals (IARC 1986, 1987, 1994). Although we expect an elevation in “AA” group animals, no significant difference was observed in AST enzyme assay. Moreover, resveratrol is a natural polyphenolic compound having great antioxidant activity. However, no statistically significant change was observed in “25 mg RESV” group compared to controls. In addition, no statistically significant differences were observed in “RESV+AA” and “AA+RESV” group with respect to “AA” group. This shows that resveratrol cannot exhibit its protective effect on its own and against acrylamide.

As in the case of AST activities, since we expect an elevation in rabbit blood serum ALT activities against acrylamide, no statistically significant change in “AA” group was seen with respect to controls. Also, no statistically significant difference can be seen in “25 mg RESV” group with respect to control. Moreover, in “RESV+AA” and “AA+RESV” groups, there was no statistically significant change compared to “AA” group in ALT enzyme activity. Therefore, neither resveratrol nor acrylamide gave a significant difference both in ALT and AST enzyme assays.

When SDH was taken into consideration, as expected, acrylamide treatment in “AA” group resulted in 1.68-fold increase ($p<0.01$) in the SDH enzyme activities of rabbit blood serum with respect to controls. We also observed that resveratrol protected liver damage of acrylamide treated animals. This can be understood from the results demonstrating that SDH enzyme activities of rabbit blood serum resulted in statistically significant 1.60-fold ($p<0.01$) and 1.38 fold ($p<0.05$) decrease in “RESV+AA” and “AA+RESV”; respectively group with respect to “AA” group. Besides, no statistically significant change was observed in “100 mg RESV” group with respect to controls in blood serum samples. All measured alanine aminotransferase (ALT), aspartate aminotransferase (AST) and sorbitol dehydrogenase (SDH) activities of average rabbit blood serum values for both control and treated rabbits were shown in Table 3.7.

Table 3. 7 AST, ALT and SDH activities of rabbit blood serum

	SDH (U/g protein)[x]	ALT[#] (U/mg protein)[y]	AST[#] (U/mg protein)[y]
Treatments	Average (Mean±SEM)		
Control[1]	2,11 ± 0,44 (N=8)	0.62 ± 0.06 (N=4)	0.84 ± 0.06 (N=4)
AA[2]	3,54 ± 0,51 (N=5)[a**]	0.58 ± 0.06 (N=5) [a]	0.74 ± 0.09 (N=5) [a]
25 mg RESV[3]	Not determined	0.50 ± 0.05 (N=4) [a]	0.73 ± 0.09 (N=4) [a]
RESV+AA[4]	2,21 ± 0,40 (N=4)[b**]	0.48 ± 0.14 (N=4) [b]	0.79 ± 0.15 (N=4) [b]
AA+RESV[5]	2,56 ± 0,38 (N=4)[b*]	0.62 ± 0.09 (N=4) [b]	0.97 ± 0.15 (N=4) [b]
100 mg RESV[6]	2,38 ± 0,42 (N=3)[a]	Not determined	Not determined

[a] no change with respect to controls [b] no change with respect to "AA" group

[a**] p<0.01 with respect to controls [b*] p<0.05 with respect to "AA" group

[b**] p<0.01 with respect to "AA" group

[#] values taken from M.Sc. thesis "*In vivo* Interaction of Carcinogenic Acrylamide with Cytochrome P450 Isozymes and Phase I Enzymes In Rabbit Liver, Kidney and Lung", Mine Nuyan, 2008

[y] The unit in ALT and AST is described as the amount of enzyme in 1 mL of serum that will lower the absorbance by 0.001 in 1 minute under the described method of Reitman and Frankel (1957).

[x] One unit of SDH will convert 1.0 µmole of D-fructose to D-sorbitol per minute at pH 7.2 at 32ºC according to the method of Rose and Henderson (1975).

[1] Control rabbits were injected with physiological saline

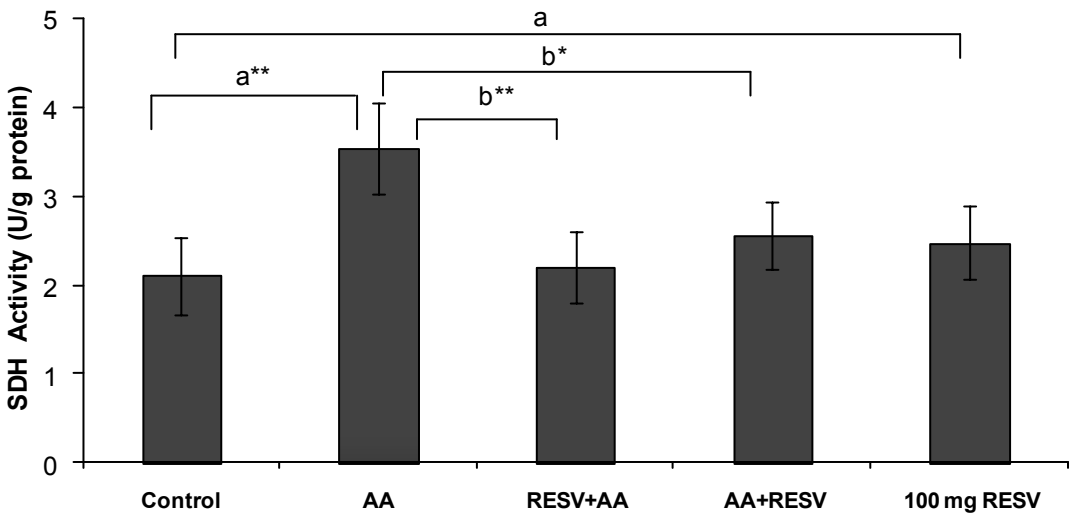
[2] "AA": Rabbits were administered with acrylamide subcutaneously (s.c.) at a dose of 100 mg/kg b.w. on days 1,5 and 8

[3] "25 mg RESV": Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8

[4] "RESV+AA": Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8 a.m.; 6 hours following administration of resveratrol, rabbits were administered with acrylamide subcutaneously at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[5] "AA+RESV": Rabbits were subcutaneously administered with acrylamide at a dose of 100 mg/kg b.w. on days 1,5 and 8 a.m., 6 hours following administration of acrylamide, rabbits were administered with resveratrol at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[6] "100 mg RESV": Rabbits were intragastrically administered resveratrol at a dose of 100 mg/kg b.w. resveratrol at day 8.



[a] no change with respect to controls a** p<0.01 with respect to controls

[b*] p<0.05 with respect to "AA" group [b**] p<0.01 with respect to "AA" group

Figure 3. 8 SDH activities of rabbit blood serum

Figure 3.8 is constructed from the data given in Table 3.7.

In addition to rabbit blood serum, SDH activity measurement was determined in rabbit liver cytosol of both treated and control animals and given in Table 3.7. As in the case of SDH activity in blood serum, we obtain a statistically significant 1.27-fold ($p<0.05$) increase in "AA" group with respect to control. Moreover, 1.59-fold ($p<0.05$) and 1.33-fold ($p<0.05$) decrease was observed in "RESV+AA" and "AA+RESV"; respectively with regard to "AA" group. Although there did not exist any significant change in "25 mg RESV" group with respect to control group, a significant 3.13-fold ($p<0.001$) decrease was observed with respect to control group. Sorbitol dehydrogenase (SDH) activities of average rabbit liver cytosol values for both control and treated rabbits were shown in Table 3.8.

Table 3. 8 Sorbitol dehydrogenase activities of liver cytosol

Treatments	Sorbitol Dehydrogenase Activities (U/g) in Liver Cytosol
	Rabbits Average (Mean±SEM)
Control[1]	193.70 ± 38.3 (N=6)
AA[2]	246.0 ± 41.7 (N=4)[a*]
25 mg RESV[3]	154.9 ± 35.8 (N=4)[a]
RESV+AA[4]	160.7 ± 32.9 (N=4)[b*]
AA+RESV[5]	185.07 ± 13.20 (N=4)[b*]
100 mg RESV[6]	61.82 ± 10.86 (N=4)[a***]

[a] no change with respect to controls [a*] $p<0.05$ with respect to controls

[a***] $p<0.001$ with respect to controls [b*] $p<0.05$ with respect to controls

[1] Control rabbits were injected with physiological saline

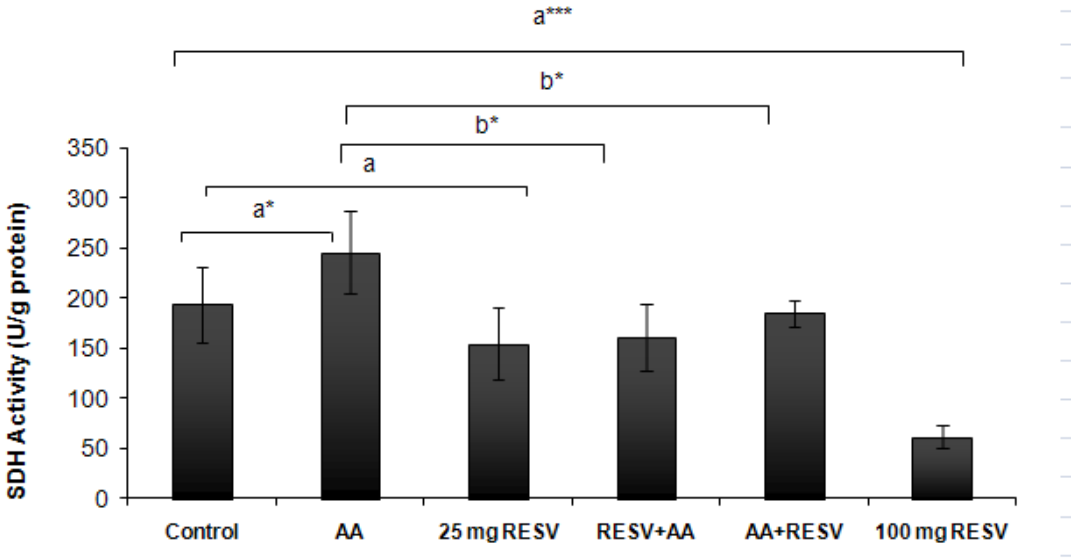
[2] "AA": Rabbits were administered with acrylamide subcutaneously (s.c.) at a dose of 100 mg/kg b.w. on days 1,5 and 8

[3] "25 mg RESV": Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8

[4] "RESV+AA": Rabbits were intragastrically administered with resveratrol at a dose of 25 mg/kg b.w. on days 1,5 and 8 a.m.; 6 hours following administration of resveratrol, rabbits were administered with acrylamide subcutaneously at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[5] "AA+RESV": Rabbits were subcutaneously administered with acrylamide at a dose of 100 mg/kg b.w. on days 1,5 and 8 a.m., 6 hours following administration of acrylamide, rabbits were administered with resveratrol at a dose of 100 mg/kg b.w. on days 1,5 and 8 p.m.

[6] "100 mg RESV": Rabbits were intragastrically administered resveratrol at a dose of 100 mg/kg b.w. resveratrol at day 8.



[a] no change with repect to controls [a*] $p<0.05$ with respect to controls

[a***] $p<0.001$ with respect to controls [b*] $p<0.05$ with respect to controls

Figure 3. 9 SDH activities of rabbit liver cytosol

Figure 3.9 is constructed from the data given in Table 3.8.

3.5 *IN VITRO* STUDIES

3.5.1 Effect of Resveratrol on Glutathione S-Transferase (GST) Activity

Rabbit liver cytosolic glutathione S-transferase activity in the presence of different concentration of resveratrol was determined by the method of Habig *et al.* (1974). A range of resveratrol concentration changing from 1 µM to 1 mM was used to investigate the effect of resveratrol acid on rabbit cytosolic GST activity.

Figure 3.10 showed that rabbit liver cytosolic glutathione S-transferase activity was inhibited by increasing the concentration of resveratrol. The enzyme reaction was inhibited at all concentrations of resveratrol used. The IC50 value of resveratrol as the concentration of resveratrol giving 50% inhition was estimated to be 239,9 µM when the assay was carried out using 1 mM CDNB. Rabbit liver cytosolic glutathione S-transferase activity was almost completely inhibited above 1 mM resveratrol concentration.

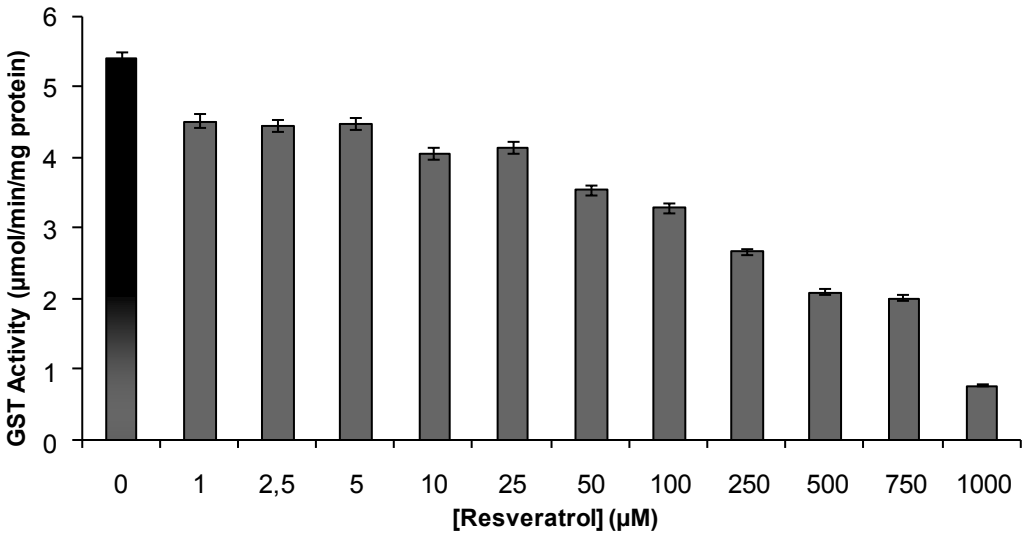


Figure 3. 10 Effect of resveratrol on rabbit liver cytosolic GST activity

The reaction medium contained indicated amount of resveratrol in addition to standard assay mixture constituents given under 'Methods'. The reaction the reaction was started by adding 150 µL 1000X diluted liver cytosol into the reaction mixture in a final volume of 3.0 mL. IC50 was calculated to be 239,9 µM.

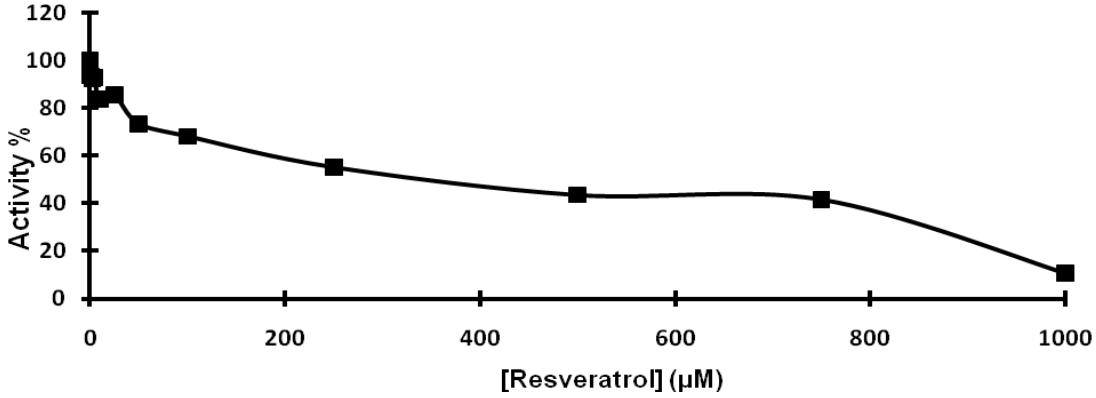


Figure 3. 11 Effect of resveratrol on rabbit cytosolic GST activity as percentage

Michealis - Menten, V versus [S], and Lineweaver – Burk, 1/V versus 1/[S], plots in the presence of three different concentrations of resveratrol were shown in Figure 3.12 and Figure 3.13, respectively. The Lineweaver - Burk plot indicated that Michealis – Menten constant (K_m) remained unchanged by the presence of different concentrations of resveratrol, while V_{max} (maximum velocity) decreased with increasing resveratrol concentration. The apparent K_m value was found to be 0.099 µM for CDNB in all concentrations of resveratrol present in the reaction medium. However, V_{max} values of the enzyme reaction were decreased from 2,78 µmol/min/mg (no resveratrol) to 1,77 µmol/min/mg (100 µM resverarol) with increasing resveratrol concentration. Figure 3.14 shows Dixon plot, 1/V versus 1/[resveratrol], in the presence of different fix concentrations of CDNB (from 0,1 mM to 0,25 mM). From the intersecting point of the three lines, the K_i value was determined to be 175 µM for liver cytosolic GST. K_i values remained same while V_{max} value differed for each concentration of CDNB. Therefore the plot was suggesting the inhibitor manner to be apparently noncompetitive.

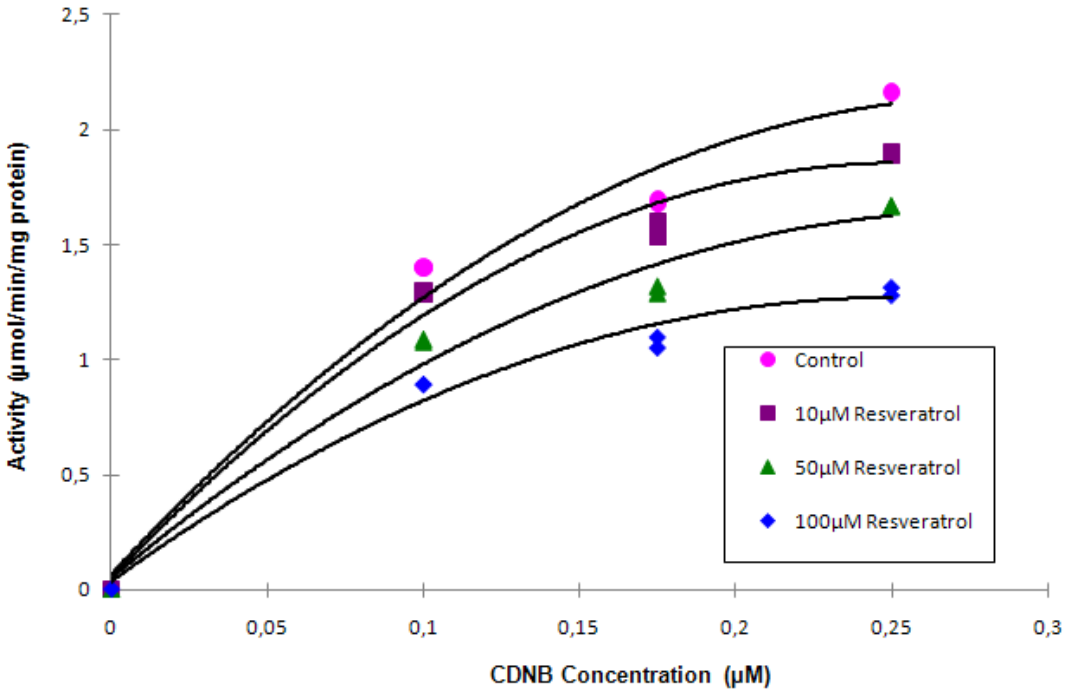


Figure 3. 12 Michaelis - Menten plot for rabbit liver cytosolic GST activity in the presence of substrate CDNB. Effect of resveratrol concentration on rabbit liver cytosolic GST activity was measured using different concentrations of CDNB (0.1 mM - 0.25 mM).

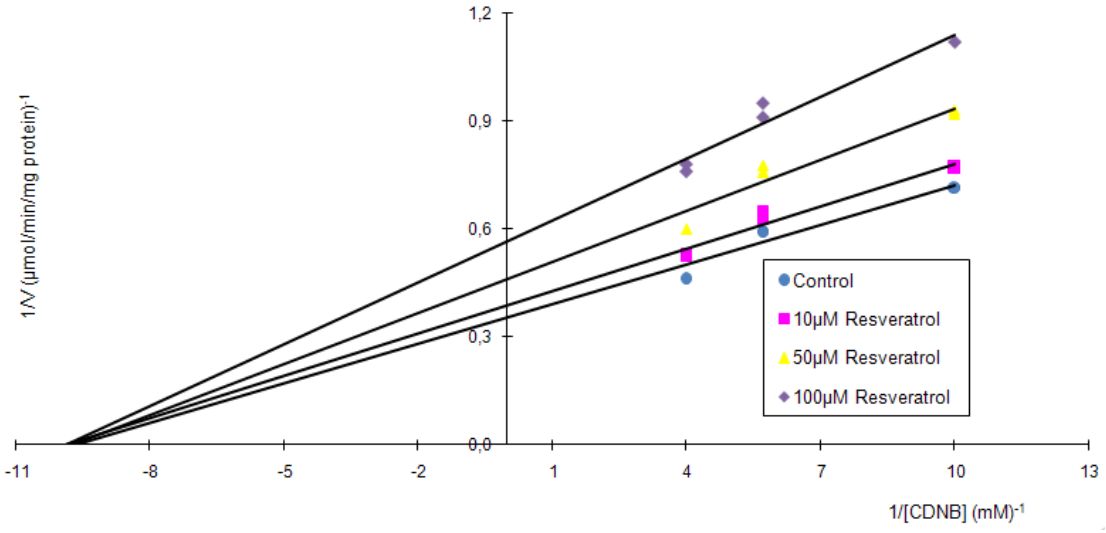


Figure 3. 13 Lineweaver-Burk plot for GST activity of rabbit liver cytosol with different concentrations of resveratrol in the presence of substrate CDNB. The apparent K_m was 0,102 µM and V_{max} values of the reaction were decreased from 2.78 µmol/min/mg to 1.77 µmol/min/mg increasing resveratrol concentration.

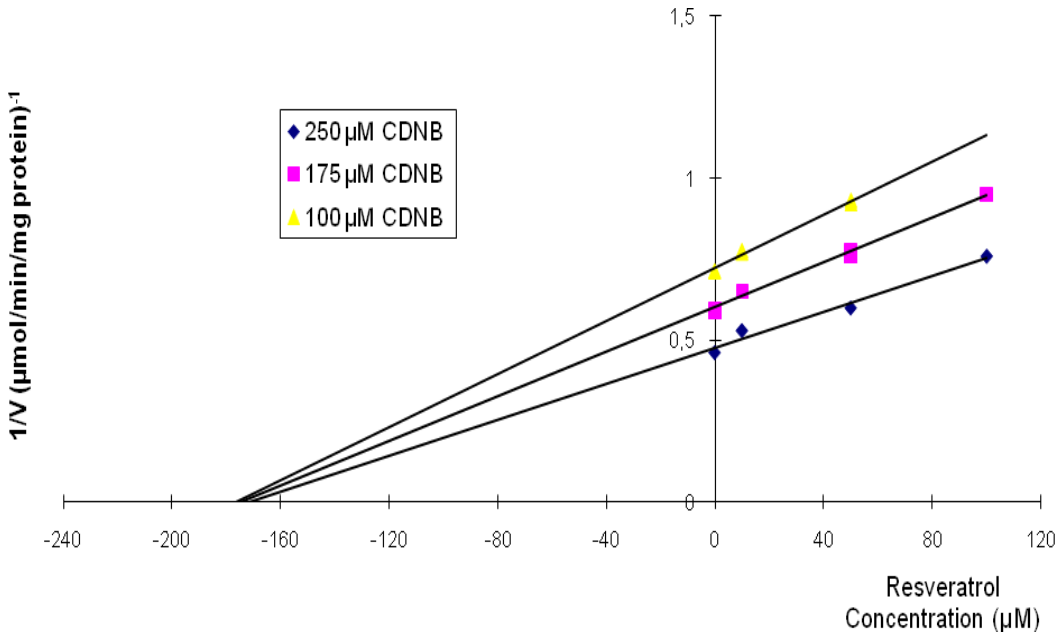


Figure 3. 14 Dixon plot for rabbit liver cytosolic activity 1/V versus [resveratrol], in the presence of substrate CDNB. The apparent K_i value for resveratrol was calculated to be 175 µM.

Michealis - Menten, V versus [S], and Lineweaver – Burk, 1/V versus 1/[S], plots in the presence of three different concentrations of resveratrol were shown in Figure 3.15 and Figure 3.16, respectively. The Lineweaver - Burk plot indicated that V_{max} (maximum velocity) remained unchanged by the presence of different concentrations of resveratrol, while Michealis – Menten constant (K_m) increased with increasing resveratrol concentration. The apparent V_{max} value was found to be 0.296 µmol/min/mg for GSH in all concentrations of resveratrol in the reaction medium. However, K_m values of the enzyme reaction were increased from 0,081 µM (no resveratrol) to 0,139 µM (50 µM resverarol) with increasing resveratrol concentration. Figure 3.17 shows Dixon plot, 1/V versus 1/[resveratrol], in the presence of different fix concentrations of GSH (from 0,05 mM to 0,20 mM). From the intersecting point of the three lines, the K_i value was determined to be 55 µM for liver cytosolic GST. K_i values remained same while K_m value differed for each concentration of GSH. Therefore the plot was suggesting the inhibitor manner to be apparently competitive.

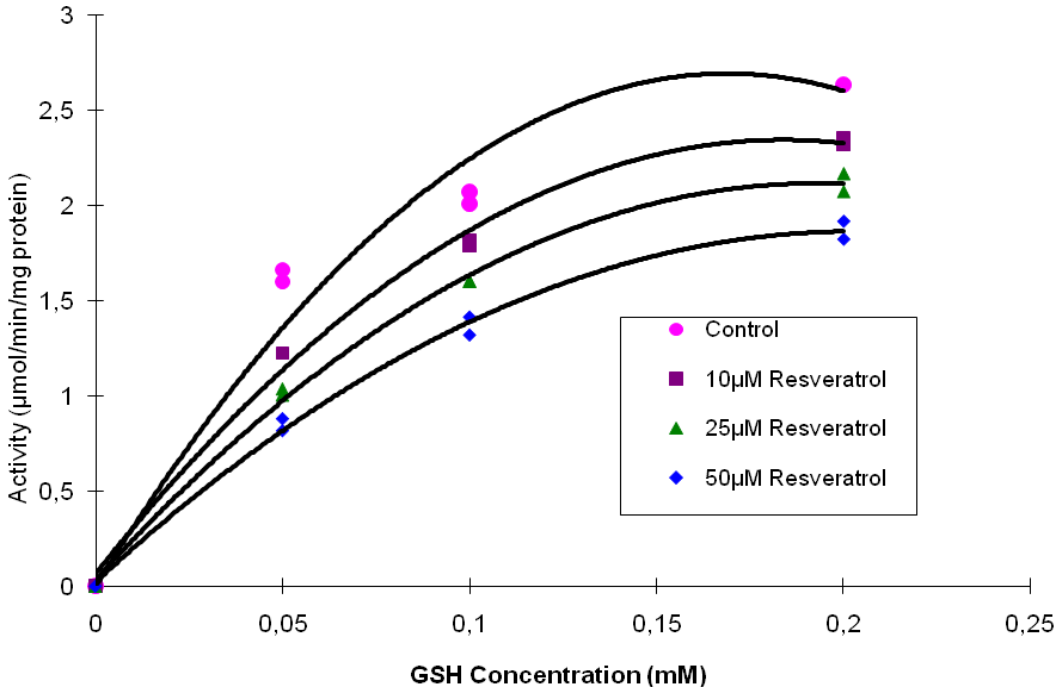


Figure 3. 15 Michaelis - Menten plot for rabbit liver cytosolic GST activity in the presence of substrate GSH. Effect of resveratrol concentration on rabbit liver cytosolic GST activity was measured using different concentrations of GSH (0.05 mM - 0.20 mM).

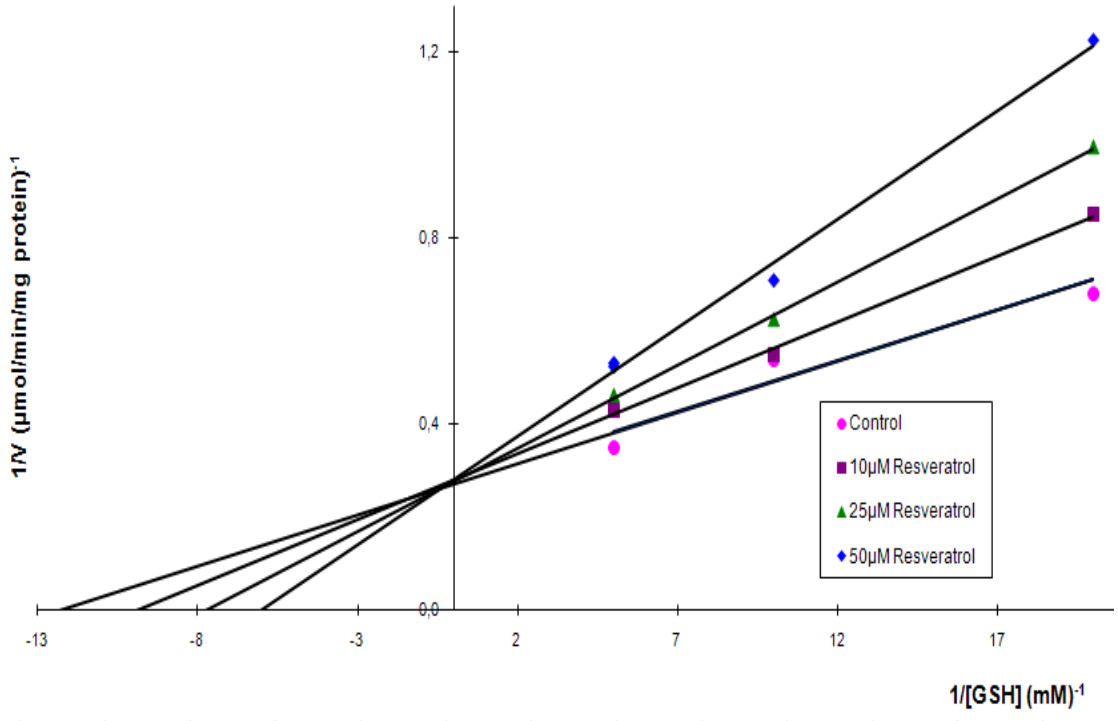


Figure 3. 16 Lineweaver - Burk plot for GST activity of rabbit liver cytosol with different concentrations of resveratrol in the presence of substrate GSH. The V_{max} was 0.296 µmol/min/mg and K_m values were increased from 0.081 µM to 0.139 µM with increasing resveratrol concentration.

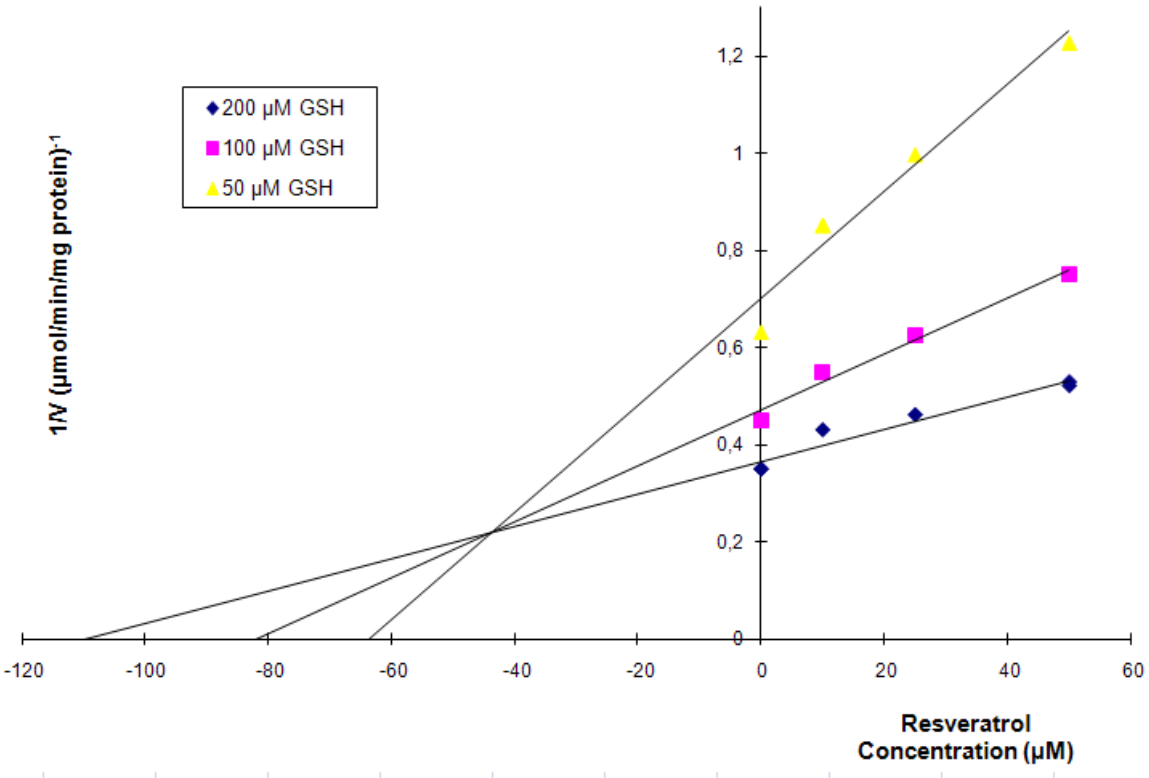


Figure 3. 17 Dixon plat for rabbit liver cytosolic activity 1/V versus [resveratrol], in the presence of substrate GSH. The apparent K_i value for resveratrol was calculated to be 55 µM.

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CHAPTER 4

DISCUSSION

A large number of natural products have been in use since ancient times and some are still under scrutiny for their therapeutic potential, both in terms of disease prevention and treatment (Canistro *et al*., 2009). However it is not yet so much known which ones have curative and preventive properties against diseases and also the fate of these polyphenols including metabolism and action mechanism in human body is under question. Resveratrol, a naturally occuring polyphenolic phytoalexin found abundantly in the skin of grapes and red wine has been referred to show protective effects in cardiovascular diseases and cancer due to its antioxidant properties (Sener *et al.*, 2006; Cai *et al*., 2003; Aggarwal *et al*., 2004). Thus the aim of the present study is to investigate the effect of resveratrol on cytosolic enzymes particularly antioxidant enzymes. Moreover, *in vitro* effect of resveratrol on glutathione-S transferase which is a Phase II enzyme was also investigated for the first time in this study to observe the enzyme inhibition by resveratrol. In addition to resveratrol; acrylamide, an industrially produced compound, is known as an animal neurotoxin, a reproductive toxin and a carcinogen formed in baked or fried carbohydrate rich food (Rosen and Hellenas, 2002; Tareke *et al*., 2002; Weiss, 2002). One important route of phenolic compounds to exert their antioxidant effect is to modulate antioxidant enzymes such as glutatione peroxidase under exposure of a toxic chemical. Thus, *in vivo* protective effects of resveratrol on acrylamide induced toxicity was also investigated on rabbit liver, kidney and lung antioxidant enzymes for the first time in the present study. Furthemore, sorbitol dehydrogenase has been considered as a very specific indicator of acute liver damage and disease (Rose and Henderson, 1975). In this study, sorbitol dehydrogenase

was characterized in terms of substrate, cofactor and enzyme concentration in rabbit liver cytosolic fraction. Moreover, acrylamide toxicity and protective role of resveratrol were also invetigated in sorbitol dehydrogenase activity of rabbit liver and serum samples.

Resveratrol is a natural and antifungal polyphenolic compound (trans 3,5,4-trihydroxy stilbene present in various food products particularly in grapes, mulberries, red wine, root extract of weed, rasperries, blueberries, Scots pine, Eastern white pine and knotweed having antioxidant, antiplatelet, angiogenic, anti-inflammatory, estrogenic, cardioprotective, cell growth modulatory, anticarcinogenic, antihypersensitive and immunomodulatory properties (Sılan *et al.,* 2007; Sener *et al.,* 2006; Kirimlioglu *et al.,* 2008; Athar *et al.,* 2009; Penumastha *et al.,* 2006; Ferrero *et al.,* 1998; Ray *et al.,* 1999; Russo *et al.,* 2001; Sgambato *et al.,* 2001; Surh *et al.,* 1999; Bertelli *et al.,* 1999). In addition to the afore mentioned properties, resveratrol is also known to have effects on the drug metabolizing enzymes. As it is indicated in the thesis of Mine Nuyan, 2008; resveratrol shows an inhibitory effect on acrylamide induced toxicity in liver microsomal CYP2E1 enzyme activity and protein levels. Furthermore, resveratrol was found to inhibit cytochrome P4501A1 (CYP1A1) and 1A2 (CYP1A2) drug metabolizing enzymes (Chang *et al*., 2000; Chun *et al*., 1999; Ciolino and Yeh, 1999; Mikstacka *et al.,* 2002).

Since acrylamide is a toxic compound, it is aimed that resveratrol exerts its free radical scavenging effect and antioxidant property on acrylamide-induced toxicity (Belguendouz *et al.,*1997; Chanvitayapongs *et al.,* 1997; Hebbar *et al*., 2005). Thus in the present study the protective effects of resveratrol on acrylamide-induced toxicity investigated in terms of antioxidant enzymes. Rabbits were administered with resveratrol intragastrically at a dose of 25 mg/kg b.w on days 1, 5 and 8 (presented as “25 mg RESV” group). Rabbits were intragastrically administered with 100 mg/kg b.w. resveratrol at day 8 (presented as “100 mg RESV” group). The combined effects of acrylamide and resveratrol were also investigated in two groups. In

“RESV+AA” group, rabbits were administered with resveratrol intragastrically at a dose of 25 mg/kg b.w at days 1, 5 and 8; and then 6 hours following administration of resveratrol, rabbits were injected with acrylamide subcutaneously (s.c.) at a dose of 100 mg/kg b.w on days 1, 5 and 8. In “AA+RESV” group, rabbits were injected with acrylamide at a dose of 100 mg/kg b.w on days 1, 5 and 8; and then 6 hours following last injection of acrylamide, rabbits were administered with a single dose of 100 mg/kg b.w. resveratrol intragastrically on day 8. In the sixth group (“100 mg RESV”) a single dose of 100 mg/kg b.w. resveratrol was administered to the animals at day 8.

Catalase is an important antioxidant enyzme having a role of decomposition of H_2O_2 into water and oxygen. According to the present study results, no statistically significant change was observed in liver, lung and kidney catalase activities of “AA”, 25 mg RESV” and “100 mg RESV” groups with respect to controls. In addition, no statistically significant change was observed in the “AA+RESV” and “RESV+AA” groups with respect to “AA” group. On the contrary, resveratrol is considered to exert protective effects in cardiovascular diseases and cancer, possibly partly due to antioxidant properties (Cai *et al*., 2003; Aggarwal *et al*., 2004). Several studies were carried out to observe the protective effect of resveratrol; both *in vivo* and *in vitro* resveratrol administration induces the expression of antioxidant enzymes SOD, CAT and GPx activities (Mokni *et al*., 2007; Sılan *et al*., 2007; Upadhyay *et al*., 2008; Cao and Li, 2004). On the other hand, Eybl and collaquaes showed that resveratrol pretreatment by administration of resveratrol by gastric gavages dispersed in 0.5% methyl cellulose as 20 mg/kg b.w. for 3 days, increases 89% of the activity of catalase compared with cadmium (Cd) induced toxicity group in liver. However, treatment only with resveratrol did not cause any influence on the activity of catalase in the livers compared to control mice.

In this study, a significant 1,24-fold ($p<0.01$) fall was found in GPx activity in all rabbits including the samples of liver cytosol in “AA” group compared to controls. On the other hand, although resveratrol is a red wine constituent having antioxidant properties, no statistically significant change was observed in alone “25 mg RESV” and “100 mg RESV” groups compared with control. On the contrary, the results demonstrated the protective effect of resveratrol that GPx activity of rabbit liver cytosols resulted in statistically significant increase 1.20-fold ($p<0.05$) and 1.40-fold ($p<0.05$) in “RESV+AA” and “AA+RESV” group with respect to “AA” group, respectively. When kidney is taken into consideration, no statistically significant change was observed in “AA” treated animals with respect to controls. There was a significant increase 1.36-fold ($p<0.001$) and 1.34-fold ($p<0.01$) in “25 mg RESV” and “100 mg RESV” group, respectively. In addition, the results demonstrated the protective effect of resveratrol that GPx activity of rabbit kidney cytosols resulted in statistically significant increase 1.29-fold ($p<0.001$) and 1.28-fold ($p<0.01$) in “RESV+AA” and “AA+RESV” group with respect to “AA” group, respectively. The activity was returned to normal level by resveratrol in acrylamide treated animals (Table 4.1). This finding can prove the protective effect of resveratrol against acrylamide.

Table 4. 1 The effects of acrylamide and resveratrol treatment on GPx enzyme activities of liver cytosolic fraction

	Liver GPx Activity(µmol/min/mg)	Change (fold)	**Kidney** GPx Activity(µmol/min/mg)	Change (fold)
Control	0.220 ± 0.006		0.221 ±0.013	
AA	0.177 ± 0.013	1.24 ↓ a*	0.237 ± 0.007	↔
25 mg RESV	0.211 ± 0.031	↔	0.301 ± 0.006	1.36 ↑ a***
100 mg RESV	0.193 ± 0.010	↔	0.295 ± 0.019	1.38 ↑ a**
RESV+AA	0.213 ± 0.011	1.20 ↑ b*	0.306 ± 0.007	1.29 ↑ b***
AA+RESV	0.247 ± 0.025	1.38 ↑ b*	0.305 ± 0.017	1.28 ↑ b**

[a] no change with respect to controls

[a**] p<0.01 with respect to controls

[a***] p<0.001 with respect to controls

[b*] p<0.05 with respect to “AA” group

[b**] p<0.01 with respect to “AA” group

[b***] p<0.001 with respect to “AA” group

In addition, according to the study done by Eybl, 2006 that the GPx activity in Cd-exposed mice pre-treated with resveratrol group significantly elevated (p<0.001) GPx activity compared with Cd-exposed level. In groups treated with antioxidants only, resveratrol treatment did not influence GPx activity in the livers compared to control group (Eybl *et al*., 2006). In similar, treatment only with daily intraperitoneal dose of resveratrol (10 mg/ kg) does not make any effect compared to control rats (Sılan *et al*., 2007). Although an elevation is expected in catalase and glutathione peroxidase activities of resveratrol tretated animals, the results of the present study demonstrated that no activity change in catalase and glutathione peroxidase activities by resveratrol administration in these dose regimens. However, in GPx activity resveratrol showed its protective effect against carcinogenic acrylamide and neutralize the oxidative stress in liver of rabbits.

The lack of effect of resveratrol in both chemoprevention and antioxidation is due to the low bioavailibility of resveratrol. Moreover, it was examined that the bioavailability of resveratrol was likely to be insufficient to provide effective levels in the corresponding organs (Wenzel *et al*., 2005). In addition, it is found out that intragastric administration of 20 mg t-RES/kg to rabbits peaked very quickly but in the low µM range (e.g. 1.1 ± 0.8 µM after 2,5 min; 0.03 ± 0.01 µM after 60 min.). Furthermore, only 1.5% of the resveratrol orally administered reaches the plasma compartment (Asensi *et al*., 2002). This low bioavailibility of resveratrol can be due to i) high tendency of resveratrol to be involved in conjugation reactions ii) complex transformation reaction resveratrol undergoes iii) rapid metabolism of resveratrol by the liver iv) lipophilic nature of resveratrol v) resveratrol does not accumulate in the extravascular tissues (Wenzel *et al*., 2005; Canistro *et al*., 2009; Asensi *et al*., 2002; Wenzel and Somoza, 2005). In this respect, it should be noted that in several trials, well-known natural compounds such as vitamins or antioxidant micronutrients, failed to protect against cancer (Omenn *et al*., 1996; Paolini *et al*., 1999; Neuhouser *et al*., 2003; Bairati *et al*., 2005).

Furthermore, a decrease is expected in catalase activities of acrylamide tretated animals, the results of the present study demonstrated that no activity change in catalase by acrylamide administration in these dose regimens. Acrylamide metabolism in the body cannot fully understood however the main route of acrylamide is the formation of epoxide glycidamide by CYP2E1. Actually it was suggested that mutagenicity and carcinogenicity of acrylamide is due to the conversion of acrylamide to epoxide glycidamide, by liver CYP2E1 (Besaratinia and Pfeifer, 2004; Adler *et al*., 2000; Manjanatha *et al*., 2006; Ghanayem *et al*., 2005b; Rice, 2005). In addition, it is also known that, besides the CYP2E1 metabolism of acrylamide, another pathway in the metabolism of acrylamide is the acrylamide conjugation with GSH. Thus, no change in CAT activities may be due to this second metabolism. This can be ensured from the data coming from the GPx activity in acrylamide treated animals.

Glutathione reductase (GR) is one of the key role playing enzyme in response to oxidative stress. GR is essential for the GSH redox cycle which maintains adequate levels of reduced cellular GSH. A high GSH/GSSG ratio is essential for protection against oxidative stress. According to the present study results, no statistically significant change was observed in liver GR activities of “AA” group with respect to controls. However, rabbit liver GR enzyme activity resulted in 1.26 fold ($p<0.01$) and 1.31 fold ($p<0.05$) significant increase in “25 mg RESV” and “100 mg RESV” group, respectively; compared to controls. In addition, no statistically significant change was observed in the “AA+RESV” and “RESV+AA” groups with respect to “AA” group. When kidney is taken into consideration, there does not exist significant activity change was observed in glutathione reductase of “AA”, “25 mg RESV” and “100 mg RESV” groups with respect to controls in kidney cytosol of treated rabbits. Moreover, no statistically significant change was observed in the “AA+RESV” and “RESV+AA” groups with respect to “AA” group in kidney cytosolic fraction of treated rabbits. Although there does not exist any elevation in kidney of resveratrol treated animals, there exists an induction of GR in liver of “25 mg RESV” and “100 mg RESV” groups compared to control. Thus, this result can demonstrate the existence of tissue specificity. Another important point is that resveratrol sometimes does not produce any significant change in GSH, GPx and GR levels as expressed in this study, too (Upadhyay *et al*.,2008). Lack of significant change in alone resveratrol treated animals is in accordance with the animal models proposed for resveratrol-mediated chemprotection (De la Lastra and Villegas, 2007). The biotransformation of resveratrol in the body is so quick so that it is less biologically active due to their esterified hydroxy groups (De la Lastra and Villegas, 2007). Probably resveratrol is effective in regulating these markers once animals counteract with enhanced oxidative stress and toxicity (Upadhyay *et al*., 2008).

Other than antioxidant enzymes, reduced glutathione in the body helps to protect cells from reactive oxygen species such as free radicals and peroxides (Pompella *et al*., 2003). The low molecular weight reduced glutathione (GSH) is a tripeptide which contains Gly-Cys-Glu residues, and it is a non-enzymatic participant of intracellular antioxidant system. The results demonstrated that as expected GSH content of rabbit liver cytosol resulted in statistically significant 1.61-fold ($p<0.05$) decrease in “AA” group with respect to “control. In addition, no statistically significant change was observed in the “25 mg RESV” group and “100 mg RESV” group with respect to controls and no statistically significant difference was observed in “AA+RESV” and “RESV+AA” group with respect to “AA” group in liver samples. Moreover, the results demonstrated that GSH content of rabbit kidney cytosol resulted in statistically significant 1.63-fold ($p<0.05$) decrease in “AA” group with respect to controls due to the carcinogenic property of acrylamide. As expected, the acrylamide toxicity can be observed as a decrease in GSH content in kidney cytosols of treated rabbits. Also, the results indicated a significant increase 1.30-fold ($p<0.05$) and 1.41-fold $p<0.05$) in “RESV+AA” and “AA+RESV” groups, respectively in comparison with “AA” group proofing the protective effect of resveratrol. Thus, the protective effect of resveratrol can be demonstrated over introduced acrylamide. In addition, although resveratrol has been considered as a potent antioxidant, no statistically significant difference was observed in “25 mg RESV” group and “100mg RESV” group with respect to controls due to the low bioavailability of resveratrol being insufficient to provide effective levels in the kidney (Table 4.2). It can be suggested from this point that resveratrol can be a potent antioxidant and free radical scavenger due to elevation of GSH content (Kırımlıoglu *et al*., 2008; Upadhay *et al*., 2008; Sılan *et al*., 2007, Şener *et al*., 2006).

Table 4. 2 The effects of acrylamide and resveratrol treatment on GSH content of liver and kidney cytosolic fraction

	Liver GSH Content (nmol/mg)	Change (fold)	**Kidney** GSH Content (nmol/mg)	Change (fold)
Control	49.48 ± 9.90		23.47 ± 4.71	
AA	30.77 ± 7.73	1.68 ↓ a*	13.90 ± 0.89	1.69 ↓ a*
25 mg RESV	38.40 ± 18.63	↔	17.77 ± 3.21	↔
100 mg RESV	37.37 ± 2.24	↔	20.14 ± 5.18	↔
RESV+AA	41.23 ± 13.34	↔	18.31 ± 2.39	1.30 ↑ b*
AA+RESV	22.83 ± 5.71	↔	19.55 ± 3.21	1.41 ↑ b*

^{a*} $p<0.05$ with respect to controls ^{b*} $p<0.05$ with respect to “AA” group

Malondialdeyhde (MDA) a secondary product of lipid peroxidation, is released as a consequence of the toxic effect of reactive oxygen species (ROS) subjected to liver injury and used as a biomarker for oxidative stress (Sener *et al*., 2003). Increased concentrations of MDA in tissues and plasma reflect lipid peroxidation levels, which are quite well known as a marker of hepatocyte injury. According to the present study results, although we expect an elevation due to toxic metabolite acrylamide, no statistically significant change was observed in all rabbits including the samples of liver cytosol in “AA” group compared to controls. On the other hand, in spite of the potential antioxidant resveratrol, no statistically significant fall was observed in “25 mg RESV” group and “100 mg RESV” group compared to controls. In addition, the results demonstrated that no statistically significant difference were observed in all rabbits including the samples of liver cytosols in “RESV+AA” group and “AA+RESV” group when compared to “AA” group. Although we found a reduction in glutathione levels in “AA” group in kidney cytosolic fraction when compared with control, there was no evidence for lipid

peroxidation against acrylamide in MDA analysis. Besides, the decrease in glutathione content was not always associated with increase of lipid peroxidation. There are some studies that acrylamide does not effect on lipid peroxidation (Srivastava *et al*., 2001; Zödl *et al.,*2007). Therefore, the GSH depletion was either insufficient to cause significant oxidative damage or, acrylamide might have induced the activity of other antioxidative enzymes (Yousef and El-Demerdash, 2006).

Many enzymes have been investigated in the past and several are used today, but, mainly because of inadequate specificity or sensitivity or technical difficulty, there is no just one single enzyme which its elevated activity in the blood is considered as a specific indicator of hepatic disorder. Alanine and aspartate aminotransferases (ALT and AST), alkaline phosphatase, lactate dehydrogenase (LDH) are the most frequently used laboratory tests for the detection of liver damage. Especially, both ALT and AST levels are known as reliable tests for liver damage. According to the present study results, sorbitol dehydrogenase can be accepted as one of the specific biomarker enzymes of liver cell damage and parenchymal hepatic diseases. Sorbitol dehydrogenase has been identified in several human and animal tissues. It is located primarily in the cytoplasm and mitochondria of the liver, kidney and seminal vesicles. SDH activity in serum is usually low but increases during acute episodes of liver damage (Dooley *et al*., 1979).

Since SDH is an important biomarker of hepatic damage, firstly we characterized the enzyme in terms of substrate, cofactor and enzyme content concentration in rabbit liver cytosolic fraction. We found the optimum fructose concentration to be 200 mM at 32 ºC in rabbits using potassium phosphate buffer. Rose and Henderson stated that activities were greatest at fructose concentration of 500 mM at 37 ºC in humans using Tris-HCl buffer. These findings clearly would be corrolated as optimal for 32 ºC. Gerlach found that concentration of 400 mM fructose is optimal at 25 ºC in human using TEA buffer. In the present study, concentrations of fructose exceeding 200 mM increasingly show the effects of substrate inhibition In addition, because of

the viscosity of the fructose solutions required, there is difficulty in obtaining rapid, effective mixing in the cuvette at higher concentrations of fructose (Gerlach, 1959).

The Km value considering D-fructose as a substrate is relatively high and indicates low affinity of the enzyme for its substrate. The first study done concerning human subjects, the Km value in the serum and in liver was determined as 350 mM and 380 mM, respectively (Rose an Henderson, 1975). Another study indicates that the Km value of SDH in human liver is 140 mM being D-fructose as substrate (Maret and Auld, 1988). The difference in Km values in human can be due to the variance of the population or the method applied for the measurement of SDH. In the present study, the Km value was first calculated in liver of New Zealand rabbits as 55,5 mM. This result is similar with findings of Rehg and Torack that the Km value was determined in rat brain as 77 mM using D-fructose as the substrate. As it can be understood from the Km values, there exist some differences among species due to specificity of the enzyme.

Sorbitol dehydrogenase activity was greatest at NADH final concentration of 141 µM. This finding is relatively low in comparison with other studies. However, it was not possible to obtain the highest enzymatic activity by increasing the NADH concentration using liver cytosol of rabbit at 32°C. At that NADH final concentration, enzyme reached saturation and afterwards cofactor inhibition took place.

After characterization of sorbitol dehydrogenase enzyme in terms of substrate, cofactor and enzyme concentration, we have also investigated in this study whether acrylamide, resveratrol and their combined treatments effect liver damage marker enzyme, sorbitol dehydrogenase, activity in rabbit liver and serum or not. As expected, acrylamide treatment in “AA” group resulted in 1.68-fold increase ($p<0.01$) in the sorbitol dehydrogenase enzyme activities of rabbit blood serum with respect to controls due to the toxic nature of acrylamide. We also observed that resveratrol protected liver damage

caused by acrylamide. This can be understood from the results demonstrating that SDH enzyme activities of rabbit blood serum resulted in statistically significant 1.60-fold decrease ($p<0.01$) in “RESV+AA” group and 1.38-fold decrease ($p<0.05$) in “AA+RESV” group with respect to “AA” group.

Besides, no statistically significant change was observed in “100 mg RESV” group with respect to controls in serum samples. In addition to rabbit blood serum, SDH activity measurement was determined in rabbit liver cytosol of both treated and control rabbits. As it is observed in blood serum, acrylamide treatment in “AA” group resulted in 1.27-fold increase ($p<0.05$) in the sorbitol dehydrogenase enzyme activities of rabbit liver cytosol with respect to controls. Additionally, no statistically significant change was observed in “25 mg RESV” group with respect to control group. Besides, we can observe the protective effect of resveratrol on its own and against carcinogenic chemical acrylamide in rabbit liver cytosolic fraction. SDH activity of “100 mg RESV” group in rabbit liver cytosol samples was decreased 3.13-fold ($p<0.001$) significantly with respect to controls. In addition, the results demonstrated that SDH activity resulted in statistically significant decresase 1.59-fold ($p<0.05$) and 1.33-fold ($p<0.05$) in “RESV+AA” and “AA+RESV” group with respect to “AA” group showing the preventive effect of resveratrol. The effects of acrylamide, resveratrol and their combined treatments on sorbitol dehydrogenase activities were summarized in Table 4.3.

Table 4. 3 The effects of acrylamide and resveratrol treatment on sorbitol dehydrogenase enzyme activities of serum and liver cytosolic fraction

	Serum Sorbitol dehydrogenase (U/g)	Change (fold)	**Liver Cytosol** Sorbitol dehydrogenase (U/g)	Change (fold)
Control	2.11 ± 0.44		193.70 ± 38.3	
AA	3.54 ± 0.51	1.68 ↑ a**	246.0 ± 41.7	1.27 ↑ a*
25 mg RESV	Not determined	↔	154.9 ± 35.8	↔
100 mg RESV	2.48 ± 0.42	↔	61.82 ± 10.86	3.13 ↓ a***
RESV+AA	2.21 ± 0.40	1.60 ↓ b*	160.7 ± 32.9	1.59 ↓ b*
AA+RESV	2.56 ± 0.38	1.38 ↓ b*	185.07 ± 13.20	1.33 ↓ b*

a* $p<0.05$ with respect to controls a** $p<0.01$ with respect to controls

a*** $p<0.001$ with respect to “AA” b* $p<0.05$ with respect to “AA”

According to Mine Nuyan (2008), no statistically significant difference was observed in ALT and AST for not only in AA treatment but also in resveratrol treatment together with the combined effect of resveratrol and acrylamide. Unlike the transaminases, which may be of limited value as a biomarker for liver damage due to concentration variations across species, measurement of SDH has become a preferred marker of hepatic damage in both clinical veterinary medicine and drug safety studies, due to its high degree of organ specificity (Dooley, 1984; Bernard and Divers, 1989). We think that measurement of serum SDH activity is of considerable clinical value, because we find it to be much more sensitive indicator of acute hepatic damage than is either measurement of alanine aminotransferase (EC 2.6.1.2) or aspartate aminotransferase (EC 2.6.1.1).

Phase II enzymes are generally accepted as possessing important protective properties due to their detoxification action. Glutathione S-transferases are a multifunctional superfamily of detoxification proteins that catalyse the conjugation of glutathione to chemical toxins (McLellan and Wolf, 1999). They play an important role in the detoxification of electrophilic xenobiotics such as, drugs, toxins and carcinogens (Duvoix *et al*., 2003). Moreover, gutathione S-transferase activity measurement is also used as a marker of oxidative stress (Rodriguez-Ariza *et al*.,1993;Martínez-Gómez *et al*.,2006). The induction of antioxidant enzyme activities represents a cellular defense mechanism to neutralize toxic effects of reactive oxygen species. Considerable attention has been focused on identifying naturally occuring substances having an inhibitory role in carcinogenesis (Surh, 1998). Phenolic compunds particulary those present in dietary and medicinal plants, have been reported to possess substantial anticarcinogenic and antimutagenic activities (Koo *et al.,* 2004). Therefore, GST activity has been shown to be modulated by natural plant products (Wortelboer *et al*., 2003; Zhang *et al*., 2003). In this respect, this is the first study focusing on the interaction of the polyphenolic compound resveratrol with rabbit liver glutathione S-transferases (GSTs).

In this study in vitro effect of resveratrol, polyphenol in red wine, produced from the secondary metabolism of plants, for its ability to modulate rabbit liver cytosolic glutathione S-transferase activity was investigated. It was shown that resveratrol is a potent inhibitor of the rabbit cytosolic glutathione S-transferase activity in liver concerning both substrates CDNB and GSH.

Resveratrol was found to be a potent inhibitor with IC50 of 239,9 μM on the activity of rabbit liver cytosolic glutathione S-transferase. Resveratrol has distinct inhibitory mechanism on cytosolic GST activities of rabbit liver in terms of different type of substrates. Moreover, resveratrol is found as a noncompetitive inhibitor of rabbit liver GST being CDNB as the substrate while it is a competitive inhibitor for liver GST activity being GSH as the substrate.

Inhibition kinetics and mechanisms were studied in the presence of different concentrations of resveratrol and substrates (CDNB and GSH) using Michaelis – Menten, V versus [S], Linewear - Burk, 1/V versus 1/[S], and Dixon, 1/V versus 1/ [resveratrol] plots. It was shown that resveratrol has distinct inhibitory mechanism on enzyme activities depending on the type substrate shown in Table 4.4.

Table 4. 4 K_i values and type of inhibition of glutathione S-transferase in different type of substrates

Inhibitor	K_i^{GSH} (µM)	K_i^{CDNB} (µM)	Type of Inhibition	
			GSH Site	CDNB Site
Resveratrol	55	175	Competitive	Noncompetitive

In liver, resveratrol was shown to be a noncompetitive inhibitor of GST with K_i of 175 µM being CDNB as the substrate (K_m remained unchanged while V_{max} decreased). In liver tissue, resveratrol binds other site of GST apart from catalytic site and binding causes a conformational change in GST resulting decrease of V_{max}. However, resveratrol was found to be competitive inhibitor of hepatic GST with K_i of 55 µM being GSH as the substrate (V_{max} remained unchanged while K_m increased). This result is correlated with the findings of Hayeshi and collequaes that noncompetitive inhibition of M2-2 isoform of GST against ellagic acid at CDNB site and competitive inhibition of P1-1 isoform of GST against ellagic acid at GSH site was observed (Hayeshi *et al*., 2007). Moreover, according to Serdar Karakurt (2008); noncompetitive inhibiton of GST at CDNB site in liver cytosols of rabbit was observed against hydrolysable antioxidant tannic acid. Actually, in the literature there can be found many GST inhibition studies against different compounds with differing K_i values and type of inhibition. This can be due to the experimental model

(*in vitro, in vivo*), the method of animal treatment (injection, dietary) and the species' and tissues' specific response.

GSTs have been shown to be overexpressed in tumor cells hence it increases the resistance for chemotherapeutic drugs. Our study has revealed that the natural phenolic product, resveratrol inhibited rabbit liver GSTs *in vitro*. Inhibition of GST may have a crucial importance for treatment of tumor cells in which chemotherapeutic drug resistance are increased due to high expression of GST. On the other hand, resveratrol's inhibitory effect on GST activity of normal cells may cause problems for detoxification system of those cells. Therefore it was suggested that resveratrol may be introduced to the tumor cells as a combinational therapy with other chemotherapeutic drugs in order to increase their efficacy.

In vivo effects of acrylamide and/or resveratrol, alone or in combination, in different doses and conditions on rabbit liver, kidney, and lung cytochrome P450 (CYPs) enzymes and NQO1 were investigated in our laboratory (Nuyan, 2008). Microsomal CYP2E1-dependent *p*-nitrophenol hydroxylase, NDMA N-demethylase and aniline 4-hydroxylase activities were found to be significantly increased in acrylamide-treated rabbit liver (1.80-3.0 fold) and kidney (1.6-fold). Rabbit liver and kidney CYP2E1 protein levels (determined by western blot analyisis) also increased approximately 2-fold due to acrylamide treatment. In rabbit liver, resveratrol was found significantly effective in decreasing both acrylamide-induced CYP2E1-dependent enzyme activities (approximately 1.5-1.80 fold) and CYP2E1 protein levels (approximately 1.5-1.70 fold). Additionally, resveratrol significantly decreased acrylamide-induced CYP2E1 protein level (2-2.5 fold) in rabbit kidney. However, no significant change was observed in rabbit lung CYP2E1-dependent enzyme activities and CYP2E1 protein levels due to acrylamide, resveratrol or their combined treatments."

Total GST and GST-Mu activities of rabbit kidney (1.5-fold, respectively) and total GST activity of rabbit lung (1.6-fold) were increased significantly only in

resveratrol treated group. NQO1 enzyme activity of rabbit kidney was significantly increased by acrylamide treatment (1.6-fold).

The results of the above study (Nuyan, 2008) have demonstrated for the first time that acrylamide induces rabbit liver and kidney CYP2E1-dependent enzyme activities and CYP2E1 protein levels. The induction of CYP2E1 enzyme activity and protein level by acrylamide treatment can stimulate formation of other toxic compounds and procarcinogens metabolized by CYP2E1 which in turn further potentiates the risk of hepatotoxicity, mutagenicity and carcinogenicity. The results of this study have also suggested that resveratrol may have protective effects on acrylamide induced CYP2E1 related toxicity.

CHAPTER 5

CONCLUSION

To sum up, in the present study, in vivo effects of acrylamide and resveratrol and their combined effects were explored concerning antioxidant enzyme activities in liver and kidney for the first time. In addition, *in vivo* effects of acrylamide and resveratrol and their combined effects were investigated on reduced glutathione content as the non-enzymatic antioxidant and biomarkers used to measure chemical toxicity including lipid peroxidation (MDA). An important biomarker of oxidative and hepatic damage – sorbitol dehydrogenase – was characterized in terms of substrate, cofactor and enzyme concentration. Furthermore, *in vivo* effects of acrylamide and resveratrol and their combined effects were investigated on SDH enzyme activities of rabbir blood serum and liver cytosol. Moreover, the possible *in vitro* effect of plant polyphenolic compound resveratrol for its ability to modulate glutathione-S transferase enzyme activities was elucidated.

New Zealand male rabbits were treated with acrylamide and resveratrol, separately in two different doses and conditions. Their combined effects were also investigated. While, acrylamide treatment significantly decreased the glutathione peroxidase (GPx) activity in liver (1.24-fold), it was significantly increased (1.20 – 1.40-fold) by combined effect of resveratrol and acrylamide in liver and kidney. Furthermore, alone resveratrol administration increased (~1.37 – fold) GPx activity in kidney. Although, glutathione reductase (GR) was found to be significantly increased (~1.30-fold) in two different dose of resveratrol treated rabbit liver, it was not changed in acrylamide and their combined treatments. Despite, glutathione (GSH) content was decreased around 1.6 fold as a result of acrylamide treatment in rabbit liver and kidney cytosols, GSH level was returned to

normal levels by resveratrol tretment in rabbit liver and kidney. Furthermore, acrylamide treatment significantly increased the SDH activity in blood serum (1.68-fold) and in liver (1.27-fold) with respect to control. On the other hand, resveratrol treatment brought this activity nearly normal level in acrylamide treated rabbits.. Besides, sorbitol deydrogenase (SDH) was found to be decreased (3.13-fold) significantly in rabbit liver cytosol as a result of single dose of 100 mg/kg b.w. resveratrol treatment. Moreover, catalase activity and MDA level were not affected from either resveratrol or acrylamide and with their combination effect in investigated rabbit organs.

An important liver damage marker enzyme other than ALT and AST, SDH was characterized in terms of substrate, cofactor and enzyme concentration in rabbits which have been not investigated before and found to be 200 mM, 141 µM and 0.5 µL, respectively in rabbit liver. Furthermore, the Km value was first calculated in liver of New Zealand rabbits as 55,5 mM.

In addition to these, *in vitro* effects of resveratrol on GST activity was also studied throughout this study. Resveratrol was shown to be a noncompetitive inhibitor for liver cytosolic GST against substrate CDNB with K_i of 175 µM. On the other hand, resveratrol was shown to be a competitive inhibitor for liver cytosolic GST against substrate GSH with K_i of 55 µM.

The results of the present study have demonstrated for the first time that resveratrol induced some of the antioxidant enzyme activities and as well nonenzymatic antioxidants in rabbit liver and kidney. The results of GPx, GR, SDH activities and GSH level have also suggested that resveratrol may have protective effects on acrylamide induced hepatoxicity and renal toxicity. Therefore, it may be a therapeutic approach for the oxidative stress-related diseases such as cancer. However, further *in vivo* studies are required to clarify the effect of resveratrol on both acrylamide-induced toxicity and bioavailability in the body.

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