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An *in vivo* and *in silico* approach to elucidate the Tocotrienol-mediated fortification against infection and inflammation induced alterations in antioxidant defense system

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Abstract. – *Background:* **Tocotrienol (Tocomin) are naturally occurring analogues of vitamin E family and has been reported to possess a potent free radical scavenging activity. In the present study we have initially investigated protective role of tocotrienol against infection and inflammation induced alterations in tissues antioxidant defense system, as well as speculated, via in silico docking studies, that tocotrienol can act by directly binding to antioxidant enzymes.**

Materials and Methods: **Syrian hamsters were injected with bacterial lipopolysaccharide (LPS, 200 µg), zymosan (20 mg), or turpentine (0.5 ml) to mimic acute infection, acute systemic inflammation, and acute localized inflammation, respectively, which are responsible for the generation of plenty of free radicals that causes oxidative stress. Tocomin (10 mg) was administered daily for 10 days before and 12 h after lipopolysaccharides (LPS) or 24 h after turpentine or zymosan injection. Molecular docking studies were performed using Autodock 4.0.**

Results: **Our results show a significant decrease in the activities of antiperoxidative enzymes, glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), glutathione-s-transferase (GST), as well as reduced glutathione (GSH), in liver and kidney of LPS, turpentine or zymosan stressed hamsters. Feeding of 10 mg Tocomin to stressed hamsters was quite effective in reversing/normalizing the altered levels of enzymatic and nonenzymatic antioxidants in liver and kidney. In order to explore the interaction between tocotrienol and antioxidant enzymes a molecular docking study was performed. The results showed good interaction in term of binding energy and inhibition constant in the following order GR >CAT > SOD >GST >GPx.**

Conclusion: **Our *in vivo* and *in silico* results for the first time indicate that tocotrienol significantly alleviate the condition of oxidative stress not only by its potent free radical scavenging properties but also may be by interacting directly and strongly with antioxidant enzymes as proved by molecular docking simulations.**

Key Words:

Tocotrienol, Antioxidant enzymes, Oxidative stress, Docking.

Abbreviations

GR = Glutathione Reductase
GPx = Glutathione Peroxidase
CAT = Catalase
SOD = Superoxide Dismutase
GST = Glutathione-s-transferase
GSH = Reduced Glutathione

Introduction

Oxidative stress has been implicated in various pathological conditions involving cardiovascular diseases, cancer, neurological disorders, diabetes mellitus, ischemia/reperfusion, other diseases and ageing. These diseases fall into two groups: (1) the first group involves diseases characterized by pro-oxidants shifting the thiol/disulphide redox state and impairing glucose tolerance-the so-called "mitochondrial oxidative stress" conditions (cancer and diabetes mellitus); (2) the second group involves disease characterized by "inflammatory oxidative conditions", leading to atherosclerosis and chronic inflamma-

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tion[1]. Reactive oxygen species (ROS) or oxygen free radicals, are products of normal cellular metabolism and is well recognized for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems[2].

Infection and inflammation elicits acute phase response (APR) that represents first line of defense against injury as well as bacterial and parasitic infection and also responsible for the activation of macrophages and neutrophils as target cells, which produces a plenty of the damaging oxygen free radicals in the system[3,4]. In addition, the excessive production of ROS associated with inflammation, leads to a condition of oxidative stress, which results an imbalance in antioxidant enzymes status. These enzymes are important in defending the body against free radicals as well as toxic substances by converting them to a form that can be readily excreted. Therefore, any changes in these enzymes could be potentially detrimental to the host by altering these defense mechanisms. Considerable evidences implicate the role of oxygen free radicals in the pathogenesis of endotoxemia and sepsis leading to organ dysfunction[4]. However, in sepsis, an increased activation of neutrophils results in the generation of increased amounts of ROS leading to host tissue damage, which itself uncouples electron transport system and thus, generates even more ROS. Such ROS may directly injure cells, proximal to oxidant generating cells and may inactivate any proteolytic enzymes involved during inflammation, due to the production of lipopolysaccharides (LPS) from Gram-negative bacteria.

Endotoxemia is known to decrease hepatic/plasma glutathione (GSH) levels and inhibit its synthesis and conjugation pathways as well as down-regulating the activity of glutathione peroxidase (GPx), glutathione-s-transferase (GST), superoxide dismutase (SOD), and catalase (CAT). In rats, however, the underlying mechanism is unknown[5-8]. It has been reported that endotoxin injection also caused renal tissue damage and decreased the SOD, GPx and CAT activities compared to control rats[9,10]. Furthermore, when zymosan was injected into animals, it induces inflammation by inducing a wide range of inflammatory mediators. These include activated components of the complement system, prostaglandins and leukotrienes, platelet aggregation factor, oxygen radicals, and lysosomal enzymes[11]. Van Bebber et al[12] administered a combination of SOD and CAT to rats treated with zymosan and found that, although lipid peroxidation was significantly decreased, mortality and organ damage remained unchanged. Demling et al[13] showed that after 24 h of zymosan administration in rats, lung CAT acivity was significantly decreased. Belmonte et al[14] showed that after 1 or 7 days of turpentine oil injection to rats, the GSH content in liver were significantly decreased in comparison to normal control rats. Proulx and Du Souich[15] observed that turpentine-induced inflammatory reaction causes oxidative stress in the liver characterized by a decrease in activity of enzymatic scavengers i.e. CAT, GPx, SOD and of reduced GSH. Proulx et al[16] have reported that subcutaneous administration of turpentine oil to rabbits induced oxidative stress, which reduced the hepatic activities of enzymatic scavengers of ROS.

Few scattered studies have reported the role of some antioxidants such as vitamins A, C and E, in the attenuation of oxidative stress caused by infection or inflammation[7-10]. The tocotrienol isomers (α-, β-, γ- and δ-) are naturally occurring analogues of tocopherol isomers (vitamin E) found mainly in cereal grains and palm oil. Tocotrienol differ from tocopherols by possessing three double bonds in the phytyl side chain. However, several reports have shown tocotrienol to be more potent than tocopherols[16-18]. Since, tocotrienol was shown to be a putative biological antioxidant by its radical scavenging activity[19,20], the present study was initially carried out to evaluate the *in vivo* ameliorative role of tocotrienol against infection and inflammation induced hepatic and renal alterations in antioxidant defense system, as well as *in silico* docking studies was done to predict the interaction between tocotrienol and antioxidant enzymes.

Materials and Methods

Drug and Chemicals

Twenty five percent palmvitae oil suspension of tocotrienol (Tocomin) containing 6.4% d-α-tocotrienol, 1% d-β-tocotrienol, 10.2% d-γ-tocotrienol, 3.2% d-δ-tocotrienol and 5.7% d-α-tocopherol as well as RBD palm olein were supplied as a gift from Carotech BHD, Chemor, Malaysia. Lipopolysaccharides (*E. coli*, 055:B5) and zymosan A were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oil of turpentine

was purchased from Vasco Drug Laboratories (Aligarh, India). All other chemicals and reagents used in this study were of analytical grade.

Animals

Male golden hamsters, weighing about 175-200 g were purchased from Central Drug Research Institute, Lucknow, India, and acclimatized for 7 days. The protocol of the study was approved by the animal Ethical Committee of the J. N. Medical College, A M University, India. The hamsters were given standard pelleted rodent chow (Ashirwad Industries, Chandigarh, India) and water *ad libitum*.

Induction of Infection and Inflammation in Hamsters

For the induction of infection and inflammation mediated oxidative stress, hamsters were injected with LPS, turpentine oil or zymosan, respectively. Lipopolysaccharide, 200 μg in 0.5 ml saline and zymosan, 20 mg in 0.5 ml warm (50^0C) phosphate buffered saline (PBS), pH 7.0, were injected intraperitoneally, whereas, turpentine oil, 0.5 ml, was injected subcutaneously. Hamsters in control group were injected intraperitoneally with 0.5 ml saline only. Subsequently, food was withdrawn from both control and treated hamsters[22].

Treatment Schedule

Tocomin (1 g) was prepared in ethanol and palmvitae oil (1:20). Tocomin suspension was administrated through gastric intubation in two divided doses (morning and evening) of 0.5 ml each/hamster/day. In normal control group, five hamsters were given 1.0 ml palmvitae oil/hamster/day, through gastric intubation, 10 days before and 24 h after saline injection. Five hamsters in each LPS, turpentine or zymosan control group were administered 1.0 ml palmvitae oil/hamster/day, 10 days before and 12 h after LPS injection or 24 h after turpentine or zymosan injection. Five hamsters in each LPS, turpentine or zymosan Tocomin treated group were given total 10 mg (5 mg each in morning and evening) Tocomin/day, 10 days before and 12 h after LPS injection or 24 h after turpentine or zymosan injection. The dose of tocotrienol was adjusted according to previous published reports[22].

Assessment of Oxidative Stress in Liver and Kidney

Post Mitochondrial Supernatant Preparation

At indicated times after LPS, turpentine or zymosan injection, hamsters in each group were anaesthetized and liver and kidney from each hamster were promptly excised and chilled in ice-cold saline. Each liver and kidney were cut into pieces, and homogenized with 10% (w/v) of chilled 0.1 M sodium phosphate buffer, pH 7.4, containing 1.17% KCl. The volume of each homogenate was recorded and centrifuged at 1,000 rpm for 10 min at 4°C. The homogenate was used to estimate malondialdehyde (MDA), reduced glutathione (GSH) and glutathione peroxidase (GPx) in liver and kidney. The liver and kidney homogenates were again centrifuged at 12,000 rpm for 20 min at 4°C to get post-mitochondrial supernatant (PMS), which was used to assay CAT, SOD, GR and GST.

Estimation of Lipid Peroxidation

Lipid peroxidation products, conjugated diene (which measure the initial phase of lipid peroxidation), lipid hydroperoxide (intermediate product of lipid peroxidation) and MDA (which measure the degradation phase of lipid peroxidation), in tissues were measured by different methods. Conjugated diene was measured according to the method of Corongiu et al[23]. Quantification of lipid hydroperoxide was done according to the method of Nourooz-Zadeh et al[24]. The method of Ohkawa et al[25] was used for the determination of MDA content in liver and kidney homogenates.

Determination of Antioxidant Enzymes Activity in Erythrocytes, Liver and Kidney

The enzymatic activity of CAT in erythrocytes hemolysate and post- mitochondrial supernatant (PMS) of liver and kidney was measured by the procedure described by Sinha[26]. Superoxide dismutase in erythrocytes hemolysate and PMS fraction of liver and kidney was determined by the method as described by Kakkar et al[27] Glutathione peroxidase activity in erythrocytes hemolysate and liver and kidney homogenate was assayed by Hafeman et al[28]. The method of Carlberg and Mannervik[29] was employed for the determination of GR activity in erythrocytes hemolysate and liver and kidney PMS fraction.

Glutathione-S-transferase (GST) activity was measured in PMS fraction of liver and kidney by the method of Habig et al[30]. Reduced glutathione (GSH) in tissue homogenate was assayed according to the method of Jollow et al[31] while in packed erythrocytes it was determined essentially by the method of Ellman[32] as modified by Sedlack and Lindsay[33].

Protein Estimation

The protein was determined by the method of Bradford[34], using bovine serum albumin as standard. Aliquots of liver and kidney homogenates and PMS were first precipitated with 10% trichloroacetic acid (TCA). The protein pellets were dissolved in 0.5 N NaOH and suitable aliquots were used for protein determination.

Molecular Docking Simulations

Docking study of Tocotrienol (CID: 9929901) with different antioxidant enzymes namely CAT (PDB_ID:1DGB), GST (PDB_ID:10GS), GR (PDB_ID:1BWC), SOD (PDB_ID:1MFM) and GPx (PDB_ID:2F8A) were performed in order to find specific binding model using AutoDock 4.0[35,36], a software that uses an empirical scoring function based on the free energy of binding[37]. Among the stochastic search algorithms offered by AutoDock suite, we choose the Lamarckian Genetic Algorithm (LGA) which combines global search (Genetic Algorithm alone) to local search (Solis and Wets algorithm[38]. The Ligand (Tocotrienol) was downloaded from PubChem (http://pubchem.ncbi.nlm.nih.gov) and by using their Smiles ID, the input files in the format suitable for AutoDock 4.0 was generated at the Corina server (http://www.molecular-networks.com/products/corina). Energy minimization of ligand and receptors were done by Discovery Studio. The grid parameter file of each receptor was generated using AutoDock 4.0. A grid-box was generated that was large enough to cover the entire receptor binding site and accommodate ligand to move freely. The number of grid points in x, y, and z-axes were 60×60×60 Å. The distance between two connecting grid points was 0.375 Å. The center of the ligand in the X-ray crystal structure was used as the center of the grid-box. For receptor structures that do not have ligand in the binding site, the center of the binding site was estimated from the structure and taken as the center of the grid-box. AutoDock4 and a Lamarckian Genetic Algorithm (LGA)[39] were used for receptor-fixed ligand-flexible docking calculations. Ten search attempts (ga_run parameter) were performed for ligand. The maximum number of energy evaluations before the termination of LGA run was 2,500,000 and the maximum number of generations of the LGA run before termination was 27000. Other docking parameters were set to the software's default values. After complete execution of AutoDock Ten conformations of ligand in complex with the receptor were obtained, which were finally ranked on the basis of binding energy and inhibition constant (Ki). The resulting conformations were visualized in the Discovery Studio Visualizer.

Statistical Evaluation

Statistical analysis of data was done by employing two-tailed Student's *t*-test as described by Bennet and Franklin[40].

Results

Impact on Liver and Kidney Lipid Peroxidation Products

As illustrated in Table I, conjugated diene, lipid hydroperoxide and MDA in liver and kidney were significantly increased in LPS, turpentine or zymosan administered hamsters when compared to normal control values. Pretreatment of these stressed hamsters with Tocomin mediated a decrease in liver and kidney conjugated diene, lipid hydroperoxide and MDA and restored these values close to corresponding normal values in each tissue. These results demonstrate that increased formation of conjugated diene, lipid hydroperoxide and MDA in liver and kidney of the above stressed hamsters were significantly reduced after Tocomin pretreatment.

Regulatory Effect of Tocomin Pretreatment on Antioxidant Defense System in LPS, Turpentine or Zymosan Stressed Hamsters

Infection and inflammatory diseases are associated with oxidative stress due higher ROS, such as superoxide radical, hydroxyl radical, hydrogen peroxide and/or deficiency in the antioxidant defense systems. An impaired radical scavenger

Table I. Effect of Tocomin pretreatment on liver and kidney lipid peroxidation products in three group of stressed hamsters. Normal control, fed 0.5 ml palmvitae oil/hamster/day, 10 days before and 24 h after saline injection; LPS Tocomin treated, fed 10 mg Tocomin/hamster/day, 10 days before and 12 h after LPS injection; Turpentine or Zymosan Tocomin treated, fed 10 mg Tocomin/hamster/day, 10 days before and 24 h after turpentine or zymosan injection.

Group	Liver			Kidney		
	Conjugated diene	Lipid hydroperoxide	MDA	Conjugated diene	Lipid hydroperoxide	MDA
Normal control	5.9 ± 0.023*	1.5 ± 0.001	3.5 ± 0.015	3.076 ± 0.029	0.303 ± 0.0037	2.9 ± 0.017
LPS control	7.5 ± 0.025* (+25.3%)[a]	2.4 ± 0.006 (+56.8%)[a]	4.79 ± 0.018 (+36.0%)[a]	4.02 ± 0.057 (+30.78%)[a]	0.45 ± 0.0017 (+47.85%)[a]	3.7 ± 0.038 (+25.4%)[a]
LPS Tocomin treated	7.02 ± 0.018* (-6.6%)[a]	1.8 ± 0.001 (-25.1%)[a]	3.78 ± 0.017 (-21.1%)[a]	3.7 ± 0.029 (-7.23%)[c]	0.4 ± 0.0035 (-10.49%)[a]	3.2 ± 0.031 (-14.6%)[a]
Turpentine control	7.0 ± 0.025* (+16.8%)[a]	2.1 ± 0.008 (+36.7%)[a]	4.15 ± 0.013 (+17.9%)[a]	3.7 ± 0.030 (+21.2%)[a]	0.4 ± 0.0034 (+32.34%)[a]	3.5 ± 0.024 (+18.8%)[a]
Turpentine Tocomin treated	6.5 ± 0.021* (-6.6%)[a]	1.78 ± 0.009 (-15.2%)[a]	3.8 ± 0.026 (-8.2%)[a]	3.5 ± 0.056 (-5.09%)	0.34 ± 0.0042 (-15.71%)[a]	3.1 ± 0.023 (-12.0%)[a]
Zymosan control	7.0 ± 0.035* (+16.9%)[a]	2.1 ± 0.008 (+37.0%)[a]	4.15 ± 0.019 (+18.07%)[a]	3.7 ± 0.024 (+20.62%)[a]	0.39 ± 0.006 (+31.35%)[a]	3.5 ± 0.030 (+17.8%)[a]
Zymosan Tocomin treatedd	6.5 ± 0.018* (-6.8%)[a]	1.78 ± 0.004 (-15.4%)[a]	3.8 ± 0.011 (-8.61%)[a]	3.5 ± 0.035 (-5.01%)	0.35 ± 0.0162 (-13.06%)[c]	3.1 ± 0.031 (-10.74%)[a]

*Values are means (nmole/mg protein) ± SD from homogenate of pooled liver or pooled kidney of 5 hamsters in each group. Significantly different from normal control at [a]$p<0.001$. Significantly different from their respective control at [a]$p<0.001$ and [c]not significant.

function has been linked to the decreased activity of enzymatic and nonenzymatic scavengers of free radicals. Therefore, the status of antioxidant enzymes, such as CAT, SOD, GPx, GST and GR including GSH concentrations in erythrocytes, liver and kidney of LPS, turpentine or zymosan stressed hamsters are highly important.

Impact on Erythrocytes CAT and SOD Activities

As seen in Table II, the erythrocytes CAT and SOD activities in LPS stressed hamsters were significantly decreased by 41% and 25%, respectively, in comparison to corresponding normal control values. Feeding of Tocomin for 10 days before, and 12 h after LPS injection significantly prevented this decrease in CAT and SOD activities and increased them by 33% and 23%, respectively, when compared to corresponding normal values of LPS stressed hamsters. After 24 h of turpentine injection, a significant reduction of 12% in both the CAT and SOD activities was seen, when compared to corresponding normal control values. Treatment with Tocomin for 10 days before and 24 h after turpentine injection blocked this decrease and increased both the CAT and SOD activities by 8%, in comparison to

Table II. Effect of Tocomin pretreatment on erythrocytes CAT and SOD activities in three groups of stressed hamsters.

Group	CAT (U/g Hb)[†]	SOD (KU/g Hb)[‡]
Normal control	380.65 ± 2.06*	2.32 ± 0.028
LPS control	226.44 ± 2.09* (-40.51%)a	1.75 ± 0.011 (-24.56%)[a]
LPS Tocomin treated	301.89 ± 4.86* (+33.32%)[b]	2.15 ± 0.012 (+22.85%)[b]
Turpentine control	333.40 ± 3.97* (-12.41%)[a]	2.05 ± 0.021 (-11.63%)[a]
Turpentine Tocomin treated	358.93 ± 6.07* (+7.65%)[b]	2.21 ± 0.0158 (+7.80%)[b]
Zymosan control	311.41 ± 3.35* (-18.18%)a	2.15 ± 0.018 (-7.327%)[a]
Zymosan Tocomin treated	355.30 ± 3.88* (+14.09%)[b]	2.22 ± 0.038 (+3.25%)[b]

[†]One unit of enzyme activity is defined as the µmoles of H_2O_2 decomposed/min/g Hb. [‡]One unit of enzyme activity is defined as the amount of enzyme required to inhibit O.D. at 560 nm of chromogen production by 50% in one minute. *Values are means ± SD from pooled erythrocytes hemolysate of 5 hamsters in each group. Significantly different from Normal control at [a]$p<0.001$. Significantly different from their respective controls at [b]$p<0.001$.

corresponding stressed control values. Similarly, in zymosan stressed hamsters, CAT and SOD activities were significantly decreased by 18% and 7%, respectively, in comparison to corresponding normal control values. Pretreatment of these hamsters with Tocomin prevented this decrease and elevated these enzyme levels by 14% and 3%, respectively.

Effect on Erythrocytes GPx, GR and Reduced GSH

The data summarized in Table III, showed a significant increase of 48%, 40% and 36% in the GPx activity of LPS, turpentine or zymosan stressed hamsters, respectively, in comparison to normal control values. Tocomin pretreatment to LPS, turpentine or zymosan stressed hamsters was associated with a significant reduction in GPx activity by 18%, 20% and 18%, respectively. On the other hand, GR activity and GSH content in LPS stressed hamsters were significantly decreased by 46% and 67%, respectively. Pretreatment with Tocomin significantly prevented this decrease in erythrocytes GR activity and GSH content and increased these values by 66% and 54%, respectively, when compared to corresponding values in LPS control group. Consistent with the decrease in GR activity and GSH content in LPS control group, a reduction of 23% and 48% in GR activity and GSH content was observed in Turpentine stressed hamsters. Tocomin pretreatment to these hamsters blocked this decrease and restored GR activity and GSH concentration in erythrocytes by 21% and 45%, respectively, in comparison to corresponding values in turpentine stressed hamsters. In comparison to corresponding normal control values, GR activity and GSH content in zymosan stressed hamsters were decreased by 38% and 40%, respectively. Pretreatment of these hamsters with Tocomin prevented this decrease in GR activity and GSH content and increases them by 41% and 36%, respectively, in comparison to corresponding zymosan stressed control values. These results indicate that the erythrocytes CAT, SOD, GR activities and GSH content was significantly decreased, whereas, GPx activity was significantly increased in LPS, turpentine or zymosan stressed hamsters. Furthermore, dietary Tocomin (tocotrienol) being a potent antioxidant, prevented this decrease or increase in enzyme activities as well as GSH content.

Impact on Liver and Kidney CAT and SOD Activities

Consistent with the decline in the enzymatic activities of erythrocytes CAT and SOD in LPS, turpentine or zymosan stressed hamsters; liver and kidney CAT and SOD activities were also decreased in these hamsters (Figures 1 and 2). In

Table III. Impact of Tocomin pretreatment on erythrocytes GPx, GR and GSH activities in three groups of stressed hamsters.

Group	GPx (U/g Hb)†	GR (U/g Hb)‡	GSH (µmole/g Hb)
Normal control	0.811 ± 0.014*	41.35 ± 1.006	1.046 ± 0.174
LPS control	1.198 ± 0.14* (+47.71%)[a]	22.13 ± 1.628 (-46.48%)[a]	0.342 ± 0.224 (-67.30%)[b]
LPS Tocomin treated	0.986 ± 0.009* (-17.69%)[c]	36.72 ± 1.663 (+65.92%)[c]	0.528 ± 0.139 (+54.38%)[d]
Turpentine control	1.134 ± 0.011* (+39.82%)[a]	31.94 ± 2.358 (-22.75%)[a]	0.541 ± 0.275 (-48.28%)[b]
Turpentine Tocomin treated	0.903 ± 0.008* (-20.37%)[c]	38.75 ± 1.567 (+21.32%)[c]	0.785 ± 0.259 (+45.10%)[d]
Zymosan control	1.106 ± 0.009* (+36.37%)[a]	25.53 ± 1.722 (-38.25%)[a]	0.626 ± 0.237 (-40.15%)[b]
Zymosan Tocomin treatedd	0.908 ± 0.008* (-17.90%)[c]	35.90 ± 2.632 (+40.61%)[c]	0.849 ± 0.086 (+35.62%)[d]

†One unit of enzyme activity is defined as the µmoles of oxidized glutathione formed/min/g Hb. ‡One unit of enzyme activity is defined as the µmoles of NADPH oxidized/min/g Hb. *Values are means ± SD from pooled erythrocytes hemolysate of 5 hamsters in each group. Significantly different from Normal control at [a]$p<0.001$ and [b]$p<0.005$. Significantly different from their respective controls at [c]$p<0.001$ and [d]$p<0.05$.

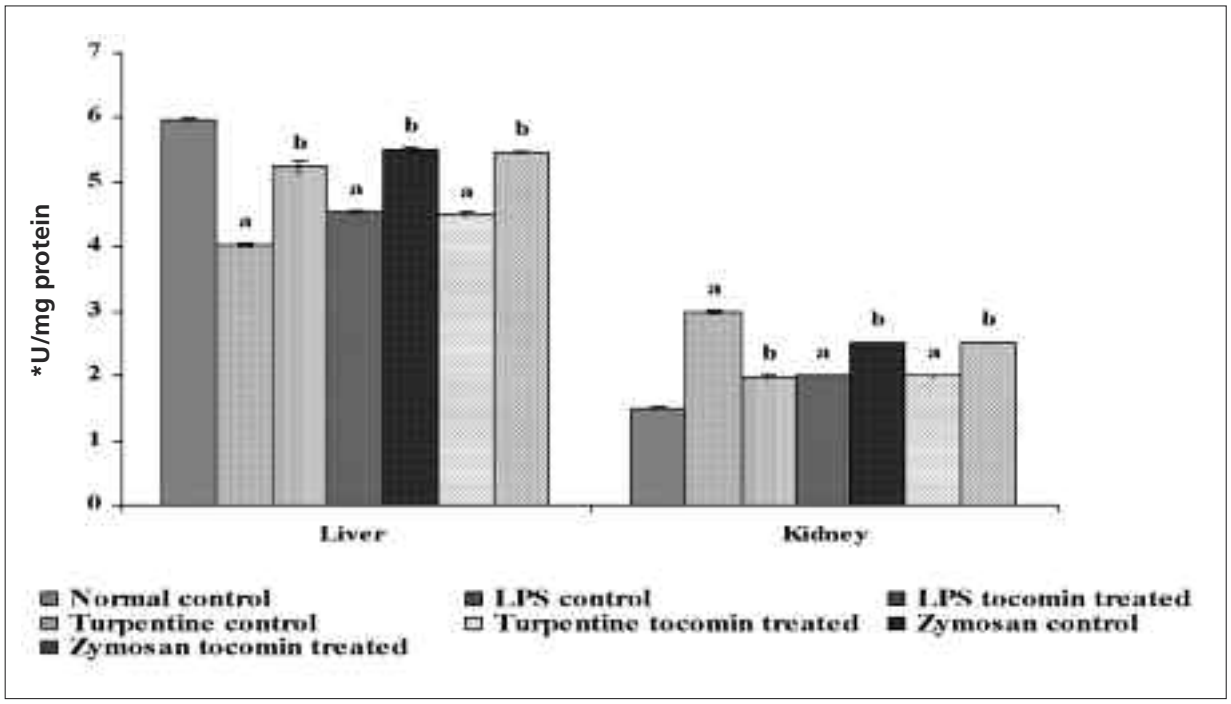

Figure 1. Impact of Tocomin pretreatment on liver and kidney CAT activity in three groups of stressed hamsters. One unit of enzyme activity is defined as the μmoles of H_2O_2 decomposed/min/mg protein. *Values are means (U/mg protein) ± SD from PMS fraction of pooled liver or pooled kidney of 5 hamsters in each group. [a]Each value is significantly different from normal control at $p<0.001$. [b]Each value is significantly different from their respective control at $p<0.001$.

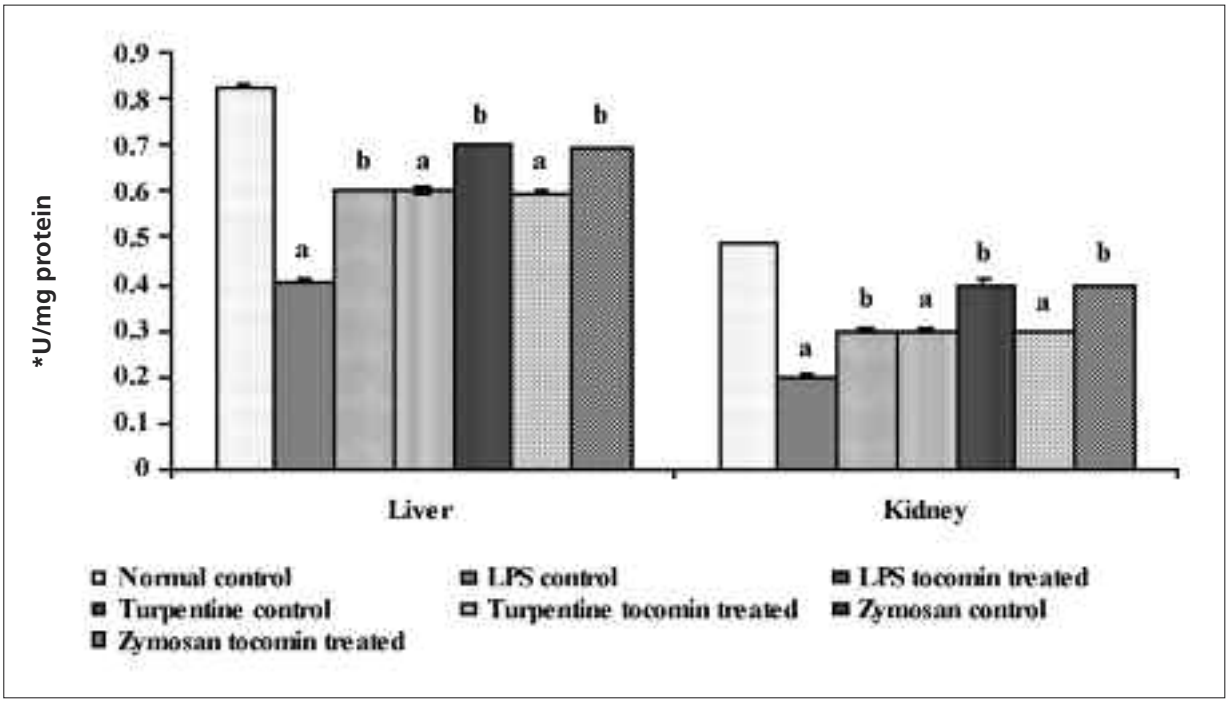

Figure 2. Effect of Tocomin pretreatment on liver and kidney SOD activity in three groups of stressed hamsters. One unit of enzyme activity is defined as the amount of enzyme required to inhibit O.D. at 560 nm of chromogen production by 50% in one minute. *Values are means (U/mg protein) ± SD from PMS fraction of pooled liver or pooled kidney of 5 hamsters in each group. [a]Each value is significantly different from normal control at $p<0.001$. [b]Each value is significantly different from their respective control at $p<0.001$.

LPS, turpentine or zymosan stressed hamsters, a significant decrease of 32%, 24% and 25% in hepatic CAT activity were seen, while kidney CAT activity also exhibited a significant decrease of 50%, 33% and 33%, respectively, when compared to corresponding normal control values. Administration of Tocomin to these stressed hamsters significantly prevented the decrease in hepatic and renal CAT activity and partially restored these values in the range of 21%-33%. Similarly, hepatic SOD activity in LPS, turpentine or zymosan stressed hamsters were significantly decreased by 51%, 28% and 27%, respectively, whereas a 59%, 38% and 39% decrease were observed in kidney SOD activity, respectively. Pretreatment of these stressed hamsters with Tocomin significantly blocked the decrease in hepatic and renal SOD level and showed an increase in the range of 17%-51%.

Impact on the Regulation of Liver and Kidney GPx, GR, GST and Reduced GSH Level

Figure 3 summarizes the results of GPx, GR and GST activities in liver and kidney of LPS, turpentine or zymosan stressed hamsters without and with Tocomin pretreatment. In LPS stressed hamsters, the enzymatic activities of GPx, GR and GST in liver were significantly decreased from the values of 64.2, 10.31 and 151.9 U/mg protein in normal control to 25 (61%), 5.99 (42%) and 69.9 (54%) U/mg protein, respectively, whereas, in kidney, a significant decrease of 61%, 66% and 50% was observed in GPx, GR and GST activities, respectively. As evident, pretreatment of LPS stressed hamsters with Tocomin resulted in a significant increase of 81%, 35% and 57% in liver and 88%, 103% and 50% in kidney GPx, GR and GST activities, respectively, when compared to corresponding tissue values in LPS group. In turpentine control hamsters, a significant decline in hepatic GPx (45%), GR (22%) and GST (41%) activities were observed, whereas, kidney GPx, GR and GST activities were reduced by 34%, 33% and 25%, respectively, in comparison to corresponding tissue normal control values. Pretreatment of these hamsters with Tocomin mediated a significant decline of 43%, 12% and 34% in liver and 27%, 25% and 11% in kidney GPx, GR and GST activities, respectively, when compared to corresponding tissue values in turpentine control group. After 24 h of zymosan injection, hepatic GPx, GR and GST activities were significantly decreased by 45%, 22% and 41%, whereas, kidney GPx, GR and GST activities were also significantly decreased by 34%, 34% and 25%, respectively. Pretreatment of zymosan stressed hamsters with Tocomin resulted in a significant increase of 42%, 11% and 18% in liver GPx, GR and GST activities, respectively. Similarly, an increase of 26%, 25% and 10% in kidney GPx, GR and GST activities, respectively, was seen, when compared to corresponding values in zymosan control group.

Reduced glutathione, a major non-protein thiol plays a crucial role in coordinating the body's antioxidant defense processes. Perturbation of GSH status of a biological system can lead to serious consequences. As evident from Figure 4, hepatic GSH contents in LPS, turpentine or zymosan stressed hamsters were significantly reduced by 33%, 20% and 20%, respectively, while a 63%, 26% and 26% reduction were observed in kidney GSH level when compared to normal control value. Tocomin pretreatment to these stressed hamsters prevented this decrease and exhibited a significant increase from 8%-66%, with maximum effect in LPS group.

In summary liver and kidney CAT, SOD, GPx, GR and GST enzymes as well as GSH content, which constitute a mutually supportive team of defense against ROS, are significantly decreased in LPS, turpentine or zymosan stressed hamsters. In addition, feeding of dietary tocotrienol (Tocomin) for 10 days before and 12 h after LPS or 24 h after turpentine or zymosan injection substantially quenches these free radicals (ROS), thus positively normalizing the above enzyme levels, indicating an antioxidant effect of dietary tocotrienol.

Molecular Docking Results

The docking simulation technique was performed by using AutoDock program to show molecular interactions of tocotrienol with antioxidant enzymes namely CAT, GST, GR, SOD and GPx. Tocotrienol was docked into each of five different protein targets. The lowest energy docked conformation of the most populated cluster (the best cluster) was selected and taken into account. The binding energy and inhibition constant (Ki) of tocotrienol with different antioxidant enzymes are illustrated in Table IV. The results showed a better interaction of tocotrienol with GR in terms of inhibition constant and binding energy, whereas the other antioxidant enzymes also showed a good interaction in the fol-

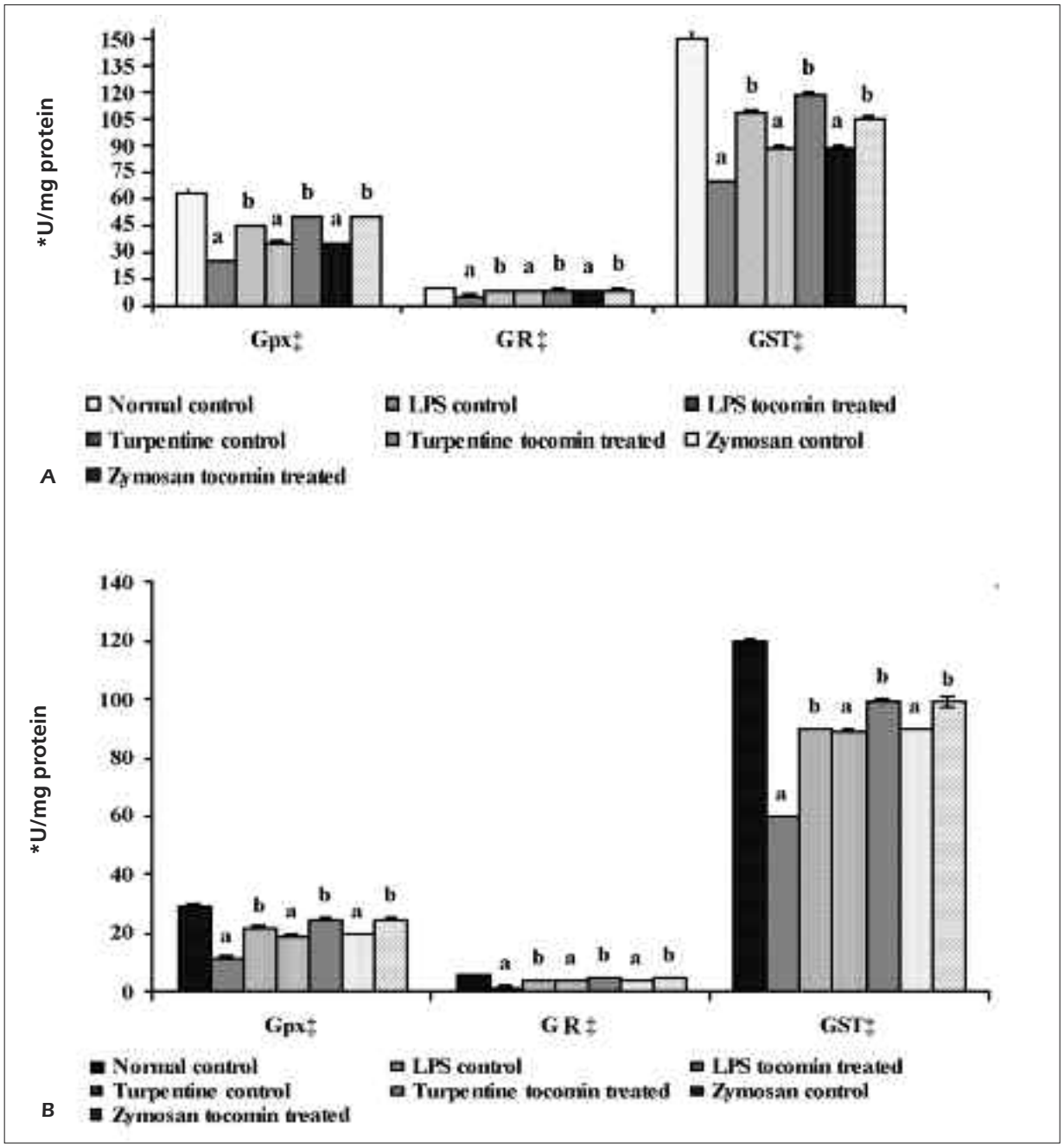

Figure 3. ***A,*** Impact of Tocomin pretreatment on liver GPx, GR and GST activities in three groups of stressed hamsters. GPx‡: One unit of enzyme activity is defined as nmole oxidized glutathione formed/min/mg homogenate protein. GR‡: One unit of enzyme activity is defined as nmole NADPH oxidized/min/mg PMS protein. GST‡: One unit of enzyme activity is defined as the nmole of 1-chloro 2, 4-dinitrobenzene (CDNB) conjugate formed/min/mg PMS protein. *Values are means (U/mg protein) ± SD from homogenate/PMS of pooled liver or pooled kidney of 5 hamsters in each group. [a]Each value are significantly different from normal control at $p<0.001$. [b]Each value are significantly different from their respective control at $p<0.001$. ***B,*** Impact of Tocomin pretreatment on liver GPx, GR and GST activities in three groups of stressed hamsters. *Values are means (U/mg protein) ± SD from homogenate/PMS of pooled liver or pooled kidney of 5 hamsters in each group. [a]Each value is significantly different from normal control at $p<0.001$. [b]Each value is significantly different from their respective control at $p<0.001$.

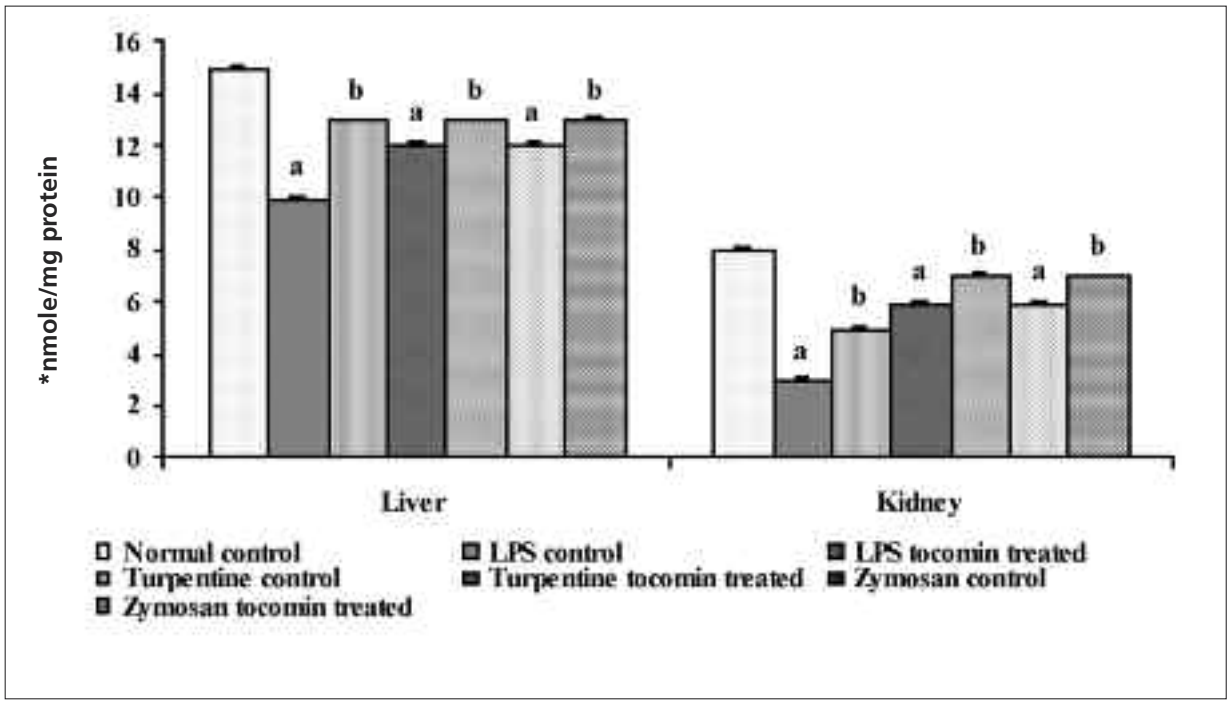

Figure 4. Effect of Tocomin pretreatment on liver and kidney GSH activity in three groups of stressed hamsters. *Values are means (nmole/mg protein) ± SD from homogenate of pooled liver or pooled kidney of 5 hamsters in each group. [a]Each value is significantly different from normal control at $p<0.001$. [b]Each value is significantly different from their respective control at $p<0.001$.

lowing order: CAT > SOD >GST >GPx. Figure 5 showed the docked structure of tocotrienol with CAT, GST, GR, SOD and GPx in which Thr-361 residue of CAT, Tyr-49 of GST, Asp-441 of GR, Asn-86 of SOD and Glu-88 of GPx found to be involved in H-bond formation with tocotrienol that further proved its binding stability.

Discussion

The acute phase response (APR) represents the initial line of defense against injury as well as bacterial and parasitic infections. Lipopolysaccharide, turpentine or zymosan, which elicits the APR, has been shown to reduce the expression of certain conjugation enzymes as well as antioxidant enzymes. Cytokines, secreted from macrophages in response to infection and inflammation, have been implicated in the suppression of activity of these enzymes in addition to their role in oxidative stress. These enzymes are important in defending the body against free radicals as well as toxic substances by converting them to a form that can be readily excreted. Therefore, any changes in these enzymes could be potentially detrimental to the host by altering

Table IV. Molecular Docking results of tocotrienol with different antioxidant enzymes.

Sr. No	Proteins	Ligand	Binding Energy (Kcal/mole)	Inhibition Constant (Ki) (nM)
1	CAT	Tocotrienol	-9.18	29.55
2	GST	Tocotrienol	-8.69	67.40
3	GR	Tocotrienol	-10.33	4.25
4	SOD	Tocotrienol	-8.70	67.20
5	Gpx	Tocotrienol	-7.69	90.09

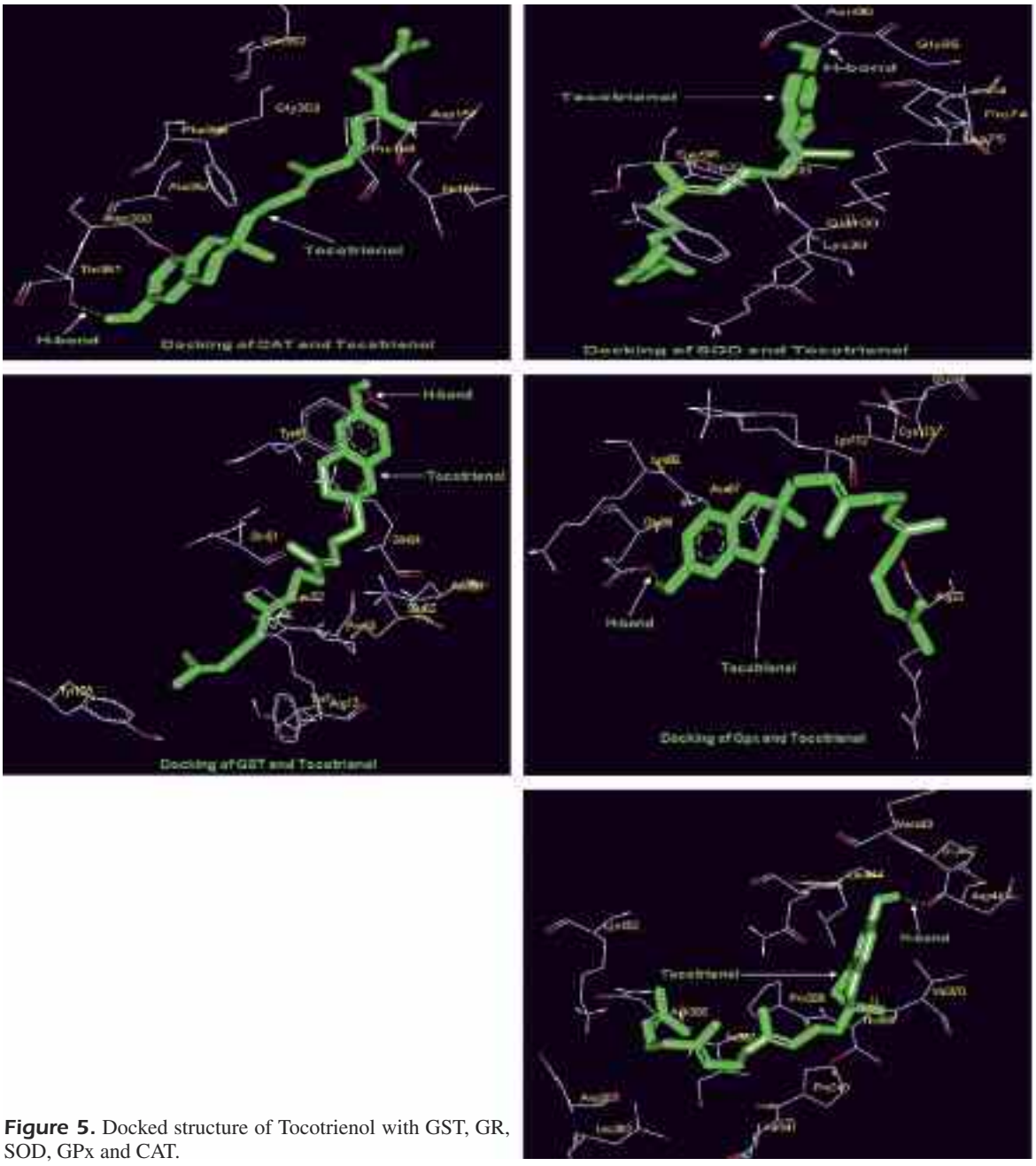

Figure 5. Docked structure of Tocotrienol with GST, GR, SOD, GPx and CAT.

these defense mechanisms. Normal cellular metabolism involves the production of ROS[42], low levels of ROS are vital for proper cell functioning, while excessive *in vivo* generation of these products can adversely affect cell functioning[42,43]. Malondialdehyde is one of the final products of lipid peroxidation in human cells, and an increase in ROS causes overproduction of MDA, which is considered a surrogate marker of oxidative stress[44,45]. Hepatic and renal conjugated diene, lipid hydroperoxide and MDA was significantly increased in hamsters after LPS, turpentine or zymosan administration. A similar increase in MDA content has been reported[9,10]. The increase in lipid peroxidation products is associated with a significant decline in total antioxidants capacity. The earlier suggests increased production of oxidants while the latter indicates diminished antioxidant defense. Both the changes indicate an existence of profound oxidative stress.

The major intracellular antioxidant enzyme, SOD, specifically converts superoxide radicals to hydrogen peroxide, and CAT as well as GPx detoxifies hydrogen peroxide to water[46]. Glutathione peroxidase protects against free radical injury by reducing the peroxide concentration via a glutathione dependent reduction process, thereby reducing the amount of peroxides available to produce cellular damage. Reduced glutathione is a major intracellular nonenzymatic antioxidant and has many biological functions, including maintenance of membrane protein and lipoprotein SH groups in the reduced form, the oxidation of which can otherwise cause altered cellular structure and function[47]. Therefore, as the balance between free radical production and antioxidant defenses is lost, the resultant oxidative stress through a series of events deregulates the cellular functions leading to various pathological conditions. An antioxidant compound might contribute partial or total alleviation of such damage. An impaired ROS scavenging function has been linked to the decreased activity of enzymatic and nonenzymatic scavengers of free radicals. Our results demonstrate a significantly lower CAT and SOD activities in erythrocytes from LPS, turpentine or zymosan treated hamsters. In addition, the activity of GR and intracellular GSH content in erythrocytes was also significantly reduced, while GPx activity was significantly increased. This decrease in GSH level during APR may be due to the combined effect of inhibited GR and reduced NADPH supply. Our results showing an increased erythrocytes GPx activity in stressed hamsters apparently further reduces the GSH content. In addition, the increased GPx activity represents a compensatory mechanism to degrade H_2O_2. These results are consistent with the findings of Yerer et al[48] showing a decrease in CAT activity and an increase in GPx activity in LPS treated rats. Dietary Tocomin given 10 days before the onset of infection and inflammation significantly improved the integrity of erythrocytes membrane as shown by improved protection as well as reversal of SOD, CAT, GPx, and GR including GSH content to near normal level.

As discussed above, oxidative stress causes impairment in enzymatic and nonenzymatic defense system that may lead to cellular damage because of their decreased capacity to remove the generated free radicals. In addition, changes in some conjugative enzymes, such as GST have been reported following endotoxin treatment that could alter an animal's response to drugs or other compounds that are metabolized by these enzymes. Glutathione-S-transferase is an important enzyme involved in conjugation reaction catalyzing the detoxification of a variety of endogenous and exogenous compounds. Our results show a significant decrease in the activities of antiperoxidative enzymes, CAT, SOD, GPx, GR and GST in liver and kidney during APR, induced by the administration of LPS, turpentine or zymosan to hamsters. In principle, the decreases observed in the activities of the above antioxidant enzymes in liver and kidney are consistent with several scattered reports, where only one of the three above APR inducers was used in rat, rabbit or mice model. A decrease in the hepatic SOD, CAT, GPx and GST activities in LPS treated rats has previously been reported[5-8]. In zymosan treated mice, a decrease in liver CAT activity was previously reported by Deitch et al[49], while a significant decrease in hepatic CAT, SOD and GPx activities in turpentine treated rabbits has been reported by Proulx and Du Souich[15]. A couple of reports showed a decrease in kidney SOD, CAT and GPx activities in LPS treated rats[9,10]. In addition, vitamin E treatment decreased lipid peroxidation, increased enzymatic antioxidant activities and also prevented the renal tissue damage in LPS treated rats[10].

Glutathione is found in relatively high concentrations in liver and is usually the most abundant intracellular thiol. Glutathione protects against oxidative damage in systems that scavenge radicals, eliminate lipid peroxidation products, preserve thiol-disulfide status of proteins, and repair oxidant damage[50]. In this study, the decrease in hepatic and kidney GSH levels in stressed hamsters may be due to the effect of inhibited GR activity and also apparently due to reduced supply of NADPH. In addition, since GSH also acts as substrate and cosubstrate in essential enzymatic reactions of GPx and GST, inhibition of their activities may also be due to decreased levels of GSH during APR in liver and kidney. Support to these results is obtained by the finding of Portoles et al[51] where endotoxin exposure to rats caused a significant decrease in total glutathione with significant increase in GSSG levels, an indicator of increased oxidative stress. Our results are consistent with other reports indicating a reduction in levels of hepatic GSH in LPS treated rats[6-8,52,54] and in turpentine treated rats[14] and rabbits[15]. Tocomin administration to hamsters, 10 days before the onset of infection and inflammation significantly blocked the oxidative stress, as

evidenced by a significant increase in hepatic and kidney SOD, CAT, GPx, GR and GST activities including GSH and MDA content and restored these levels close to corresponding normal control values. Restoration of overall antioxidant defense system by Tocomin may also be beneficial in preventing LPS, turpentine or zymosan induced oxidative hepatic and renal tissue damage.

Our results indicating strong antioxidant impacts of Tocomin in LPS, turpentine or zymosan stressed hamsters are in agreement with earlier findings[53] indicating an inhibition in the formation of conjugated dienes and MDA by Tocotrienol rich fraction (TRF) or individual tocotrienol and tocopherols when fed to rats along with an atherogenic diet. These results also indicate that γ-tocotrienol exerts a significantly more potent impact as compared to α-tocopherol. Support to our results is also obtained from another study[54] where feeding of a mixture of tocotrienol along with an atherogenic diet to rabbits was associated with a significant reduction in the formation of serum lipid peroxides. The antioxidant activity of the α-tocotrienol (T3) homologue has been shown to be more than 3-fold greater than that of α-tocopherol (T)[55]. Using an *in vitro* liposome system, antioxidant activities for several tocotrienol were 4- to 33-fold higher than that for α-T[56]. The possible mechanism for this superior efficacy of tocotrienol compared to tocopherols has been reported elsewhere[55].

As discussed above, the amelioration in antioxidant enzymes by tocotrienol was due to its potent antioxidant activity, but in order to explore that whether amelioration in these enzyme activity was due to direct interaction between tocotrienol and antioxidant enzymes or not, and if so to analyze how this interaction occur. Therefore, in response to our results, we investigated whether tocotrienol can directly bind to these enzymes and cause the observed increase in the activity of the enzyme. Our results illustrated that by using *in silico* docking studies, we can easily predict the probable ligand-binding site for tocotrienol with GR, CAT, SOD, GST and GPx. For the first time, docking results of tocotrienol showed significant interaction with GR, CAT, SOD, GST as well as GPx in terms of binding energy and inhibition constant in the following order: GR >CAT > SOD >GST >GPx (Table IV). Furthermore, the observed differences in binding pattern of tocotrienol towards different antioxidant enzymes have been explained through their different binding site occupancy as illusttrated in Figure 5. The docked structure of tocotrienol with CAT, GST, GR, SOD and GPx (Figure 5) in which Thr-361 residue of CAT, Tyr-49 of GST, Asp-441 of GR, Asn-86 of SOD and Glu-88 of GPx found to be involved in H-bond formation with tocotrienol indicating an high conformational stability. Our *in silico* results are consistent with *in vivo* results, illustrating that better interaction of tocotrienol with antioxidant enzymes may be responsible for increase in GR, CAT, GST and SOD activity which were also proved by *in vivo* data (Table II and III). Whereas GPx showed minimum binding energy *in silico* which is again consistent with our *in vivo* results, indicating an minute increase in enzymatic activity (Table III), which might be due to poorer binding of tocotrienol with GPx. Our results are in agreement with earlier reports where analogues of grape seed procyanidin extract, catechin and curcumin as ligands exhibit higher docking potential with Cu/Zn SOD, GST and GSH, respectively, and speculated that better interaction of these natural antioxidants with antioxidant enzymes can be responsible for increase in their enzymatic activity[57-59].

In conclusion, this study strongly suggests the protective effect of tocotrienol pretreatment on tissue defence system in infection and inflammation challenged hamsters. Our results indicate a potent antioxidant mechanism of tocotrienol, which was justified by *in vivo* study showing a significant amelioration in altered level of antioxidant defense system. We have also confirmed by docking simulation that the changes in GR, CAT, SOD, GST and GPx enzyme activity are attributable to direct interaction between tocotrienol and the enzyme, and we have found its probable binding site by *in silico* studies. However, further experiments will be necessary if the role of tocotrienol in the molecular mechanisms underlying this regulation is to be determined.

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