PROTECTIVE EFFECT OF *PHYLLANTHUS FRATERNUS* AND *HEMIDESMUS INDICUS* AGAINST ALLYL ALCOHOL INDUCED HEPATOTOXICITY

THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

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DECLARATION

I, R.Sailaja, declare that the work presented in my thesis has been carried out by me under the supervision of Prof. O.H.Setty, and has not been submitted for any degree or diploma of any other University.

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CERTIFICATE

This is to certify that R.Sailaja has carried out the research work embodied in the present thesis entitled "PROTECTIVE EFFECT OF *PHYLLANTHUS FRATERNUS* AND *HEMIDESMUS INDICUS* AGAINST ALLYL ALCOHOL INDUCED OXIDATIVE STRESS" under my supervision and guidance for the full period prescribed under the Ph.D ordinance of this University. I recommended her thesis for the submission for the degree of Doctor of Philosophy of this University.

Supervisor

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ABBREVIATIONS

AAT	Aspartate amino transferase
ADH	Alcohol dehydrogenase
ADP	Adenosine 5′- Di phosphate
AlAT	Alanine amino transferase
ALP	Alkaline phosphatase
ANS	1-Aniline-8-napthalene sulfonate
ATP	Adenosine 5′-Tri phosphate
BSA	Bovine serum albumin
CCCP	Carbonyl cyanide p-triflouro methoxy phenyl hydrazone
CDNB	1-Chloro 2,4-dinitro benzene
DMPO	5,5′-Dimethyl-1-pyrroline-N-oxide
DNPH	2,4-Dinitrophenyl hydrazine
DTNB	5,5′-Dithiobis-(2nitro benzoic acid)
EDTA	Ethylene diamine tetra acetic acid
FAD	Flavin adenine dinucleotide
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidised)
GST	Glutathione transferase
H_2O_2	Hydrogen peroxide
H_3PO_4	Orthophosphoricacid
HEPES	N-2-Hydroxy piperazine-N-2-ethane sulphonic acid
HNE	Hydroxy nonenal
KCl	Potassium chloride
LDH	Lactate dehydrogenase
LPO	Lipid peroxidation
MDA	Malondialdehyde
MDH	Malate dehydrogenase
MOPS	3-(N-Morpholino) propane sulphonic acid
NAD	Nicotinamide adenine di nucleotide
NADH	Nicotinamide adenine di nucleotide (reduced)
NADPH	Nicotinamide adenine di nucleotide (phosphate) (reduced)
NF	Nuclear factor
P/O	Phosphate to Oxygen ratio
PAGE	Polyacrylamide gel electrophoresis
PMSF	Pheny methane sulphonyl fluoride
PUFA	Polyunsaturated fattyacids
RCR	Respiratory control ratio
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SMP	Submitochondrial particles
SOD	Superoxide dismutase

TBA	Thiobarbituric acid
TBARS	Thio barbituric acid reactive substance
TBS	Tris buffer saline
TCA	Trichloroaceticacid
TRIS	Tris (hydroxy methyl) aminomethane 2-Amino-2-(hydroxyl methyl-Amino-2-(hydroxyl methyl) propane-1,3-diol

CONTENTS

INTRODUCTION

Plants and plant products are part of the vegetarian diet and a number of them exhibit medicinal properties. Several Indian plants are also being used in Ayurvedic and Siddha medicines. The medicinal properties of several herbal plants have been documented in ancient Indian literature and the preparations have been found to be effective in the treatment of diseases (Handa et al., 1996). The reports indicate that there is an inverse relationship between the dietary intake of antioxidant-rich foods and the incidence of human diseases (Sies, 1993; Halliwell, 1997). Hence search for new synthetic and natural antioxidants is essentially important. Although initial research on antioxidants was mostly on isolated pure compounds, recent focus is more on natural formulations (Hagerman et al., 1998; Haramaki and Packer, 1995). It has been found that compounds in their natural formulations are more active than their isolated form (Khopde et al., 2001).

Mitochondria are regarded as the power houses of the cell. They carry out the most important function, i.e., oxidative phosphorylation. Mitochondria in different cell types vary widely in size, shape and number. Each mitochondrion has an outer membrane that is freely permeable to large molecules and an inner membrane that is relatively impermeable and contains the respiratory chain. The inner compartment of the mitochondria, enclosed by the inner membrane, is the matrix in which Krebs cycle takes place. NADH and $FADH_2$ (that are generated from the Krebs cycle) act as electron donors to the electron transport chain. Proton extrusion across the inner mitochondrial membrane generates an electrochemical proton gradient, which drives ATP synthesis. Oxidative phosphorylation is the process by which the energy of oxidation is coupled to the synthesis of ATP.

The respiratory chain comprises of four enzyme complexes located on the inner mitochondrial membrane (Wallace, D.C., 1992).
Complex I [NADH: Ubiquinone oxido reductase]: It is the largest and contains atleast 45 polypeptides, seven of which are encoded by mitochondrial DNA. NAD linked substrates feed reducing equivalents into the chain via Complex I which passes electrons down the chain to ubiquinone.
Complex II [Succinate: Ubiquinone oxido reductase]: It consists of 5 polypeptides that are encoded by nuclear DNA. It accepts reducing equivalents from succinate and transfers to ubiquinone.
Complex III (Ubiquinone: cytochrome C reductase): It consists of 11 subunits with one subunit (cyt.b) encoded by mitochondrial DNA.
Complex IV (Cytochrome C oxidase): It consists of 13 polypeptides 3 of which are encoded by mitochondrial DNA.
ATP Synthase: It is composed of 12 subunits, two of which are encoded by mitochondrial DNA.

The gastrointestinal tract of higher animals and human species is the port of entry of variety of naturally occurring organic plant and animal poisons and a wide variety of chemicals in the form of drugs, pollutants and poisons (Mason et al., 1965). Before being distributed to the body through blood they are first directed into the liver, which plays a key role in the metabolism and elimination of several drugs. Liver cells are equipped with a active detoxification system called the mixed function oxidase which

metabolically alters a variety of xenobiotics and guards the organism against potentially harmful drugs. In chronic liver desease particularly in cirrhosis, hepatic drug metabolism may be altered by changes in hepatic blood flow or in the activity of drug metabolising enzymes resulting in modification of the intensity of therapeutic and toxic effects (Wilkinson and Shand, 1975).

The mitochondrial respiratory chain and free radicals:

Free radicals are the molecules having an unpaired electron in the outer orbit. They are highly unstable and very reactive. Examples are superoxide, hydroxyl, peroxyl, alkoxyl, hydroperoxyl, nitric oxide and nitrogen dioxide. Oxygen and nitrogen free radicals can be converted to other non-radical reactive species, such as hydrogen peroxide, hypochlorous acid, hypobromous acid and peroxynitrite. Hydrogen peroxide reacts with superoxide anions resulting in the formation of hydroxyl radical through the Haber-Weiss Fenton reaction which is highly reactive and would react with biomolecules like proteins, lipids and nucleic acids present in the vicinity (Winston and Cederbaum, 1983). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in animals and humans under physiologic and pathologic conditions (Evans and Halliwell, 2001).

Oxygen free radicals or, more generally, reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are products of normal cellular metabolism. ROS and RNS are well recognised for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial depending upon their extent of generation to living systems (Valko et al., 2006). ROS are produced as byproducts of cellular metabolism, primarily in mitochondria. Small, physiological amounts of ROS are of cellular requirement, because they are involved in signaling pathways such as inducing and regulating a variety of cellular activities, including cytokine secretion, growth, differentiation and gene expression (Halliwell and Gutteridge 1999, Hensley et al., 2000) and in the defense against invading pathogens. However ROS in excess have the potential to induce significant biological damage and hence cell possess many antioxidant systems to scavenge ROS. Under normal physiologic conditions there is a balance between formation and neutralization of ROS. Under conditions of oxidative stress ROS production is increased. ROS induce oxidative damage to all biomolecules like DNA, proteins, and lipids. Oxidative stress has been implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, other diseases and ageing (Dalle-Donne et al., 2006).

The mitochondrial electron transport chain is a source of superoxide. Under physiologic conditions, approximately 1-3% of oxygen consumed by body is converted to superoxide and other ROS. Thus although molecular oxygen is absolutely essential for aerobic life, it can be toxic under certain conditions. This phenomenon is termed as oxygen paradox (Gilbert, 2000). There is a link among mitochondrial metabolism rate and elimination of ROS and age related changes on mitochondrial function (Balaban *et al*., 2005). A superoxide radical (O_2^-) is generated when one electron is

transferred to oxygen. Superoxide radical has characteristics of both anion and radical. At 250 nm, superoxide radical reveals maximum absorption (approximately Є=2300) and the characteristic spectrum of electron spin resonance (ESR). Major sources of superoxide radical in the cells are NADPH oxidase, xathine oxidase, cytochrome P 450 systems, mitochondrial electron transport chain, and arachidonate metabolism. Superoxide is produced from both Complexes I and III of the electron transport chain. In its anionic form it is too strongly charged to readily cross the inner mitochondrial membrane. Recently, it has been demonstrated that Complex I-dependent superoxide is exclusively released into the matrix and that no detectable levels escape from intact mitochondria (Muller et al., 2004). This finding fits well with the proposed site of electron leak at Complex I, namely the iron–sulphur clusters of the (matrix-protruding) hydrophilic arm. In addition, experiments on Complex III show direct extramitochondrial release of superoxide, but measurements of hydrogen peroxide production revealed that this could only account for <50% of the total electron leak even in mitochondria lacking Cu, Zn-SOD. It has been proposed that the remaining 50% of the electron leak must be due to superoxide released to the matrix.

When free radicals and other reactive species (e.g., •OH, HOO•, $ONOO^-$) extract a hydrogen atom from an unsaturated fatty acyl chain, a carbon centered lipid radical (L•) is produced. This is followed by the addition of oxygen to L• to yield a lipid peroxyl radical (LOO•). LOO• further propagates the peroxidation chain reaction by abstracting a hydrogen atom from a nearby unsaturated fatty acid. The resulting lipid hydroperoxide (LOOH) can easily decompose to form a lipid alkoxyl radical (LO•). This series of ROS-initiated lipid peroxidation reactions with the production of lipid peroxyl and alkoxyl radicals, collectively called chain propagation, occurs in mammalian cells, such that oxygen free radicals may cause damage far in excess of their initial reaction products. Lipid peroxidation causes changes in the physical and chemical properties of membranes, thus altering their permeability and fluidity.

Oxidative stress is known to cause lipid peroxidation, DNA fragmentation, impaired cellular energy status, and disruption of ion homeostasis. Lipid peroxidation products like 4-hydroxy nonenol and malondialdehyde are highly reactive molecules that can react with α-amino group of proteins, RNA and DNA (Sorrell and Tuma., 1987). Lipid peroxidation has also been associated in a various ways with a number of normal and abnormal physiological processes. The normal process includes prostaglandin synthesis, phagocytosis and aging are abnormal conditions in which lipid peroxidation is implicated include haemolytic anemia, reproductive dysfunction, liver necrosis, muscle dystrophy, lung damage, atherosclerosis and testicular atrophy. Extensive lipid peroxidation in biological membranes causes impairment of membrane function, decrease fluidity, inactivation of membrane bound receptors and enzymes, cross linking with sulfydryl groups of enzymes etc. Thus uncontrolled lipid peroxidation mediates a variety of degenerative diseases leading to cell death.

Many different types of protein oxidative modification can be induced directly by ROS or indirectly by reactions of secondary by products of

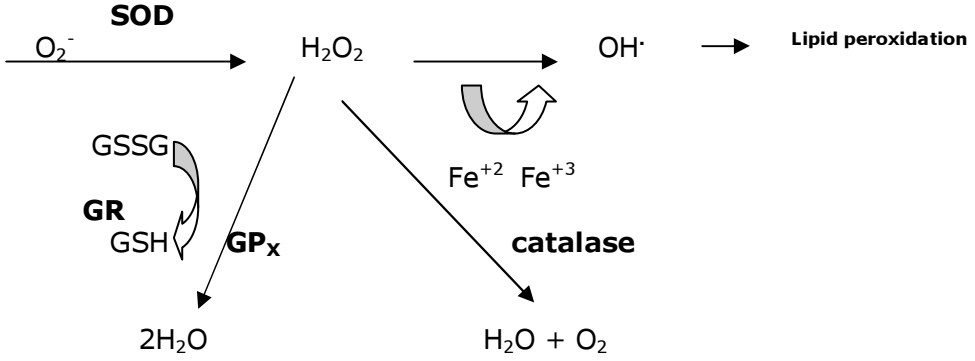
oxidative stress (Berlett and Stadtman, 1997). Cysteine and methionine are particularly prone to oxidative attack by almost all ROS. Protein modifications elicited by direct oxidative attack on Lys, Arg, Pro, Thr, or by secondary reaction of Cys, His or Lys residues with reactive carbonyl compounds, can lead to the formation of protein carbonyl derivatives (aldehydes and ketones). ROS can cause oxidation of amino acid residue side chains, formation of protein-protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation. Thus protein carbonyl content is the most general indicator and is the most commonly used marker of protein oxidation (Berlett and Stadtman, 1997; Shacter, 2000; Beal, 2002).

Modifications of the respiratory chain Complexes (I–IV) by nitration, carbonylation and HNE formation decrease their enzymatic activity in vitro. The electron leakage from respiratory chain complexes and subsequent ROS formation may cause damage to any specific subunit and contribute to a long-term mitochondrial dysfunction (Choksi et al., 2004).

Mammalian cells possess many defense mechanisms to detoxify free radicals through enzymatic and non-enzymatic reactions (Fig A). The key metabolic steps are the catalysis of superoxide to hydrogen peroxide and oxygen by superoxide dismutase, conversion of H_2O_2 to H_2O by glutathione peroxidase or to O_2 and H_2O by catalase. Since the reaction catalysed by glutathione peroxidase requires GSH as substrate and depends in part on GSSG to GSH ratio, which depends on the redox state of the cell. Radical scavenging antioxidants such as Vitamin E interrupt the lipid peroxidation reaction by transferring its phenolic hydrogen to peroxyl free radical of PUFA. Vitamin C reacts with Vitamin E radical to yield a vitamin C radical and regenerates vitamin E. Like vitamin E radical vitamin C radical is not a reactive species because its unpaired electron is energetically stable. The vitamin C radical is then converted to vitamin C by GSH which is oxidized to GSSG. This is then reduced to GSH by the NADPH dependent glutathione reductase.

Also there are proteins like haemoglobin, transferin and ceruloplasmin that bind ferrous and copper ions and prevent radical generation through Fenton reaction, and proteases, ribonucleases and lipases that preferentially degrade the modified components of protein, DNA and lipids respectively. In addition vitamins, carotenoids, flavinoids can act as chain-breaking antioxidants (Slater et al., 1975).

Fig A: Antioxidant enzymes



One of the most prominent defense systems in the liver is the presence of glutathione (GSH) which is a tripeptide found in high concentrations in all cells. It is synthesized from glutamate, cysteine, and glycine in the cytosol. Due to the presence of cysteine in its backbone, GSH is key in the regulation of disulfide bonds of proteins and in the disposal of electrophiles and oxidants (DeLeve and Kaplowitz, 1991; Hammond et al., 2001).

Glutathione (GSH), the major intracellular non protein thiol, is mainly known as a nucleophilic scavenger and an enzyme-catalyzed antioxidant in electrophilic/oxidative tissue injury. GSH plays an important role in the maintenance of the intracellular redox state. The intracellular level of GSH, which differ from one cell type to another, may be crucial for ROS-induced NF-κB response (Nanxin and Michael, 1999). The extracellular GSH catabolism may be involved in the modulation of cell signalling and activation of transcription factors.

NF-κB is a ubiquitous transcription factor that regulates inflammatory mediators, and several structural proteins that are involved in infection, inflammation, stress responses and apoptosis (Baeuerle and Henkel, 1994). The exact and complex molecular mechanisms involved in the regulation of NF- κB remain to be elucidated (Nanxin and Michael, 1999). Reactive oxygen species have been implicated as second messengers involved in the activation of NF- κB *via* tumour necrosis factor (TNF) and interleukin-1 (Poli et al., 2004 and Valko et al., 2006). H_2O_2 possess several properties that make it ideal for to act as second messenger. They are, of small size and therefore diffuse rapidly through biological membranes, and their synthesis and degradation are fast (Baeuerle et al., 1996).

Superoxide dismutases constitute the only mammalian antioxidant enzymes converting superoxide to H_2O_2. There are 3 types of mammalian SODs i.e., copper–zinc SOD (CuZnSOD), manganese SOD (MnSOD), and extracellular SOD (ECSOD). CuZn-SOD is mainly a cytosolic enzyme, but it has also been detected in cellular organelles. The distribution of CuZnSOD positions it to be the primary enzyme protecting cells against cytosolic-generated superoxide. MnSOD is synthesized in the cytosol as a precursor molecule and is transported to the mitochondria [Matsuda et al., 1990). Being mitochondrial, probably it has an important role in the oxidant resistance and apoptosis of rapidly growing cancer cells. ECSOD is synthesized in the cytosol and has a secretory leader sequence and four heparin-binding domains (one per each of four subunits) that contribute to the binding of ECSOD to extracellular matrix proteins (Marklund et al., 1982; Oury et al., 1994).

MnSOD is essential to the vitality of mammalian cells. MnSOD is a homotetramer, which contains one manganese ion per subunit (Weisiger and Fridovich, 1973). The MnSOD gene is located in chromosome 6q25. Total knockout of the MnSOD gene is perinatally lethal, leading to neurological manifestations and cardiotoxicity (Lebovitz et al., 1996; Tsan, 2001). Heterozygous mice with lowered MnSOD activity have increased mitochondrial oxidative damage [Tsan, 2001; Williams et al., 1998]. The familial form of amyotrophic lateral sclerosis, or FALS, has been shown to

be associated with defects in the gene encoding Cu,Zn-SOD (*sod* 1) (Rosen, D. R. *et al.,* 1993; Deng et al., 1993; Siddique, 1990).

CuZnSOD, especially genetic variations thereof,been linked to degenerative neuronal diseases. CuZnSOD is a homodimer with a molecular weight of 32 kDa and its gene is located in chromosome 21q22 (McCord and Fridovich, 1969; Sherman et al., 1984). CuZn-SOD knockout animals are viable (Ho et al., 1998), but fibroblasts derived from those animals proliferate more slowly (75%) than control cells and their resistance to paraquat toxicity is decreased (Huang et al., 1997), emphasizing the importance of CuZn-SOD in cell growth and survival. In mammals the highest levels have been detected in the liver, kidney, erythrocytes, and central nervous system.

Gluthathion peroxidase (GSH-Px, EC 1.11.1.9) is a selenoprotein including selenocystein residue in the active site. Three types of GSH-Px have been confirmed. Cellular glutathione peroxidase is a tetrameric protein, and is composed of four subunits containing one atom of Se. Plasma glutathione peroxidase exhibits a tetrameric as well as cellular form. A third glutathione peroxidase, namely phospholipid hydroperoxide glutathione peroxidase (PHGSH-Px), is a monomeric membrane-associated enzyme. These enzymes catalyze the reduction of H_2O_2 and a variety of organic hydroperoxides (ROOH). The decomposition efficiency of the substrates of glutathione peroxidase depends on the concentration of the reduced glutathione as a coenzyme. It is suggested that the physiological roles of GSHPx include reduction of H_2O_2 and prevention of lipid peroxidation as well as catalase (Little, 1972).

Catalase (EC 1.11.6.1) is a tetrameric hemeprotein, and is mainly confirmed in peroxysome. It is ubiquitously distributed in animal and plant cells and catalyzes the reduction of various substrates such as H_2O_2, methylperoxide (CH_3OOH), and ethylperoxide (C_2H_5OOH). Under optimum condition (Km = 1.1 M), this enzyme activity is higher than GSHPx. Patients with acatalasemia, a rare congenital disorder with abnormalities in erythrocyte catalase activity and lowered catalase levels in other tissues, appear to live a fairly normal life (Ogata, 1991).

Allyl alcohol:

Commercial name	Allylalcohol/vinyl carbinol/2-Propen-1-ol
Molecular formula	$C_3H_6O/CH_2=CHCH_2OH$
Molecular weight	58.1 gms
Physical state, appearance	Colour liquid with pungent odour

Allyl alcohol is a toxic, colorless liquid. It is an extremely hazardous substance. It is mainly used as herbicide and is used in the manufacture of food flavorings, allyl compounds, war gas, resins, plasticizers and fire retardants, allyl esters. It is an intermediate for pharmaceuticals, other organic chemicals and military poison.

Short-term (acute) effects

Severe irritation and burns of the eyes, nose and skin. Inhalation of allyl alcohol causes a build-up of fluid in the lungs (pulmonary edema), causing a severe shortness of breath and death if not treated. Ingesting allyl alcohol can cause abdominal pain, nausea, vomiting, diarrhea and/or liver damage, headache, dizziness, weakness and loss of consciousness, coma, cardiovascular failure and death.

Long-term (chronic) effects

Liver or kidney damage, depending on the route of exposure. Inhalation of allyl alcohol causes respiratory conditions such as asthma, bronchitis or emphysema, coughing, shortness of breath and lung irritation and/or damage.

Allyl alcohol (AA) is a chemical used in manufacturing processes in food flavoring and in agriculture as a weed killer (Beauchamp et al., 1985; Atzori et al., 1989). It irritates mucous membranes and is especially harmful in the liver, producing cell necrosis selectively in the periportal zone. It is metabolized by cytosolic alcohol dehydrogenase to acrolein, an unsaturated aldehyde (Rees and Tarlow, 1967). Acrolein is a powerful electrophile and reacts with nucleophiles, such as sulfhydryl groups. The thiol group of glutathione is a favored target and so glutathione is primarily involved in the reaction which leads to GSH depletion. The reaction is markedly accelerated by the activity of cytosolic glutathione S-transferase. The nucleophilic glutathione is an important protective factor of hepatic cells in the detoxification of acrolein.

The effect of acute exposure of rats to allyl alcohol (0.05 ml/kg, ip) on the activity of enzymes of hepatic phase I (cytochrome p450-linked microsomal monooxygenases, epoxide hydrolase) and phase II (glucuronyl-, glutathione-, acetyl- and sulfo transferases) biotransformation were studied in rats. Allyl alcohol reduced hepatic cytochrome p450 in liver, and the activities of ethylmorphine demethylase, benzphetamine demethylase, benzo[a]pyrene hydroxylase, and ethoxyresorufin deethylase. No significant decreases in epoxide hydrolase or glucuronyltransferase activities were observed. The activities of cytosolic conjugating enzymes (glutathione, sulfo- and acetyl transferases) also were minimally affected by toxic liver injury (Gregus et al., 1982).

Acute Exposure of allyl alcohol administered to Fischer 344 rats produce cell specific injury in centrilobular hepatocytes, periportal hepatocytes, and bile duct cells, respectively. Allyl alcohol administration increased serum alanine aminotransferase activity but had no effect on serum gamma-glutamyl transferase activity (Leonard TB et al., 1984). Acute Exposure 0.05 ml/kg of allyl alcohol administered orally to mice caused depletion of hepatic glutathione (Sieger's et al., 1977).

It has been suggested that the alkylation of nucleophilic groups of cellular macromolecules affected by acrolein after glutathione depletion is the event actually leading to cell injury (Ohno et al., 1985). However other

reports have suggested that a major role in allyl alcohol induced hepatotoxicity play lipid peroxidation (Miccadei et al., 1988; Pompella et al., 1991). Acrolein depletes glutathione content of the hepatocytes there by sensitizing the cells to the constitutive flux of active oxygen species (Miccadei et al., 1988).

Acrolein is a highly toxic aldehyde involved in a number of diseases as well as drug-induced toxicities. Formed during the combustion of organic matter, it is implicated in the pathogenesis of smoke inhalation injury to the lung (Hales et al., 1988). Acrolein also forms via hepatic biotransformation of the chemotherapeutic drugs like cyclophosphamide and Ifosfamide (Ludeman, 1999). In addition, there is growing recognition that endogenous acrolein, formed via lipid peroxidation, mediates cell damage in various diseases of old age, including Alzheimer's disease (Uchida et al., 1998a; Uchida, 1999; Lovell and Markesbery, 2001). Acrolein's pronounced toxicity reflects its reactivity as a bifunctional electrophile, ensuring that it readily attacks electron dense-centres in DNA and protein (Esterbauer et al., 1991). This reactivity underlies most of the cellular effects of acrolein, including alterations in the activity of transcription factors such as AP-1, nuclear factor κB, and Nrf2 (Horton et al., 1999; Biswal et al., 2002; Tirumalai et al., 2002); inhibition of cytokine production (Li et al., 1997); and cell death (Li et al., 1997; Kern and Kehrer, 2002).

Glutathione S-transferases (GSTs):

The glutathione-s-transferases (GSTs, EC 2.5.1.18) are an integral part of phase I (oxidation)/ phase II (conjugation) system, multifunctional proteins involved in the detoxication of reactive electrophilic compounds from exogenous (xenobiotics and their metabolites) or endogenous origin (organic peroxides, product of oxidative stress) in aerobic organisms (Jakoby, 1978).

Structural multiplicity of GST:

Although GSTs are distributed ubiquitously, they have been studied most extensively in mammalian livers, mainly because of their exceptional abundance there. In the rat liver, the GSTs constitutes up to 10 % of the total extractable proteins whereas they represent approximately 3% in the rat brain and human liver (Jakoby, 1978).
So far, seven different classes of cytosolic isozymes of GSTs are identified i.e., alpha, Mu, Pi, Theta, Sigma, Kappa and Zeta (Coggan et al., 1998; Hayes and Pulford, 1995; Mannervik et al., 1992; Board et al., 1997) and three different forms of Microsomal GSTs (Andeersson et al.,1994; Jackobsson et al., 1996). They are active as homo- or hetero- dimers containing subunit belonging to the same class (Carne et al., 1979), with the exception of microsomal GSTs that exists as homotrimer ranging from 17 to 28 kDa. Each subunit consists of two binding sites designated as G-site, for GSH binding at N-terminus, and an H-site for hydrophobic or electrophilic substances binding at the C-terminus of the protein (Armstrong, 1997). The binding domain of GSTs to GSH is exploited for the purification of GSTs employing a GSH-affinity column.

Armstrong in 1994 proposed that binding of GSH to G-site results in formation of thiolate anion (GS-) that lowers the pKa of GSH from 9.0 in aqueous solution to 6.5 in bound state. Binding of GSH to G-site is primarily facilitated by conserved tyrosine residue that stabilizes thiolate anion (GS-) by hydrogen binding.

Catalytic and Non-catalytic functions of GSTs:

Various actions of GST can be classified into two categories: (1) catalytic functions and (2) binding, transport and storage functions. The common feature of all GSTs is their ability to increase the nucleophilicity of the sulfhydryl group of GSH, which participate in various reactions. The well known catalytic functions of GST are conjugation, peroxide reduction and isomerisation.

Catalytic Functions:

1. Conjugation:

All GSTs conjugate the sulfhydryl group of GSH to the electrophilic centers of second substrates, forming a GSH thioether product. This reaction is the first step of the meracapturic acid pathway and has been reviewed extensively (Chasseaud, 1979; Jakoby and Habig, 1980; Smith et al., 1977). The second substrates include a wide variety of xenobiotics, most of which are synthetic chemical such as halonitrobenzene, or endogenously generated electrophilic intermediates, such as arene oxides, a phase one biotransformation product. The thioether product itself is excreted directly into the bile, and finally into the faeces after further metabolism in the gut (Chasseaud, 1976; Neilsen and Rasmussen, 1977). The conjugates that reach the kidney are further converted to mercapturic acid and then excreted into the urine (Kozak and Tate, 1982; Tateishi and Shinizu, 1980; Thompson and Meister, 1977). The purpose of the mercapturic acid formation appears to be inactivation of the potentially toxic electrophilic center of the substrate molecule and, at the same time, formation of a more hydrophilic conjugate for excretion. Therefore GSTs serve a key role in preventing the interaction of highly reactive chemical compounds and activated carcinogens with macromolecules such as proteins or nucleic acids (Chasseaud, 1979).

2. Oxidation-reduction:

In 1976 it was reported that some GSTs catalyze GSH-dependent reduction of organic hydroperoxides (Lawrence and Burk, 1976; Prohaska and Ganther, 1976). This glutathione peroxidise activity is referred to as glutathione peroxidise II to distinguish it from glutathione peroxidise I (EC.1.11.1.9), which was originally reported in 1957 to be tetrameric protein containing four atoms of selenium (Se) per 84,000 dalton molecular weight mass (Mills, 1957). The major biochemical difference between GSHPX I and GSHPX II and the basis for their analytical distinction is the ability of Se-GSH-PX to catalyze the reduction of both organic hydro peroxides and hydrogen peroxide, whereas the GSTs with GSHPX II activity, also referred to as non Se-GSH-Px, can only catalyze the reduction of organic hydroperoxides (Lawrence and Burk, 1976). In the selenium deficient rats there is an increased GPX II activity. The GSHPX II

catalyses the conversion of organic peroxides to corresponding alcohols. This type of reaction is thought to represent nucleophilic attack by GSH on electrophilic oxygen. It is believed to involve two steps, one of which is catalytic and proceeds via the formation of sulfenic acid of glutathione. The substrates that GSTs reduce include fatty acids phospholipids, and DNA hydroperoxides. As these compounds are generated by lipid peroxidation and oxidative damage to DNA, it is proposed that GSTs as well as other GSH-dependent enzymes help to combat oxidative stress. Detoxification of lipid peroxides by microsomal GSTs can occur *in situ* whereas detoxification by cytosolic GSTs requires prior release of FA hydroperoxides by phospholipase A_2.

3. Isomerization:

The GSTs were also discovered to have isomerase activity (Benson et al., 1977). The reaction can be carried out with two physiologically important substrates, Δ^5- androstene-3, 17-dione and maleyl acetone (Benson et al., 1977; Keen and Jakoby 1978). A transient GSH adduct is presumed to be formed in these reactions, which rearranges to GSH and the more stable isomer of the organic substrates, GSH is, therefore not consumed.

Non-catalytic functions

Ligand binding properties:

Many GST enzymes exhibit a ligand binding functions, which involves the non-covalent binding of hydrophobic ligands such as heme, bilirubin, various steroids, and conceivably some lipophilic anticancer drugs that are not the usual substrates (Bhargava et al., 1980; Homma et al., 1985). GST-mediated ligand bindings are often associated with the inhibition of GST activity by the bound ligand and appear to facilitate the intracellular transport of these lipophilic compounds (Boyer and Kenny, 1985). For example, binding of thyroid hormone to GST controls their intracellular transfer to receptors and components involved in their metabolism. It was shown previously that bile acids are capable of binding to certain forms of GSTs with affinity constants in the order of 10^{-4}-10^{-5} M^{-1}. Bile acid binding was found to inhibit 50% of cytosolic GST activity (Vessey and Zakim, 1981).

Induction of toxicity by GST

GST binding to foreign compounds does not always result in detoxification. For, example, a few GSH conjugates are relatively unstable and the reaction product is either cleaved to liberate an unconjugate metabolite that requires further detoxification (Thiolysis), or the reaction is reversible- allowing regeneration of the original electrophile (Reversible conjugation).

Thiolysis

This occurs with certain ethers, esters and organic phosphates when conjugation leads to cleavage of the substrate with only one of the two products being conjugated. In case of p-nitrophenol acetate, the herbicide fluorodifen and insecticide EPN results in the release of p-nitrophenol; presumably, the p-nitrophenol is metabolized by UDP-glucuronosyl transferase and phenol sulfotransferase. Thiolysis represent incomplete

detoxification because the conjugated cleavage product still provides threat to the cell.

Reversible conjugation

This occurs with certain cytotoxic isothiocyanate. Following reaction of benzyl, allyl, phenethyl isothiocyanate with GSH, their respective conjugates are not stable and yield the parental thiocyanate in mildly acidic media. In the case of benzyl and phenethyl isothiocyanate, GST can catalyze both forward and backward reactions but at high concentrations, the equilibrium is shifted in favour of formation of the GSH conjugates. The reversibility of this reaction means conjugates may not represent detoxification products rather, temporary storage or transport forms.

GSTs exist in multiple forms in rat liver (Habig et al., 1974; Mannervik, 1985). The major cytosolic forms consist of homo or hetero-dimers of the subunits Ya, Yc, Yb_l and Yb_2 (Hayes & Mantle, 1986). These subunits have also been named with the arabic numerals 1, 2, 3 and 4 respectively, (Mannervik, 1985). Heterodimers can only be formed between subunits that have extensive sequence homology, and the subunits that can hybridize are thought to arise from the same gene family (Ketterer et al., 1983; Hayes, 1984). The major GST isoenzymes are GST YaYa, GST YaYc, GST YcYc, GST Yb_lYb_l, GST Yb_lYb_2 and GST Yb_2Yb_2 (Hayes, 1983). The GSTs have differing non-Se-GSHpx activities, dependent upon the activity of their individual subunits, with the Ya and Yc subunits having the highest activity (Meyer et al., 1985; Mannervik, 1985). Ya and Yc catalyse the reduction of hydro peroxides, isomerisation of prostaglandin. Yb subunit involved in the formation of leukotrienes.

Medicinal plants:

Phyllanthus fraternus:

Family:
Euphorbiaceae

Habitat:
Annual herb, the stem is non-erect and 30cm tall, leafy shoot is 5-10cm long, oblong and joined to the brachlets of the stem, six sepals in the flower, distributed in India, pakisthan and introduced into Saudi Arabia, Africa and West indies (Abedin et al., 2001). It is widely distributed in the northern region of India.

Phyllanthus fraternus

Properties and uses ascribed to *P.fraternus* in traditional medicine:

The plant is bitter in taste, astringent, stomachic, diuretic and antiseptic. It is used in gastric complaints including dyspepsia, colic, diarrohea and dysentery; also employed in dropsy and diseases of urinogenital system. The plant is also said to be useful in diabetes. A decoction of the leaves is used as a refrigerant for scalp; leaves and roots are made into poultice with rice water for application on oedematous swellings and ulcers. The latex is also applied to offensive sores and ulcers, mixed with oil, it is used in opthalmia. The fresh leaves are also considered as a remedy for jaundice.

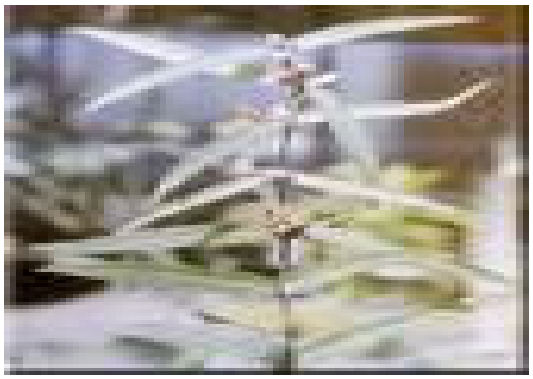
Chemical studies of the plant have reported that the leaves of *P.fraternus* contain a number of lignans and alkaloids. Phyllanthin (a bitter constituent) and hypophyllanthin (a non-bitter compound) were isolated from the leaves of *P.fraternus* and identified as lignans. Phyllanthin was found to be (+) 3, 4, 3′, 4, 9, 9′ – hexamethoxy-8, 8′– buttyrolignan with absolute (8s, 8′s) configuration (Row et al., 1964). The hexane extract of the leaves gave three additional lignans viz. niranthin, nirtetralin and phyltetralin (anjaneyulu et al, 1973). The distribution of the lignans in the leaves varied considerably with geographic location of the plant (anjaneyulu et al, 1973). The arial parts of the *P.fraternus* yielded four alkaloids, 4-methoxy securinine (phyllanthine) and 4-methoxy-norsecurinine. The ethyl acetate portion of the water soluble fraction of the ethanolic extract of the *P.fraternus* roots yielded 2 new glycoflavones, which are charecterised as 3,5,7- trihydroxy flavonal- 4′o-α-L-rhamnopyranoside (Chauhan et al., 1977). Another new compound viz., lintetralin was also isolated from *P.fraternus* (Ward et al., 1979).

Pharmacological studies on *P.fraternus*:

The aqueous extract of *P.fraternus* leaves was reported to produce hypoglycaemic action in normal as well as alloxan-diabetic rabbits. The extract lowered the blood sugar even when it was administered one hour after glucose administration. The hypoglyceamic activity of the leaf extract appeared to be higher than that of tolbutamide (Ramakrishnan et al., 1982). Petrol extract of *P.fraternus* (whole plant) showed antifungal activity against *Helminthosporium sativum* (Bhatnagar et al., 1961). An aqueous extract of the plant inhibits DNA polymerase of wood chuck hepatitis virus (WHV) and binds to the surface antigen of WHV in vitro (Venkateswaran et al., 1987). *P.fraternus* has been shown to be effective as an adjunct with other siddha drugs in the treatment of jaundice due to infective hepatitis (Ramanan and Sainani, 1961). It was reported earlier from our laboratory that mitochondrial dysfunction caused by the administration of alcohol (Sebastian and Setty, 1999) or thioacetamide (Padma and Setty, 1997) or carbon tetra chloride (Padma and Setty, 1999) could be prevented by prior administration of aqueous extract of *P. fraternus*.

***Hemidesmus indicus* (Linn.) R.Br.**

Family:
Asclepiadaceae



Hemidesmus indicus

Roots

Habitat*:*
Throughout India

Classical and Common Names*:*

Ayurvedic*:*
Saarivaa (White variety), Ananatmuula, Gopavalli, Utpalsaarivaa, Kapuuri. Krishna Saarivaa (black variety), Jambuupatraasaarivaa Ayurvedic Formulary of India accepts *Hemidesmus indicus* and *Cryptolepis buchananii Roem and Schytt* as the white and black varieties of Saarivaa**.**

Siddha:
Ninnari

English*:*
Indian Sarsaparilla

Telugu*:*
Sugandhipala, muttapulgam

Parts Used*:*
Root

Properties and Uses*:*

The drug is sweet, demulscent, diaphoretic, diuretic and tonic and is useful in the loss of appetite, fever, skin diseases, diarrhoea, and nutritional disorders and is a blood purifier.

The root is included in the Indian pharmacopoeia (1966) and the Indian pharmaceutical vodex (1955). It is sweet, demulcent, alternative, diaphoretic, diuretic, tonic and is useful in the loss of appetite, fever, skin diseases, diarrhoea, and is a blood purifier. It forms an important ingredient of some Ayurvedic preparations such as *Aswagandhadi churnam*, *Aswagandhadi leham*, *chandanasava* and others.

The plant has been mentioned in Indigenous System of Medicine as *Mutravirecham, Balya, Raktashodhak* (blood purifier), *Dahaprashaman* (soothes burning sensation) and useful in treatment of *jwar* (fever) *kushtha, pradar, agnimandya, prameha* and others*.*

The methanolic extract of *Hemidesmus indicus* roots was found to inhibit lipid peroxidation and scavenge hydroxyl and superoxide radicals in vitro. The amount required for 50 % inhibition of lipid peroxide formation was 217.5 µg / ml. The concentrations needed to scavenge hydroxyl and superoxide radicals were 73.5 and 287.5 µg / ml, respectively (Mary et al., 2003).

Active Principles and Pharmacology*:*

- Two novel pregnane glycosides, denicunine and heminine have been isolated from the dried stem of *Hemidesmus indicus.* Twigs of the plant gave a pregnane ester diglycoside named desinine.

- Roots gave β-sitosterol, 2-hydroxy-4-methoxy-benzaldehyde, α-amyrin, β-amyrin and its acetate, hexatriacontane, lupeol actacosonoate, lupeol and its acetate.

- Leaves, stem and root cultures produce cholesterol, campesterol, β-sitosterol and 16-dehydropregnenolone.

- Leaves and flowers also gave flavonoid glycoside: rutin, hyperoside and iso-quercitrin.

- The essential oil (2-hydroxy-4-methoxy-benzaldehyde) isolated from the plant possesses anti bacterial property against Gram-positive and Gram-negative bacteria.

- The aqueous ethanolic extract of the whole plant showed anti-viral activity against Ranikhet disease virus**.**

- A saponin from the drug was found to possess an anti-inflammatory activity in experimental animals. The fresh decoction of roots was found to possess a blood-purifying property.

Use in Western Herbal*:*

Hemidesmus indicus has been successfully used in the cure of venereal disease. The drug is used as an infusion, as boiling dissipates its volatile principle, for rheumatism, skin diseases and thrush. Also used in nephritic complaints and for sore mouth in children.

Sarsaparilla is used as an anti-inflammatory and cleansing agent, which can bring relief to skin problems such as eczema, psoriasis and general itchiness, and helps in rheumatism and gout treatment. It has a tonic and specifically testosterogenic action on the body leading to increased muscle bulk and has a potential use for impotence.

OBJECTIVES

OBJECTIVES OF THE STUDY

The main objective of this study is to find out the protective effect of prior administration of the aqueous extract of *Phyllanthus fraternus* and *Hemidesmus indicus* against the allyl alcohol induced oxidative stress. The study includes:

1. To develop oxidative stress by the administration of allyl alcohol in rat model to suit to our kind of experiments.

2. To establish a protective effect of prior administration of the aqueous extract of *Phyllanthus fraternus* and *Hemidesmus indicus* against the allyl alcohol induced oxidative stress.

3. To find out the mechanism by which these plant extracts exert protection.

The following parameters are used to follow the extent of oxidative stress and answer this question.

a) Measurement of membrane integrity (measured by rate of respiration, RCR and P/O) and membrane potential.

b) Liver damage measured by transaminases and from histological studies.

c) Measurement of free radical damage to like lipid peroxidation and protein oxidation.

d) Measurement of activities of antioxidant enzymes like catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase.

e) Measurement of body's important antioxidant glutathione (both reduced and oxidised form).

f) Activity and purification of glutathione transferases.

g) Measurement of superoxide radical using spin trap by ESR.

MATERIALS AND METHODS

Animals:

All the experiments were performed in colony bred rats, derived from Wistar strain, raised in animal house facility of University of Hyderabad. Rats weighing about 120±20 gms were used for the present study. They were given food (balanced pellet food supplied by Hindustan lever Ltd., India) and water *ad Libitium*. The weight of the rats was monitored for atleast 4 days before starting the experiment.

Chemicals:

Adenosine di phosphate (ADP), L-glutamic acid, L-Malic acid, Succinic acid, 1- anilino-8-napthalene sulfonate (ANS), Lactate dehydrogenase (LDH),Malate dehydrogenase (MDH),Nicotinamide adenine di nucleotide (reduced) (NADH),Carbonyl cyanide p-triflouro methoxy phenyl hydrazone (CCCP), Thiobarbituric acid (TBA), 2,4-dinitrophenyl hydrazine (DNPH), 5,5′-dithiobis-(2nitro benzoic acid) [DTNB], epinephrine, antimycin, epoxy activated sepharose 6B, reduced glutathione (GSH), glutathione reductase, Nicotinamide adenine di nucleotide phosphate (NADPH), o-phthalaldehyde, N-ethyl maleimide, Xanthine, Xanthine oxidase, 5,5′-Dimethyl-1-pyrroline-N-oxide (DMPO) were obtained from Sigma chemical company. Goat anti-rabbit IgG ALP conjugate secondary antibody, BCIP-NBT substrate for alkaline phosphatase, protein molecular weight marker were obtained from Bangalore genei. All other chemicals were obtained from standard commercial sources in India and were of analytical grade.

Glassware and solutions:

All the glassware used for the experiments were routinely cleaned by immersion in a hot chromic acid bath. They were thoroughly washed with tap water, rinsed with single and then with double distilled water. Ultrapure water was used for purification of GST and for ESR experiments.

Preparation of Plant Extracts:

Preparation of aqueous extract of *Phyllanthus fraternus*:

The whole plant of *P. fraternus* was collected from the fields during winter season, then cleaned with distilled water and air dried. The air dried whole plant including roots was homogenized at room temperature in water (5 gm/12.5 ml) using motor and pestle. The homogenate was then filtered through a cheese cloth. The dry weight of the extract was determined by gravimetric method by drying the extract in an oven. The water extract equivalent to 100 mg/kg dry weight was administered daily to each rat.

Preparation of the aqueous extract of the roots of *Hemidesmus indicus*:

Roots of *Hemidesmus indicus* are used for the aqueous extract preparation. The roots were cut into small pieces and aqueous extract prepared by boiling 30g of cut roots in 300 ml of deionised water. The extract was then filtered using cheese-cloth or a filter paper. The dry weight of the extract

was determined by gravimetric method by drying the extract in an oven. The aqueous extract equivalent to 100 mg/kg/day was administered to rats.

Treatment Schedule of Rats:

Experiments using *Phyllanthus fraternus:*

The animals were divided into four groups of four rats in each group.
Group A: Control rats which received 0.5 ml of saline intraperitoneally.
Group B: Received allyl alcohol 4-5 mg in 0.5 ml of saline (0.7 mmol per kg body weight) intraperitoneally and sacrificed after 4 h.
Group C: Received orally aqueous extract of *P.fraternus* (equivalent to 100 mg/kg) for a period of 4 days and then 0.5 ml of saline was given intraperitoneally and sacrificed after 4 h.
Group D: Received orally aqueous extract of *P.fraternus* (equivalent to 100 mg/kg) for 4 days and then allyl alcohol 4-5 mg in 0.5 ml volume of saline (0.7 mmol per kg body weight) was given and sacrificed after 4 h.
All the rats were fasted for 17h before and 4h after allyl alcohol administration.

Experiments using *Hemidesmus indicus:*

The animals were divided into four groups of four rats in each group.
Group A: Control rats which received 0.5 ml of saline intraperitoneally.
Group B: Received allyl alcohol 4-5 mg in volume of 0.5 ml in saline (0.7 mmol per kg body weight) intraperitoneally and sacrificed after 4 h.
Group C: Received orally aqueous extract of *Hemidesmus indicus* (equivalent to 100 mg/kg) for a period of 8 days and then 0.5 ml of saline was given intraperitoneally and sacrificed after 4 hr.
Group D: Received orally aqueous extract of *Hemidesmus indicus* (equivalent to 100 mg/kg) for 8 days and then allyl alcohol 4-5 mg in a volume of 0.5 ml in saline (0.7 mmol per kg body weight) was given and sacrificed after 4 h.
All the rats were fasted for 17h before and 4h after allyl alcohol administration.

Isolation of mitochondria:

Mitochondria were isolated from liver according to the method of Lawrence and Davies (1986). The isolation medium A consists of 70mM sucrose, 220mM mannitol, 2mM HEPES, 0.2mM Ethylene diamine tetra acetic acid (EDTA) and 36mg bovine serum albumin (BSA) per 100ml. The pH was adjusted to 7.4. Isolation medium B consists of 0.25M sucrose. The experimental rats were killed by stunning and livers were excised and transferred immediately to ice-cold homogenizing medium A. All the subsequent operations were carried at 4^0C. Livers were blotted with filter paper, washed twice with medium A to remove traces of blood. They were then weighed and minced finely. A 10% (W/V) homogenate was prepared in medium A using a Potter-Elvehjem homogenizer with a teflon pestle.

The pestle was driven by an electric motor at 3500 rpm and a maximum of 8 up and down strokes were given for complete homogenization. The 10% homogenate was centrifuged at 700 x g for 10min. The pellet was discarded and the supernatant was centrifuged at 7000 x g for 10min. The supernatant thus obtained contains a white fat layer which was removed with a cotton swab. The pellet containing mitochondria was suspended in 0.25M sucrose and centrifuged at 7000 x g for 10min. This step was repeated to wash the mitochondria. The final pellet was suspended in medium B and a portion was used immediately for polarographic studies and the other portion was stored in -70^{0}C for further studies. The protein content was determined by Biuret method using BSA as a standard (Gornall et al., 1949).

Measurement of oxidative phosphorylation:

Oxidative phosphorylation was measured in Hansatech oxytherm Respirometer according to the method of Estabrook, 1949. Respiration rates were measured at 25^{o}C using a oxygen electrode disc in an airtight chamber of 1 ml volume. The reaction system contained 50 mM sucrose, 50 mM Tris-Hcl, 20 mM potassium phosphate, 2 mM EDTA, 7 mM magnesium chloride, pH 7.4 and 1- 2 mg of freshly isolated mitochondrial protein. After the addition of substrate (6.25mM malate + 3.125mM glutamate or 0.02M succinate) state-3 respiration was initiated by the addition of 200 nmol and 400 nmol of ADP for succinate oxidase and NADH oxidase respectively. Respiratory control ratio (RCR) was obtained from the ratio of ADP stimulated (state-3) respiration to ADP exhausted (state-4) respiration and ADP/O = P/O ratio which was calculated according to Estabrook, 1949.

Assay of lipid peroxides by thiobarbituric acid reaction:-

In liver homogenate and mitochondria lipid peroxide level was carried out by the method of Ohkawa et al., 1979. Differential centrifugation was used to isolate mitochondria from 10% liver homogenate in 1.15% KCl. Final pellet containing mitochondria were washed with 1.15% KCl and suspended in the same medium. Reaction system contained 5 mg of mitochondrial protein, 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid (pH-3.5) and 1.5 ml of 0.67% (w/v) aqueous solution of thiobarbituric acid. The total volume was made upto 4 ml with water and the tubes were heated in a water bath at 95^{0}C for 60 min. After cooling, 1.0 ml of water and 5 ml of n-butanol were added and the tubes were vortexed and then centrifuged at 2000 X g for 10 min at room temperature. The absorbance of the organic layer was measured at 535 nm. A blank was also run simultaneously and tetra methoxy propane was used as an external standard. The extent of lipid peroxidation was expressed as nmol of malondialdehyde (MDA) formed per 100 mg protein.

Measurement of protein carbonyls:-

The concentration of protein carbonyls in mitochondria was determined using 2,4-dinitrophenylhydrazine (DNPH) (Reznick and Packer, 1994;

Bailey et al., 2001). To an aliquot of mitochondria containing 2-4mg protein, 4 ml of 10mM DNPH in 2N HCl was added and incubated at room temperature shaking intermittently for 1 h. Sample blank was also run simultaneously. After incubation the mixture was precipitated with 10% TCA and centrifuged. The precipitate was washed thrice with 4 ml of ethanol : ethyl acetate (1:1). The final protein precipitate was dissolved in 6 M guanidine hydrochloride and the absorption at 370 nm (DNPH-treated sample minus sample blank) was measured. Carbonyl content was calculated using the molar extinction coefficient value as 22,000 and expressed as nmol carbonyls per mg protein.

Determination of total sulphydryl groups:-

Total sulphydryl groups in mitochondria were determined by the method of Sedlak and Lindsay, 1968. Mitochondrial suspension containing 1 mg protein was mixed with 1.5 ml of 0.2 M tris buffer (pH-8.2) and 0.1 ml of 0.01 M 5,5′-dithiobis-(2-nitrobenzoic acid) [DTNB]. The final volume was adjusted to 10 ml with 7.9 ml of absolute methanol and color developed for 15 min and centrifuged at 3000 x g at room temperature for 15 min. A reagent blank (without sample) and sample blank (without reagent) were also run simultaneously. The absorbance of the supernatant was measured at 412 nm and total sulphydryl groups were expressed as nmols per mg protein using DTNB molar extinction coefficient as 13100.

Generation of superoxide radicals:-

Generation of superoxide radicals was measured spectrophotometrically according to the method of Dionisi et al., 1975. The reaction mixture contained 0.25 M sucrose, 50 mM HEPES pH 8.4, 1 mM epinephrine, 3 mM succinate and 0.5 mM EDTA and 0.1-0.2 mg protein of mitochondrial fragments. 2.5 µg antimycin per ml was used to initiate the radical formation. Generation of superoxide radicals was quantified spectrophotometrically at 480 nm and was expressed as nmoles of adrenochrome formed per min per mg protein using molar extinction coefficient of adrenochrome as 4020.

Assay of catalase:

Catalase activity was determined by the method of Aebi (1984). The assay mixture contained 150µl of 30mM H_2O_2 and 850µl of enzyme source. The decrease in absorbance was measured immediately at 240nm and activity was expressed as nmols of H_2O_2 consumed per min per mg protein.

Assay of Glutathione peroxidase:

Glutathione peroxidase was assayed by the method of Flohe and Gunzler (1984). The activity of enzyme was measured indirectly by determining the oxidation of NADPH, at 340nm. Reaction mixture contains 50mM potassium phosphate (pH 7.0), 0.5mM EDTA, 1mM reduced glutathione (GSH), 0.24U glutathione reductase and 0.1mg of homogenate, incubated for 10min at 37^0C.The reaction was initiated by the addition of 0.15mM NADPH. After

3min 0.15mM hydrogen peroxide was added and decrease in absorbance was measured. Results were expressed as nmols of NADPH oxidized per min per mg protein.

Assay of Glutathione reductase:

The glutathione reductase activity was assayed by following the oxidation of NADPH at 340nm according to the method of Anderson (1985). Reaction mixture contains 100mM potassium phosphate (pH 7), 1mM EDTA, 0.5mM NADPH and mitochondrial protein 0.05-0.1mg. The reaction was initiated by the addition of 5mM oxidized glutathione (GSSG). Results were expressed as nmols of NADPH oxidized per min per mg protein.

Assay of Superoxide dismutase:

Superoxide dismutase activity was determined by the inhibition of oxidation of epinephrine to adrenochrome which was monitored at 480nm using xanthine-xanthine oxidase system by the method of Dionisi et al., 1975. Reaction mixtures contained 0.01M sodium carbonate buffer, 1mM xanthine and 0.011 µM xanthine oxidase, 0.1-0.5mg protein of sample (mitochondrial fragments or sonicated mitochondria or cytosol). The reaction was initiated by the addition of 1mM epinephrine and absorbance was measured at 480nm. Results were expressed as units per mg protein. One unit is defined as amount of enzyme which inhibits oxidation of epinephrine by 50%.

Determination of Oxidized and Reduced glutathione:

Measured as per the method of Hissin and Hilf (1976) 250mg of liver was homogenized in 3.75ml of the phosphate–EDTA buffer (pH-8.0), 1ml of 25% H_3PO_4 and centrifuged at 4^0C at 100,000g for 30min and the supernatant obtained was used for the assay of reduced (GSH) and oxidized glutathione (GSSG).

Reduced Glutathione (GSH) assay: To 0.5ml of the supernatant, 4.5ml of phosphate–EDTA buffer, pH 8.0 was added. The final assay mixture contained 100µl of the diluted tissue supernatant, 1.8ml of phosphate-EDTA buffer (pH-8.0) and 100µl of o-phthalaldehyde (OPT) solution. After 15 min incubation fluorescence at 420nm was determined with the activation at 350nm.

Oxidised Glutathione (GSSG) assay: A 0.5ml of supernatant was incubated at room temperature with 200µl of 0.04M N-ethyl maleimide for 30min .To this 4.3ml of 0.1N NaOH was added. To 100µl of this mixture 1.8ml 0.1N NaOH, 100µl of OPT was added and fluorescence was determined at 420nm with activation at 350nm.
Results were expressed as µmoles per gm tissue.

Assay of Aminotransferases:

Alanine aminotransferase and aspartate aminotransferase were assayed in the serum and liver samples of control and experimental animals. Following decapitation, blood was collected and centrifuged at 3000 rpm for 10min at room temperature and the supernatant taken as serum. A 10% liver homogenate was prepared in ice-cold 0.32M sucrose using a motor driven Potter-Elvehjem homogenizer with a teflon pestle. Assay of these enzymes was performed in a Shimadzu-160A spectrophotometer. Time periods and enzyme concentrations were adjusted in such a way that a linear curve was obtained for atleast 3-5min.

Aspartate aminotransferase (AAT E.C.2.6.1.15):

The method of Bergmeyer and Bernt (1974) was adopted for the assay. The oxaloacetate formed in the reaction was converted to malate by malate dehydrogenase (MDH) and the NADH consumed in the course of the reaction, which will be proportional to AAT activity, was measured.

Liver/serum AAT

L-aspartate + α-ketoglutarate----------→ glutamate + oxaloacetate

The oxaloacetate formed in the reaction was converted to malate using malate dehydrogenase (MDH) and the NADH consumed in the course of the reaction, which will be proportional to AAT activity, was measured.

Commercial MDH

Oxaloacetate + NADH ←----------→ malate + NAD^+

Assay mixture: 160 µ moles potassium phosphate, 20 µ moles aspartic acid (pH 7.4), 18 µ moles α-ketoglutarate (pH 7.4), 0.4 µ moles NADH, 1% Triton X-100, 5µl of MDH (0.5 mg protein/ml) and 25µl serum or 40 µg of liver homogenate in a total volume of 1.0ml.
The reaction was pre incubated for 10min in the absence of α-ketoglutarate and the reaction was initiated by the addition of α-ketoglutarate and the decrease in absorbancy due to NADH oxidation was followed at 340nm for 10min. Enzyme activity was calculated using a mM extinction coefficient of 6.22 for NADH and is expressed as µ moles NADH oxidized per mg protein per hr.

Alanine aminotransferase (AlAT E.C.1.1.1.42):

A similar method was adopted for the assay of AlAT (Bergmeyer and Bernt, 1974).

Liver\serum AlAT
Alanine + α-ketoglutarate----------→ pyruvate + glutamate

The pyruvate so formed, was converted to lactate in the presence of NADH and lactate dehydrogenase (LDH).

Commercial LDH
Pyruvate + NADH ←----------→ lactate + NAD^+

Assay mixture: 160 µ moles potassium phosphate, 40 µ moles alanine (pH 7.4), 18 µ moles α-ketoglutarate (pH 7.4), 0.42 µ moles NADH, 1% Triton X-100, 5µl of MDH (0.5 mg protein/ml) and 25µl serum or 40 µg of liver homogenate in a total volume of 1.0ml. Change in absorbancy due to NADH oxidation was measured at 340nm for 10min. Activity was calculated and expressed as µ moles NADH oxidized per mg protein per hr.

Preparation of submitochondrial particles:

Submitochondrial particles were obtained from freshly prepared liver mitochondria according to the procedure of Hackenbrock and Hammom (1975). Mitochondria suspended in 0.25M sucrose (50mg protein/ml) were mixed with digitonin (0.12 mg /mg protein) and stirred at 4^0C for 15min. The suspension was centrifuged at 12,000 x g for 15min and the pellet was washed with 1ml of 0.25M sucrose. The mitoplasts thus obtained were suspended in ice-cold double distilled water (50mg protein in 25ml). The suspension was centrifuged at 10,000 x g for 10min and the pellet was suspended in a small volume (5-7ml) of ice-cold water and sonicated with MSE ultrasonicator using a microprobe. Three pulses each of 30 seconds duration were given and then centrifuged at 12,000 x g for 10min. From the supernatant fraction SMP were sedimented by centrifugation at 105,000 x g for one hour in a ultracentrifuge. The pellet containing SMP was suspended in 0.25M sucrose and used immediately or stored in liquid nitrogen depending upon the parameter that is to be assayed.

Measurement of membrane potential (ΔΨ) in submitochondrial particles:

The membrane potential (ΔΨ) was determined by the distribution of the fluorescent probe, 1-anilino-8-napthalene sulfonate (ANS) across the submitochondrial membrane (Azzi, 1971). A fluorescence increase was observed when a substrate was supplied to the membranes of the submitochondrial particles.

Submiotochondrial particles (SMP) prepared by sonic disruption of rat liver mitochondria were used for this study. The reaction system contained submitochondrial particles (380 µg protein) preincubated for 5 min in a buffered medium (containing 250mM sucrose, 10mM Tris-HCl, 5mM $MgCl_2$, pH 7.5) and 10µM ANS in a total volume of 1.0 ml. The intensity of fluorescence was measured in a fluorescence spectrophotometer. The excitation and emission wave lengths used were 350nm and 480nm respectively. 1mM succinate was added to the reaction system to energise the membrane and the fluorescence change was recorded. The addition of succinate induced an increase in ANS fluorescence. After the fluorescence change reached to a steady state, CCCP (a potent uncoupler) was added (0.3µM) to bring the membrane to deenergised state.

The difference in the ANS fluorescence of the energized and de-energised state was used for the estimation of membrane potential (ΔΨ). The internal volume of the submitochondrial particles used for the

calculation was 2 µl per mg protein (from earlier studies). Membrane potential was calculated using Nernst equation as follows:
At room temperature

$\Delta\Psi = -59\log [C_1/C_2 + (C_1-C_2)\ V/\ C_2\ v]$
C_1 = concentration of ANS inside SMP
C_2 = concentration of ANS outside SMP
V = extermal volume outside SMP
v = internal volume of submitochondrial particles

Histopathology of liver tissue:

Tissue processing and embedding:

Liver tissue was removed, fixed in 10% formaldehyde and stored at -70^{0}C for further use.

Procedure:

- About 0.25 gms of the tissue is taken and immersed into tube containing 10% formaldehyde (1hour).
- Transfer to 70% alcohol and keep for 1 hr (repeat thrice)
  Transfer to 90% alcohol and keep for 1hr (repeat twice)
- Transfer to 100% alcohol and keep for 1 hr (repeat twice)
- Transfer to xylene and keep for 30-45 min (repeat twice)
- Paraplast was melted in the incubator at 60^{0}C and tissue was kept immersed. (3 changes each 2 hours)
- Final embedding

Differential Staining Technique:

Haematoxylin Eosin Staining:

- Xylene (3 changes, 5 min each)
- 100% alcohol (2 changes, 5 min each)
- 90% alcohol (2 changes, 3 min each)
- 70% alcohol (3min)
- 50% alcohol (3min)
- Water (5min)
- Haematoxylin (6-8 min)
- Rinsed with water; kept under running tap water for 10 min
- Eosin (3-5 min)
- 70% alcohol (2min)
- 90% alcohol (two changes, 2 min each)
- 100% alcohol (5 min)
- Xylene (5 min each three changes)
- DPX Mounting

Detection of free radicals by electron spin resonance spectroscopy (ESR) using spin trap 5, 5/-Dimethyl-1-pyrroline-N-oxide (DMPO):

Preparation of mitoplasts:

Mitoplasts were prepared from isolated mitochondria by digitonin treatment as described previously (Pedersen et al., 1978). Liver from rats was excised, washed with 0.25M sucrose and homogenized in isolation buffer consisting of 210mM mannitol, 70mM sucrose, 2mM HEPES and 0.05% BSA. The homogenate was centrifuged at 800 x g for 10min, the pellet removed, and the centrifugation process repeated. The resulting supernatant was centrifuged at 8000 x g for 10 min, washed with isolation buffer and the centrifugation repeated. Mitochondria (40mg/ml) were mixed with an equal volume of isolation buffer containing 0.2% digitonin and stirred for 15min at 4^0C. The sample was diluted 6-fold with buffer and centrifuged at 10000 x g for 10min. The pellet was washed once with isolation buffer and centrifuged at 10000 x g for 10min. The final pellet was suspended in buffer (230mM mannitol, 70mM sucrose, 20mM Tris-HCl, pH adjusted to 7.4 with Mops) and used for ESR studies.

ESR:

For ESR analysis (Han et al, 2001), mitoplasts (1 mg) were placed in 200µl of buffer [230mM mannitol, 70mM sucrose, 20mM Tris-HCl, pH adjusted to 7.4 with 3-(N-Morpholino) propane sulphonic acid (Mops)] in the absence or presence of respiratory substrates or inhibitors. 5,5/-Dimethyl-1-pyrroline-N-oxide (DMPO) (160mM) was added and the ESR spectra were recorded on a JES-FA 200 ESR spectrometer. Instrument settings were as follows: microwave power, 20mW; microwave frequency, 9.42 GHz; time constant, 0.03sec; scan time, 4min; scan width, 100G.

Alcohol dehydrogenase:

Alcohol dehydrogenase (ADH) activity was determined in liver cytosol on spectrophotometer by measurement of the rate of NADH formation at 340nm, using ethanol as substrate according to the procedure of Aasmoe and Aarbakke (1999). ADH activity was assayed in 1 ml cuvette containing 0.1M glycine-NaOH buffer pH 10.4, 1.3mM NAD and 0.6mg of cytosolic protein. The reaction was started by the addition of 20mM ethanol as enzyme substrate and change in absorbance was recorded. Specific activity was expressed as the rate of NADH formation per min per mg protein.

Glutathione transferase (GST) purification – GSH agarose affinity column:-

Preparation of affinity matrix:

Affinity matrix was prepared according to Simmons and Vander jagt (1977). Briefly, 6gm of epoxy activated sepharose 6 B was washed with 500ml of double distilled water followed by 40 ml of 44mM phosphate buffer pH 7.0. The slurry was transferred to a side-armed conical flask and the volume was adjusted to 20ml with the same

buffer and nitrogen gas was passed through for 5min. To this 4ml of reduced glutathione (4mg of GSH in 4ml of distilled water, pH adjusted to 7.0 with KOH) was added and coupling was done for 24hr at 37^0C with constant stirring . The gel was washed with 100ml of distilled water followed by 100ml of 0.5M KCl in 0.1M sodium acetate, pH 4.0 and 0.5M KCl in 0.1M sodium borate buffer, pH 8.0. Finally the gel was transferred to 10mM potassium phosphate buffer, pH 7.0 containing 150mM KCl with 0.01% sodium azide and stored at 4^0C until use.

Preparation of tissue homogenates:

Rat liver tissue was homogenized in 50mM Tris-HCl buffer, pH 8.0 containing 0.25M sucrose and 1mM phenyl methane sulphonyl fluoride (PMSF) using a glass homogenizer. Homogenization was done by keeping the glass homogenizer in an ice jacket and care was taken to minimize the froth formation. The homogenate was passed through two layers of cheese cloth and then centrifuged at 10,000 X g at 4^0C for 30min. The resulting supernatant was centrifuged at 1,05,000 X g for 1hr. The supernatant obtained is referred to as crude cytosolic fraction.

GST assay:

GST activity was determined as described by Habig et al., (1981) using 1, chloro 2,4 dinitro benzene as substrate. The typical assay mixture contain 1mM CDNB (1, chloro 2,4 dinitro benzene), 1mM reduced glutathione (GSH) and 100mM sodium phosphate buffer (pH 6.5) in a volume of 1ml. Thioether formation was determined by reading the absorbance at 340nm and quantification was done using 9.6 $M^{-1}.cm^{-1}$ as the extinction coefficient. One unit of enzyme activity was defined as one micromole of product formed or one micromole of substrate consumed per minute. Specific activities were given in units per mg protein. Blank activity was measured in the absence of enzyme and subtracted from experimental values to correct for possible non enzymatic reactions.

Purification of GSTs

Gel filtration on sephadex G-150:
The crude cytosolic fraction was subjected to ammonium sulfate precipitation. Ammonium sulphate, 590g per liter was added to the crude cytosolic fraction and the preparation was centrifuged at 12,000 rpm for 30min. The precipitate was dissolved in 25mM Tris-Hcl and dialysed for 24 hr against 10 vols. of 25mM Tris-Hcl, pH-8.0. The dialysed cytosol was applied on Sephadex G-150 column previously equilibrated with 25mM Tris-Hcl, pH-8.0. Same buffer was used as eluent and 5.0ml fractions were collected at a flow rate of 1ml/min. Active fractions were pooled and loaded on affinity column.

Affinity chromatography on GSH linked epoxy activated sepharose 6B:

The pooled fractions were dialysed for 24 hr against 10 vols. of 10mM potassium phosphate buffer (pH 7.0) with 4 changes at 6h intervals to remove endogenous GSH, which may interfere with the binding of GSTs to affinity column. After dialysis sample was loaded on to the GSH-Sepharose 6B affinity column previously equilibrated with 10mM potassium phosphate buffer pH 7.0 containing 0.15M KCl and then washed with the same buffer till the protein content dropped to zero (by spectroscopic detection at 280nm). The affinity bound GSTs were then eluted with 50mM potassium phosphate buffer pH 7.5 containing 10mM GSH and 1ml fractions were collected. Active fractions were pooled, dialysed and then concentrated by lyophilisation.

Protein determination:

Protein content in the chromatographic fractions were determined spectrophotometrically by the procedure of Warburg and Christian (1941) by measuring the absorbance at 280nm and 260nm. Protein content in the samples like crude homogenate and cytosol was assayed by the method of Biuret.

SDS-PAGE analysis:

Polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) on a vertical slab gel system. The gels contained 12% acrylamide with 30:1 ratio of acrylamide to N, N, N', N'- methylene- bis-acrylamide. Samples were boiled at 100^0C for 5 min in the presence of loading dye to dissociate proteins into their individual polypeptide chains. The loading dye contained: SDS (4% w/v), 2-mercaptoethanol (2% v/v) in 0.1 M Tris-HCl, pH 6.8. The protein mixture was subjected to electrophoresis on polyacrylamide stacking gel in 0.5M Tris-HCl, pH 6.8, and 12% (w/v) resolving gel in 1.5M Tris-HCl, pH 8.8 at 100v till the dye front reaches the end of the gel. The electrode buffer (pH 8.3) contained 0.025M Tris buffer, 0.192M glycine and 0.1% SDS. 10 µg of crude extract and 1 µg of affinity purified cytosolic GSTs loaded on SDS-PAGE.

Silver staining:

Silver staining of proteins was essentially done as described by Blum et al., 1987. Briefly, gels were fixed in fixative solution (50% methanol, 12% acetic acid and 0.5% of 37 % formaldehyde) for 1hr followed by washing with 50% alcohol 3 times for 30 minutes each. Gels were treated with sodium thiosulphate (0.2gms/litre) precisely for 1 minute and washed with double distilled water to remove exess of thiosulphate. The gels were treated with freshly prepared silver nitrate (2gms/litre of double distilled water containing 0.76% of 37 % formaldehyde /litre) for 30 minutes on a shaker. Exess of silver nitrate was removed by washing thrice with doubled distilled water. And finally the gel was developed using 6% sodium carbonate containing 0.6% of 37% formaldehyde/litre. As soon as the

protein spots of required intensity appeared the reaction was stopped by the addition of 12% acetic acid and the gels were preserved in 50% ethanol.

Western blotting

Immunoblot analysis was carried out on nitrocellulose membranes according to the published procedures of Towbin et al., 1979. The cytosolic GSTs separated on SDS-PAGE were transferred on the nitrocellulose membrane. After the transfer process, the membrane was air dried for few seconds. Immediately the membrane was made wet in Tris buffer saline (TBS, pH-7.6) and thorough rinsing was done. Then the membrane was transferred into TBS-buffer, which contains 5% non-fat milk for 1hr to block the nonspecific binding sites. The blots were then probed with primary antibody for 1hr at room temperature. The unbound primary antibody was removed by washing 2 times (5 min each) with TBS, 2 times (5 min each) with TBS-T (TBS containing 0.1% tween, pH-7.6), 2 times (5 min each) with TBS. The membrane was blocked with 5% milk powder in TBS for 5 min. Then the membrane was incubated for 1 hr in TBS with 1% non fat milk containing secondary antibody Goat anti-rabbit IgG ALP conjugate. The unbound antibody was removed by washing 2 times (5 min each) with TBS, 2 times (5 min each) with TBS-T (TBS containing 0.1% tween), 2 times (5 min each) with TBS and subjected to colour development using BCIP-NBT which is substrate for alkaline phosphatase. The membrane was dried and densitometric analysis was performed.

RESULTS

Phyllanthus fraternus:

In the present study the effect of administration of allyl alcohol on the oxidative stress and the protective effect of prior administration of aqueous extract of *Phyllanthus fraternus* on allyl alcohol induced oxidative stress were studied.
The percent protective effect due to the prior administration of *Phyllanthus fraternus* was calculated as follows.
[100/100 – (Value of group B)] X (Value of group D – Value of group B)
In other words a 100% protection (on a given parameter) means that the value is back to the control level which is normalized as 100.

Studies on oxidative phosphorylation:

NADH oxidase (through glutamate and malate)

Fig.1A is a original trace of oxygen concentration obtained by oxytherm respirometer. ADP stimulated respiration is taken as state 3 and ADP exhausted respiration as state 4. Figure 1B shows the effect of administration of allyl alcohol and the protective effect of prior administration of *phyllanthus fraternus* on state 3 respiration, respiratory control ratio (RCR) and P/O ratio.

Externallyl added NADH cannot penetrate the tightly coupled mitochondria. So, glutamate and malate were used to reduce the internal NAD^+ pool and generate NADH in the matrix which gives electrons to the respiratory chian. Studies on this enzyme complex give the information on the ability of transfer of electrons through all the three sites. The P/O ratio is the index of efficiency of the system to conserve energy in the form of ATP, while RCR gives an index of the integrity of the mitochondrial membrane.
In allyl alcohol administered rats state 3 respiration, RCR and P/O was decreased by 43, 55 and 29% compared to controls and this decrease is significant. There was a significant increase in state 3 respiration and RCR in rats administered both *P.fraternus* and allyl alcohol when compared to rats administered only allyl alcohol. Administration of *P. fraternus* prior to allyl alcohol administration offered protection of 72, 40 and 80% on state 3 respiration, RCR and P/O ratios respectively. Administration of the plant extract alone did not show any change.

Studies on Succinate oxidase:

Fig 2 shows the effect of administration of allyl alcohol and the protective effect of prior administration of *phyllanthus fraternus* on state 3 respiration, respiratory control ratio (RCR) and P/O ratio with succinate as substrate.
When succinate was used as substrate (electron transfer through site II + site III) there was a decrease of 43, 52 and 20% on state 3 respiration, RCR and P/O ratios respectively due to the administration of allyl alcohol when compared to control. There was a significant increase in state 3 respiration, RCR and P/O ratios in rats administered both *P.fraternus* and allyl alcohol when compared to rats administered with only allyl alcohol. This shows that there is a significant effect due to the administration of *P.*

fraternus. Administration of *P. fraternus* prior to allyl alcohol offered protection of 77, 54 and 20% on state 3 respiration, RCR and P/O ratios respectively (Figure-2). There was no significant effect on succinate oxidation in rats administered *P. fraternus* extract alone.

Transaminases:

Aspartate amino transferase (AST):

Fig-3 and 4 shows the effect of allyl alcohol and *P. fraternus* on liver and serum aspartate aminotransferase (AST). Serum AST levels are significantly increased (625%) where as in liver homogenate AST levels are significantly decreased (56%) by the administration of allyl alcohol when compared to controls. These transaminases are known to decrease in liver and increase in serum during liver damage and are good index of the extent of the liver damage. Prior administration of aqueous extract of *P. fraternus* offered significant protection when compared to group of rats administered with allyl alcohol alone. Administration of *P. fraternus* prior to allyl alcohol offered protection against the liver damage to an extent of 60, 38% in homogenate and serum.

Alanine amino transferase (AlAT):

Fig-3 and 4 shows the activity of alanine amino transferase. The activity of AlAT decreased by 62% in liver homogenate where as it increased four folds (441%) in the serum by the administration of allyl alcohol. Administration of *P. fraternus* prior to allyl alcohol offered protection of 48, 27% in homogenate and serum. Thus the prior administration of aqueous extract of *P. fraternus* offered significant protection against the liver damage induced by allyl alcohol.

Membrane potential:

Fig-5A shows the original traces of fluorescence changes of ANS in submitochondrial particles. The addition of succinate energizes the membrane and builds the membrane potential which is followed by an increase in ANS fluorescence. The addition of CCCP, an uncoupler, deenergizes the membrane and membrane potential is dissipated which is followed by decrease in the fluorescence. The fluorescence changes were less due to allyl alcohol administration when compared to control. Fig-5B shows the effect of allyl alcohol with and without prior administration of *P.fraternus* on membrane potential. Significant decrease in membrane potential was observed by the administration of allyl alcohol. Prior administration of aqueous extract of *P. fraternus* offered protection to an extent of 62%.

Lipid peroxide level:

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *P. fraternus* on lipid peroxide level is shown in Figure-6. In both homogenate and mitochondria the lipid peroxide level is significantly increased (65 and 109% respectively) due to the administration of allyl alcohol compared to controls. There was a significant decrease in both homogenate and mitochondria in rats administered both *P.fraternus* and allyl alcohol when compared to rats

administered only allyl alcohol. Prior administration of aqueous extract of *P. fraternus* offered protection of 88 and 91% on lipid peroxides of both homogenate and mitochondria respectively. Administration of *P. fraternus* alone decreased the lipid peroxide level (28%) in the homogenate and increased (15%) in mitochondria. This change is not statisticallyl significant.

Protein carbonyls:

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *P. fraternus* on protein carbonyls is shown in Figure-7. The carbonyl content is significantly increased (118%) due to the administration of allyl alcohol. The prior administration of aqueous extract of *P.fraternus* offered complete protection (comparable to controls) and prevented the raise in the carbonyl content due to the administration of allyl alcohol in liver mitochondria.

Total sulphydryl groups:

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *P. fraternus* on total sulphydryl groups is shown in Figure-8. The total sulphydryl groups are significantly decreased (54%) due to the administration of allyl alcohol. The prior administration of aqueous extract of *P. fraternus* offered protection to an extent of 59%. The increase in rats administered both *P. fraternus* and allyl alcohol is statisticallyl significant when compared to rats administered only allyl alcohol.

Superoxide radicals:

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *P. fraternus* on the generation of superoxide radicals is shown in Figure-9. The superoxide radicals are significantly increased (220%) due to the administration of allyl alcohol. Administration of aqueous extract of *P. fraternus* prevented the raise and protected to an extent of 53% on superoxide level. There was a significant difference in rats administered both *P. fraternus* and allyl alcohol (group-D) when compared with both control (group-A) or rats administered with allyl alcohol (group-B).

Catalase:

Figure 10 shows the effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *P. fraternus* on catalase activity. In both homogenate and mitochondria the catalase activity is significantly decreased (57 and 21% respectively) due to the administration of allyl alcohol. There was a significant increase in the catalase activity in homogenate in rats administered both *P. fraternus* and allyl alcohol (group-D) when compared to rats administered only allyl alcohol (group-B). Prior administration of aqueous extract of *P. fraternus* offered protection to an extent of 40 and 57% on catalase activity in homogenate and mitochondria respectively.

Superoxide dismutase (SOD):

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *P. fraternus* on the generation of superoxide dismutase activity is shown in Figure 11. SOD activity is significantly decreased in intact mitochondria, cytosol and membrane fragments (43, 45 and 53% respectively) due to the administration of allyl alcohol. There was a significant increase in the SOD activity of intact mitochondria and membrane fragments in rats administered both *P. fraternus* and allyl alcohol (group-D) when compared to rats administered only allyl alcohol (group-B). Prior administration of aqueous extract of *P. fraternus* offered protection of 66, 29 and 60% on SOD activity of intact mitochondria, cytosol and membrane fragments respectively.

Glutathione peroxidase (GSHPx):

Figure-12 shows the effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *P. fraternus* on glutathione peroxidase activity. The GSHPX activity is significantly decreased (41%) due to the administration of allyl alcohol. The prior administration of aqueous extract of *P. fraternus* offered protection (56%) and prevented the decrease in the GPx activity due to the administration of allyl alcohol. There was a significant difference in rats administered both *P. fraternus* and allyl alcohol (group-D) when compared to rats administered only allyl alcohol (group-B).

Glutathione reductase (GR):

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *P. fraternus* on glutathione reductase activity is shown in Figure-13. The GR activity is significantly decreased (44%) due to the administration of allyl alcohol. Prior administration of aqueous extract of *P. fraternus* offered protection of 57% on GR activity. There was a significant difference in rats administered both *P. fraternus* and allyl alcohol (group-D) when compared to rats administered only allyl alcohol (group-B).

Glutathione levels:

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *P. fraternus* on levels of glutathione is shown in Figure-14. The reduced glutathione (GSH) and oxidized glutathione (GSSG) are significantly decreased (46 and 48%) due to the administration of allyl alcohol. Prior administration of aqueous extract of *P. fraternus* offered protection of 41 and 50% on GSH and GSSG levels. There was a significant difference in rats administered both *P. fraternus* and allyl alcohol (group-D) when compared to rats administered only allyl alcohol (group-B).

Alcohol dehydrogenase:

Fig-15 shows the effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *P. fraternus* on alcohol dehydrogenase activity The alcohol dehydrogenase activity is significantly increased (45%) due to the administration of allyl alcohol. Prior administration of aqueous extract of *P. fraternus* offered protection of 62% on alcohol dehydrogenase activity. There was a significant decrease in rats administered both *P. fraternus* and allyl alcohol (group-D) when compared to rats administered only allyl alcohol (group-B).

Glutathione transferase (GST):

Gel filtration on sephadex G-150 column:

Gel filtration profiles of rat liver cytosol from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*P.fraternus* administered) were given in figures 16, 18 and 20 respectively. The active fractions were pooled and loaded on affinity column.

Affinity purification of cytosolic GSTs from rat liver cytosol:

Table 1, 2 and 3 shows the purification profile of rat liver cytosolic GSTs from group-A (control), group-B (allyl alcohol administered) and group-D (*P. fraternus* and allyl alcohol administered). The crude extract of control liver showed a CDNB specific activity of 0.69 μmol.min^{-1}.mg protein^{-1}. The affinity-purified GSTs had a 21μmol.min^{-1}.mg protein^{-1} specific activity giving a 30 fold purification with a yield of 68%. The cytosolic GSTs of allyl alcohol treated rat liver showed a specific activity of 0.31 μmol.min^{-1}.mg protein^{-1}. After purification by affinity chromatography, the specific activity obtained was 8.7μmol.min^{-1}.mg protein^{-1} giving 28 fold purification with an overall yield of 62%. The cytosolic GSTs of *P.fraternus* and allyl alcohol treated rat liver showed a specific activity of 0.44 μmol.min^{-1}.mg protein^{-1}. After purification by affinity chromatography, the specific activity obtained was 14.7 μmol.min^{-1}.mg protein^{-1} resulting in a 33 fold purification with an overall yield of 60%. Affinity elution patterns of rat liver cytosolic GSTs from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*p.fraternus* administered) were given in figures 17, 19 and 21 respectively.

SDS PAGE:

The affinity purified GSTs were resolved into three subunits on SDS-PAGE with molecular weights of 25.6, 27 and 28 kDa which were designated as Ya, Yb and Yc respectively. Calibration of relative molecular weight was done by including the molecular weight standards along with affinity purified GSTs. Fig 22-A shows SDS-PAGE of affinity purified rat liver cytosolic GSTs from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*p.fraternus* administered).

Western blotting:

The cytosolic proteins as well as affinity purified GSTs were analyzed by immuno blotting after electrophoresis using polyclonal antibodies raised against affinity purified GSTs. Ya subunit was shown to be decreased significantly (fig 23i) upon allyl alcohol administration. Prior administration of aqueous extract of *P.fraternus* offered significant protection in Ya subunit. There was no significant difference in Yb and Yc subunits (fig 23 ii and iii). Fig 22-B shows wetern blot. Figure 23 shows densitometric scans of western blots.

Measurement of radicals using spin trap DMPO by ESR:

Figure-24 i, ii and iii shows original traces of ESR signals of DMPO-OH adduct of rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*P.fraternus* administered) in the presence of glutamate and malate. Figure-24 iv shows addition of superoxide dismutase completely abolished the ESR signal. Figure-25 shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*P.fraternus* administered) in the presence of glutamate and malate. ESR signal intensity of DMPO-OH adduct was statistically increased due to the administration of allyl alcohol. Prior administration of aqueous extract of *P. fraternus* offered protection of 47%. Figure-26 shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*P.fraternus* administered) in the presence of antimycin, glutamate and malate. The change in the signal intensities of DMPO-OH adduct of these 3 groups was not statistically significant in the presence of antimycin. Figure-27 shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*P.fraternus* administered) in the presence of succinate. The change in the signal intensities of DMPO-OH adduct of these 3 groups was not statistically significant. Figure-28 shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*P.fraternus* administered) in the presence of succinate and antimycin. The change in the signal intensities of DMPO-OH adduct of these 3 groups was not statistically significant in the presence of antimycin.

FIGURE 1A

Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on the rate of respiration using glutamate + malate as substrate

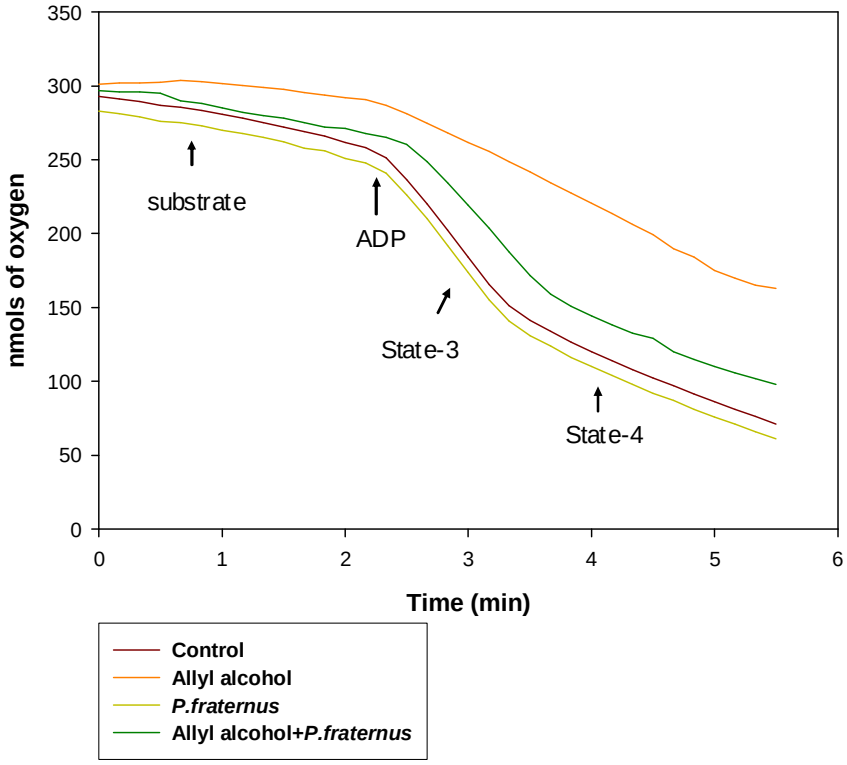
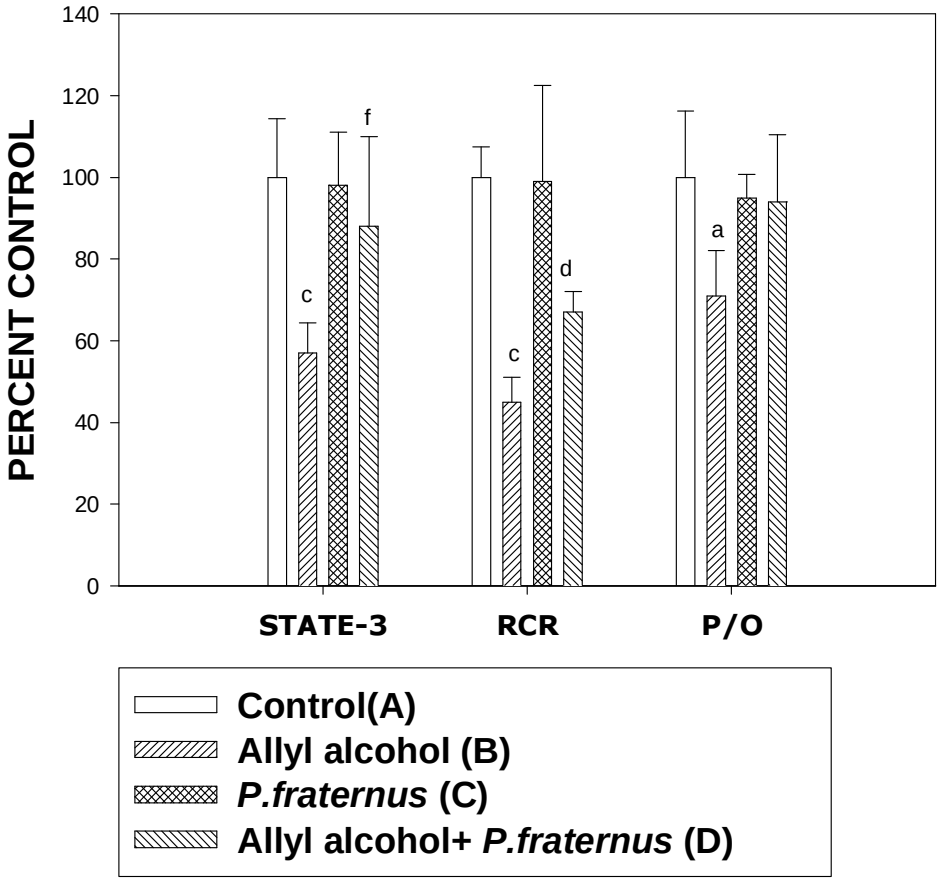


Figure 1A shows the original traces of oxygen concentration obtained by oxytherm respirometer. The arrows indicate the time point at which substrate/ADP is added. ADP stimulated respiration is taken as state 3 and ADP exhausted respiration as state 4. The experiment was done with 2mg of mitochondrial protein in 1.0ml of the buffer (50 mM sucrose, 50 mM Tris-Hcl, 20 mM potassium phosphate, 2 mM EDTA, 7 mM magnesium chloride, pH 7.4) in the air tight container. Additions were done with Hamilton syringe.

FIGURE 1B

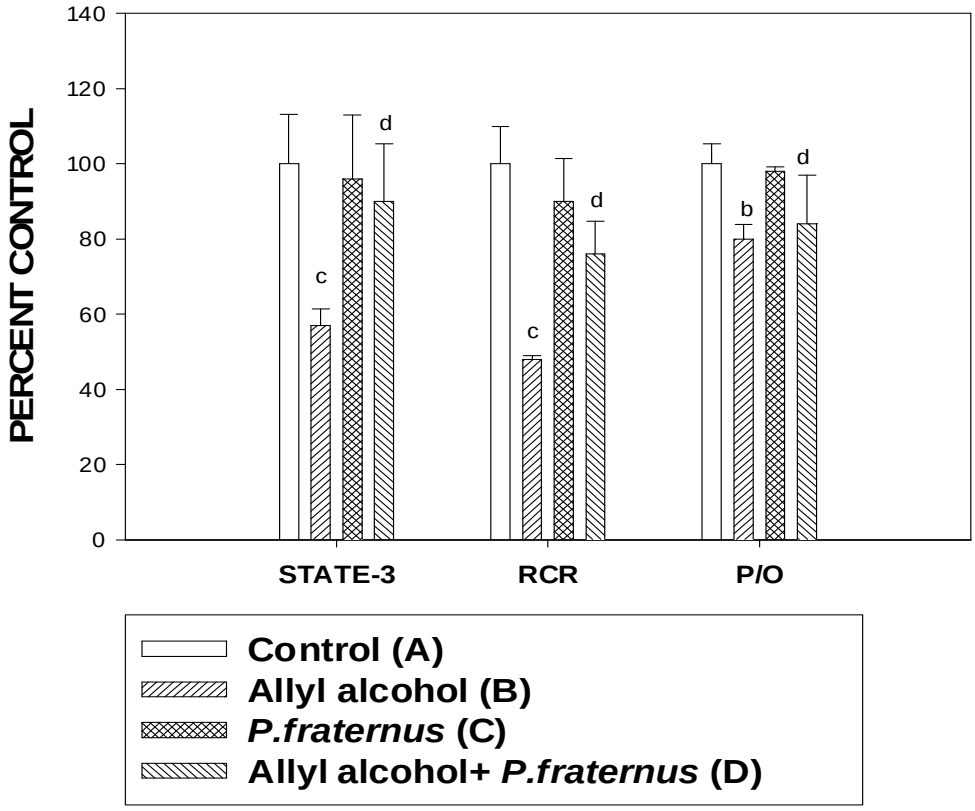
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on the rate of respiration, RCR, P/O ratio using glutamate + malate as substrate



Values are mean± SD of at least four rats in each group. 2mg protein was used for each assay. Rate of respiration was measured using glutamate + malate as substrate by the addition of 400 nmols of ADP. It is expressed as nano moles of oxygen per minute per milligram protein. RCR is the ratio of ADP stimulated state-3 to ADP exhausted state 4 respiration and P/O=ADP/O ratio. All the values are expressed relative to controls which were taken as 100. Control values for the rate of respiration, RCR, P/O ratio using glutamate and malate as substrates were 32.3±4.6, 4.1±0.3, 3.0±0.7 respectively. $^{a}p<0.05$ vs. Group-A. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. Group-B. $^{f}p<0.05$ vs. Group-B.

FIGURE 2

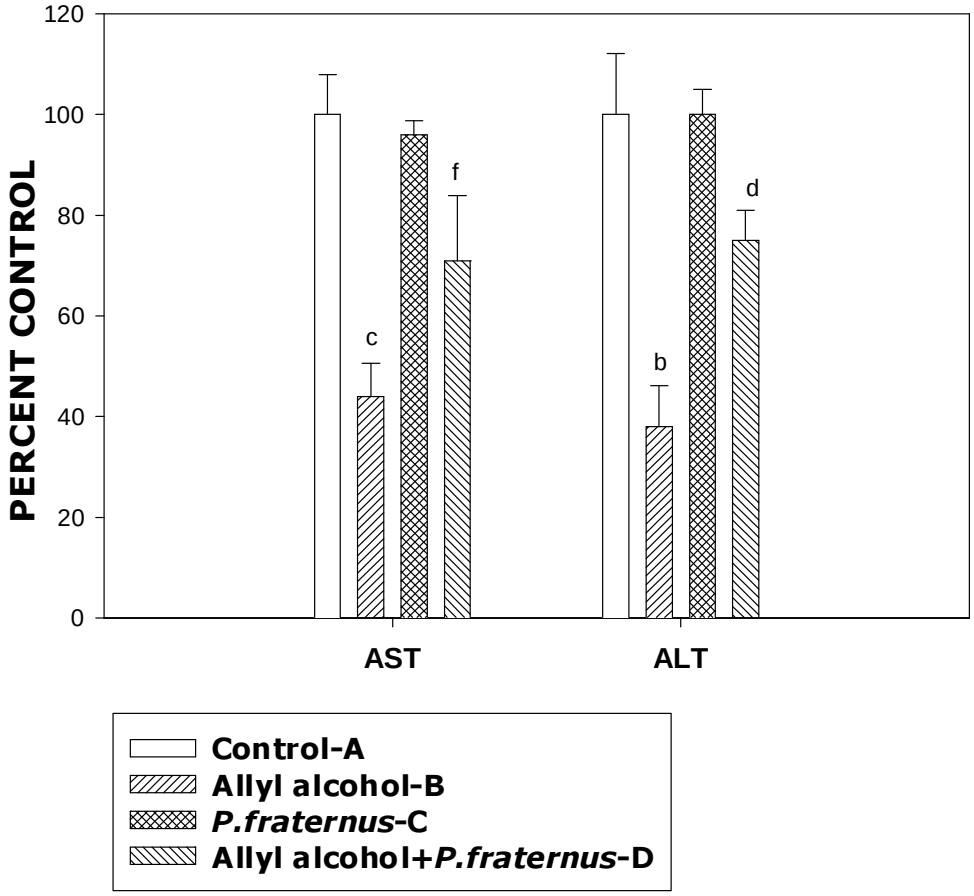
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on the rate of respiration, RCR, P/O ratio using succinate as substrate



Values are mean± SD of at least four rats in each group. 2mg protein was used for each assay. Rate of respiration was measured using succinate as substrate by the addition of 200 nmols of ADP. It is expressed as nano moles of oxygen per minute per milligram protein. RCR is the ratio of ADP stimulated state-3 to ADP exhausted state 4 respiration and P/O=ADP/O ratio. All the values are expressed relative to control which were taken as 100. Control values for the rate of respiration, RCR, P/O ratio using succinate as substrate were 53.3±6.9, 4.0±0.4, 2.0±0.1 respectively. $^{b}p<0.01$ vs. Group-A. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. Group-B.

FIGURE 3

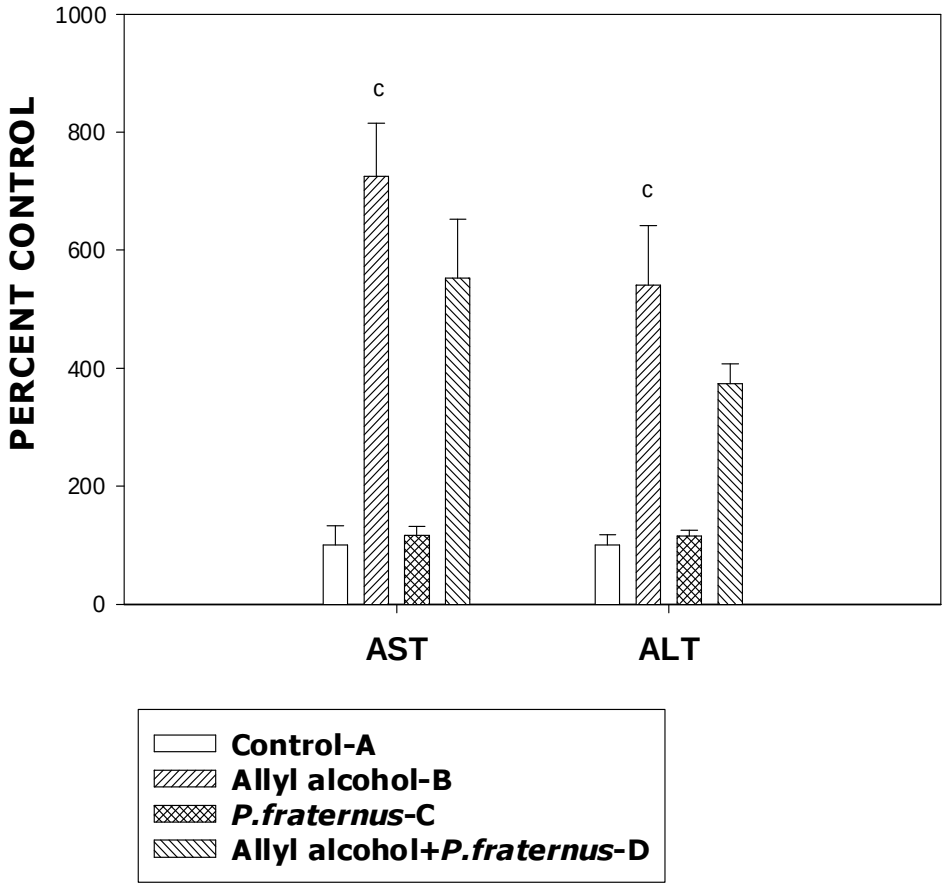
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on Aspartate amino transferase (AST) and alanine amino transferase activity (AlAT) in liver homogenate



Values are given as percent control, and are mean ± SD of atleast four animals. 40µg protein was used for each assay. Aspartate amino transferase (AST) and alanine amino transferase (AlAT) activities are expressed as µmoles of NADH oxidized per hour per mg protein. The control values of AST and AlAT in liver homogenate were 51±5.13, 3.02±0.67 respectively. $^{b}p<0.01$ vs. Group-A. $^{c}p<0.001$ vs. Group-A. $^{f}p<0.05$ vs. Group-B. $^{d}p<0.01$ vs. Group-B.

FIGURE 4

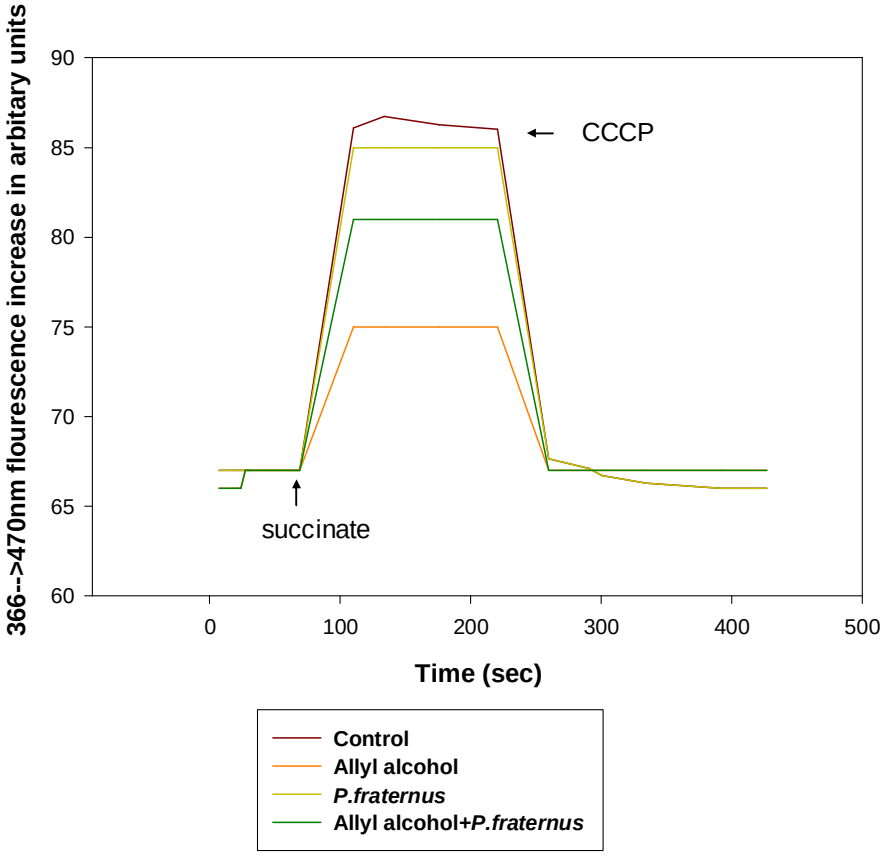
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on Aspartate amino transferase (AST) and alanine amino transferase activity (AlAT) in serum



Values are given as percent control, and are mean ± SD of atleast four animals. 25µl serum was used for each assay. Aspartate amino transferase (AST) and alanine amino transferase (AlAT) activities were expressed as µmoles of NADH oxidized per hour per mg protein. The control values of AST and AlAT in serum were 0.094±0.031, 0.015±0.002 respectively. $^{c}p<0.001$ vs. Group-A.

FIGURE 5A

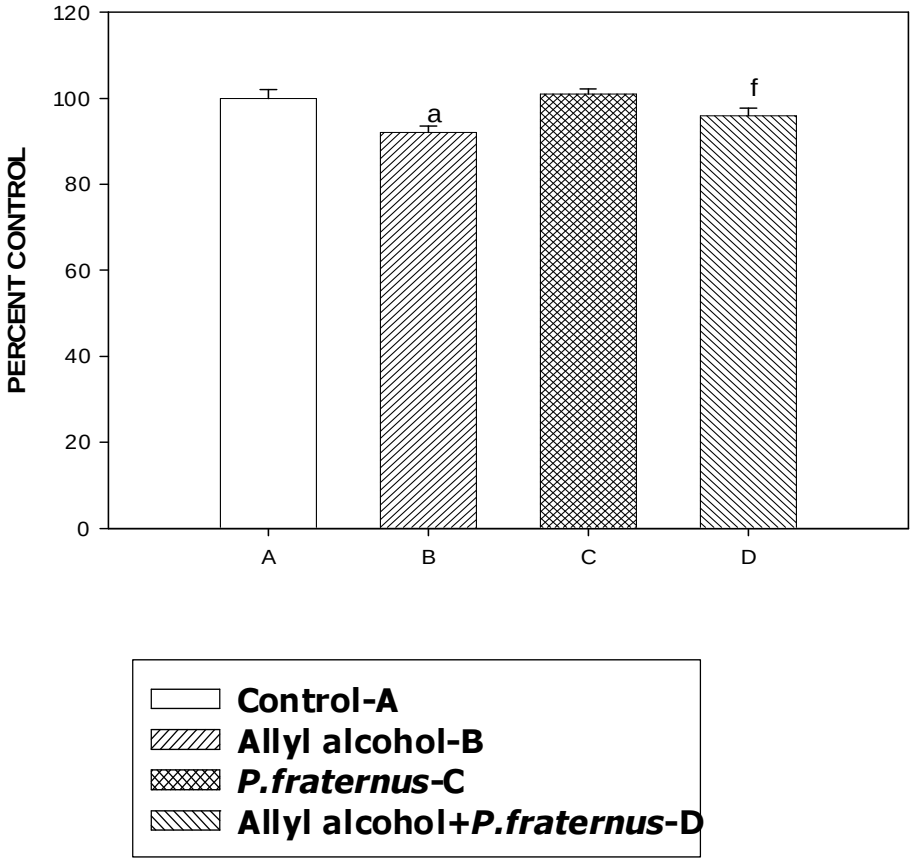
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on membrane potential in SMP



This figure shows the original traces of fluorescence changes of ANS in submitochondrial particles**.** 1mM succinate was added to the reaction system [submitochondrial particles (380 µg protein), buffered medium (containing 250mM sucrose, 10mM Tris-HCl, 5mM $MgCl_2$, pH 7.5) and 10µM ANS in a total volume of 1.0 ml] to energise the membrane and the fluorescence change was recorded. The addition of succinate induced an increase in ANS fluorescence. After the fluorescence change reached to a plateau, CCCP (a potent uncoupler) was added (0.3µM) to bring down the membrane to deenergised state. The intensity of fluorescence was measured in a fluorescence spectrophotometer. The excitation and emission wave lengths used were 366nm and 470nm respectively.

FIGURE 5B

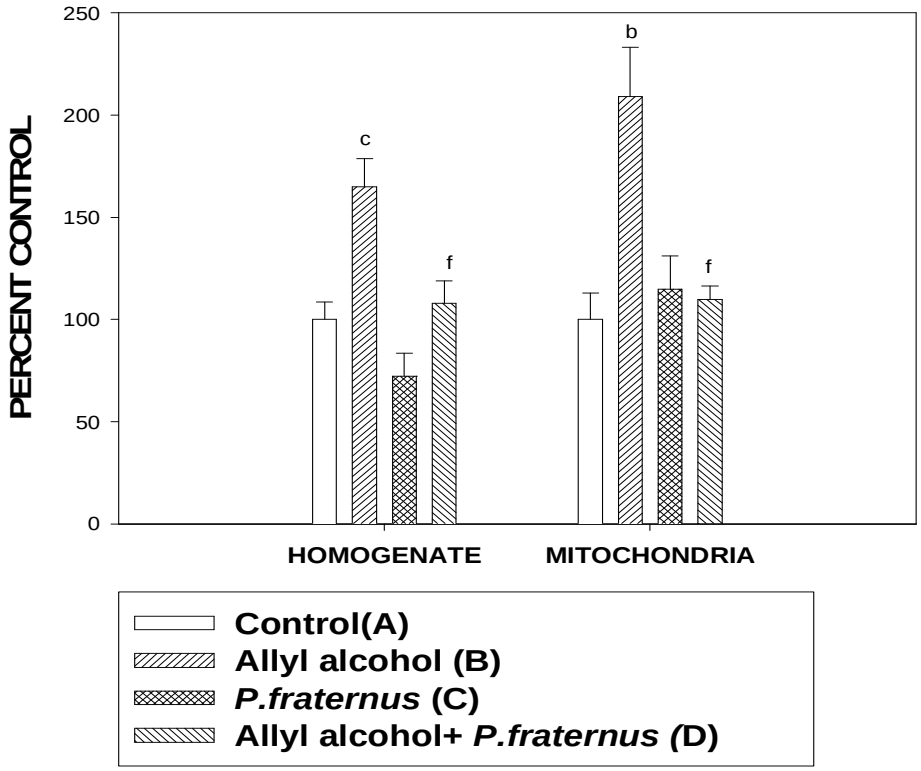
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on membrane potential in SMP



Values are given as percent control, and are mean ± SD of atleast four animals. 380µg SMP protein was used for each assay. Membrane potential is expressed as millivolts. The control value was 150±3.0. $^{a}p<0.05$ vs. Group-A. $^{f}p<0.05$ vs. Group-B.

FIGURE 6

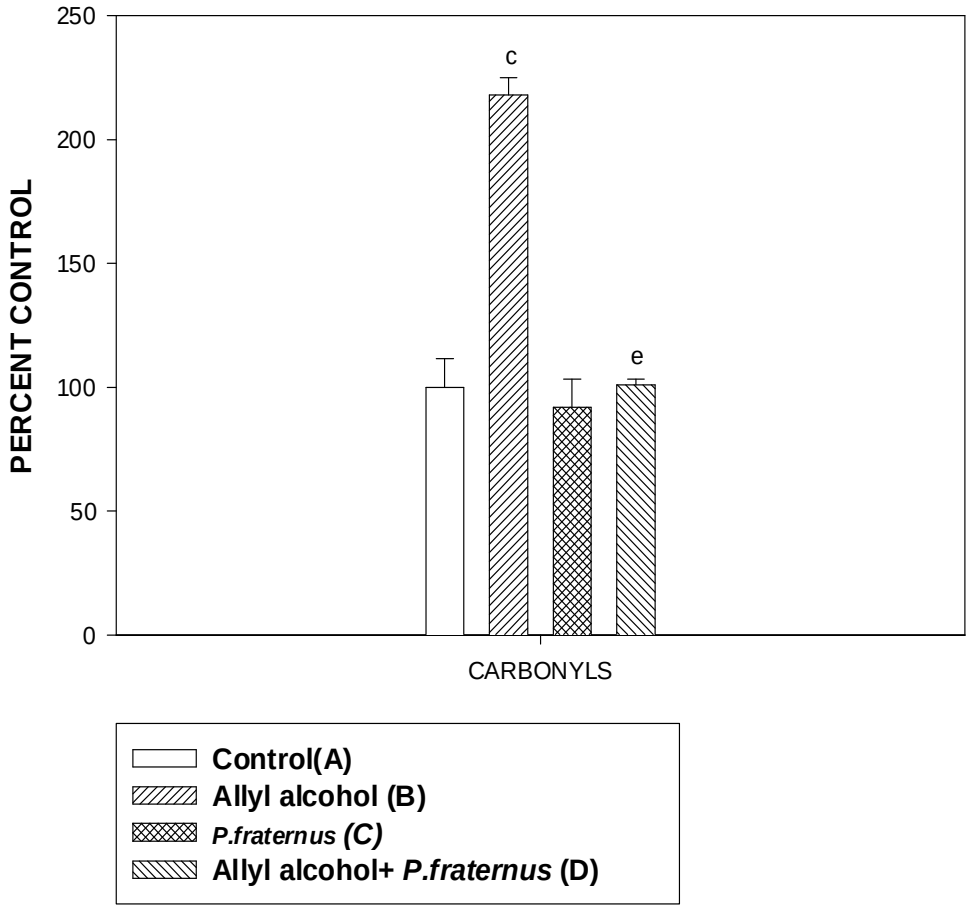
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on lipid peroxide level in liver homogenate and mitochondria



Values are given as percent control, and are mean ± SD of at least four animals. 5mg protein was used for each assay. Lipid peroxide level is expressed as nmol MDA formed per 100mg protein. The control values in homogenate and mitochondria were 115.0±10.0, 108.3±14.4 respectively. $^{b}p<0.01$ vs. Group-A. $^{c}p<0.001$ vs. Group-A. $^{f}p<0.05$ vs. Group-B.

FIGURE 7

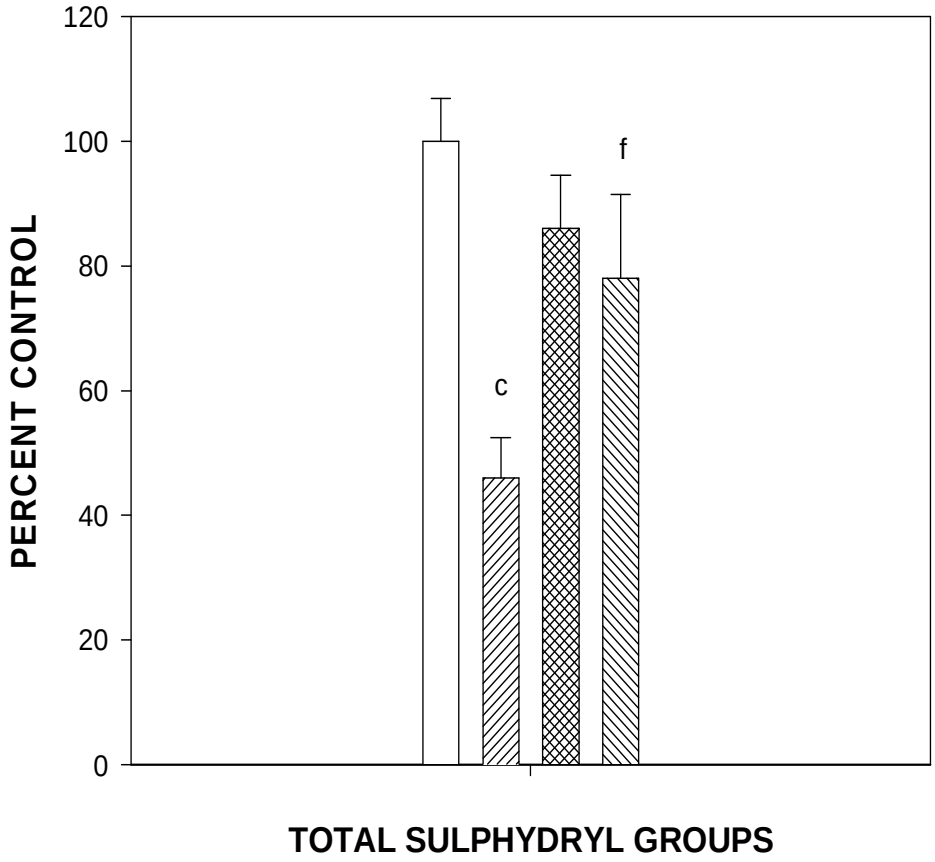
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on protein carbonyl content



The values are mean ± SD of at least four animals. 4mg protein was used for each assay. Protein carbonyl content is expressed as nmol per mg protein. The control value for the protein carbonyl content was 1.0±0.1. $^{c}p<0.001$ vs. Group-A. $^{e}p<0.001$ vs. Group-B.

FIGURE 8

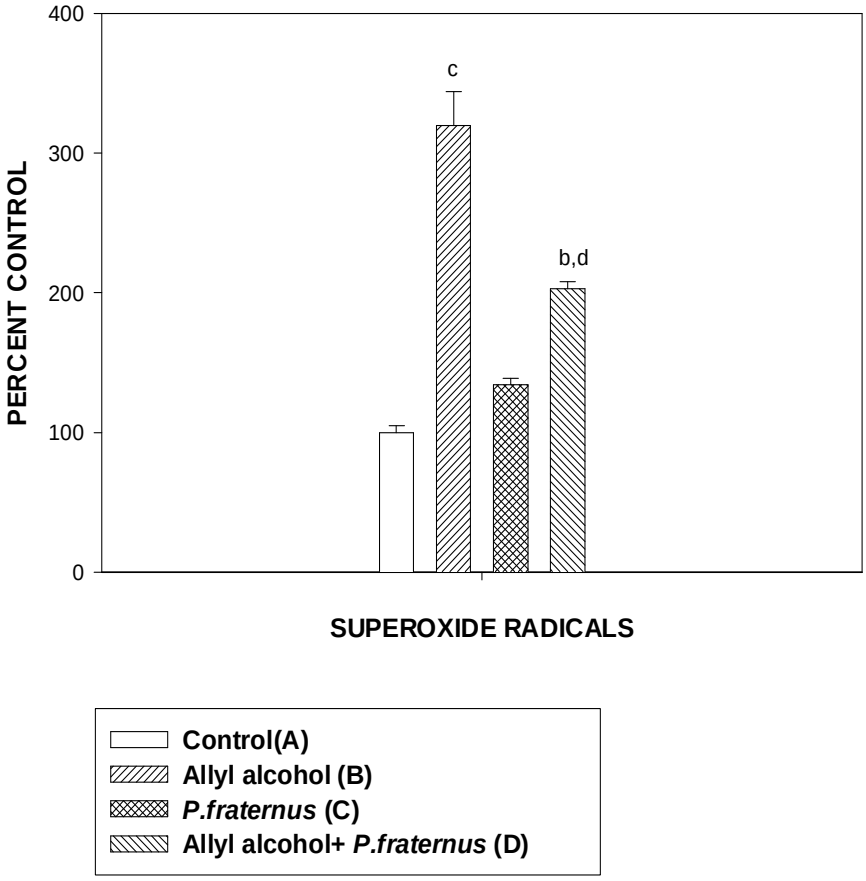
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on total sulphydryl groups in rat liver mitochondria



The values are mean ± SD of at least four animals. 1mg protein was used for each assay. Total sulphydryl groups are expressed as nmol per milligram protein. The control value for total sulphydryl groups were 8.9±0.6. $^{c}p<0.001$ vs. Group-A. $^{f}p<0.05$ vs. Group-B.

FIGURE 9

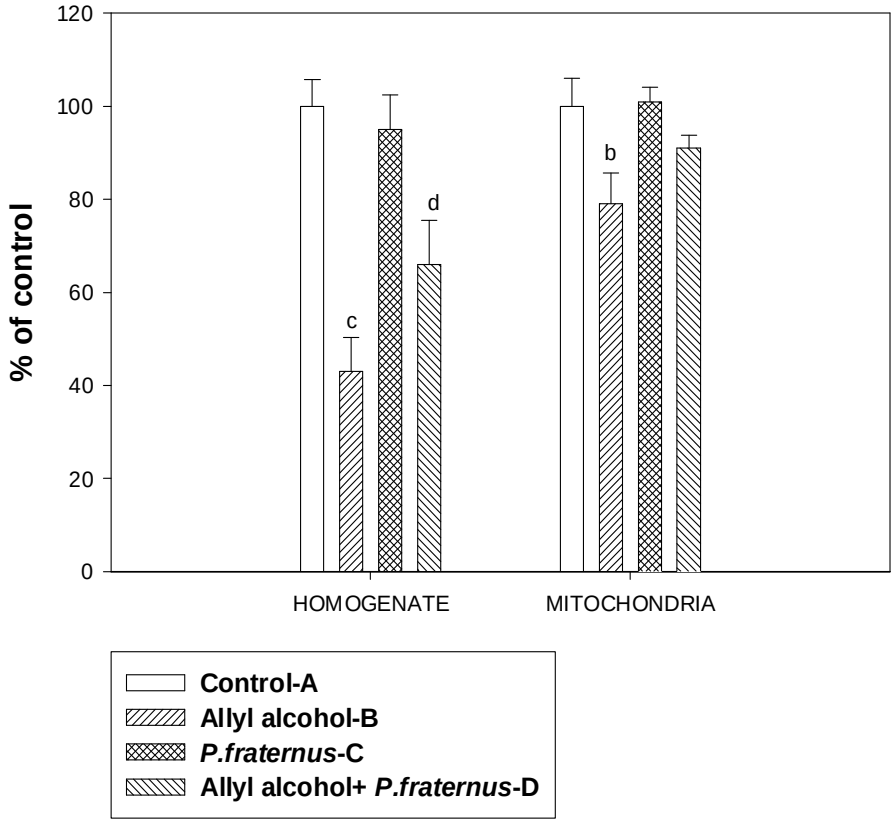
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on generation of superoxide radicals in mitochondrial fragments



The values are mean ± SD of at least four animals. 0.1mg prorein of mitochondrial fragments were used for each assay. Generation of superoxide radicals are expressed as nmol per minute per milligram protein. The control value for generation of superoxide radicals 14.5±0.7. $^{b}p<0.01$ vs. Group-A. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. GroupB.

FIGURE 10

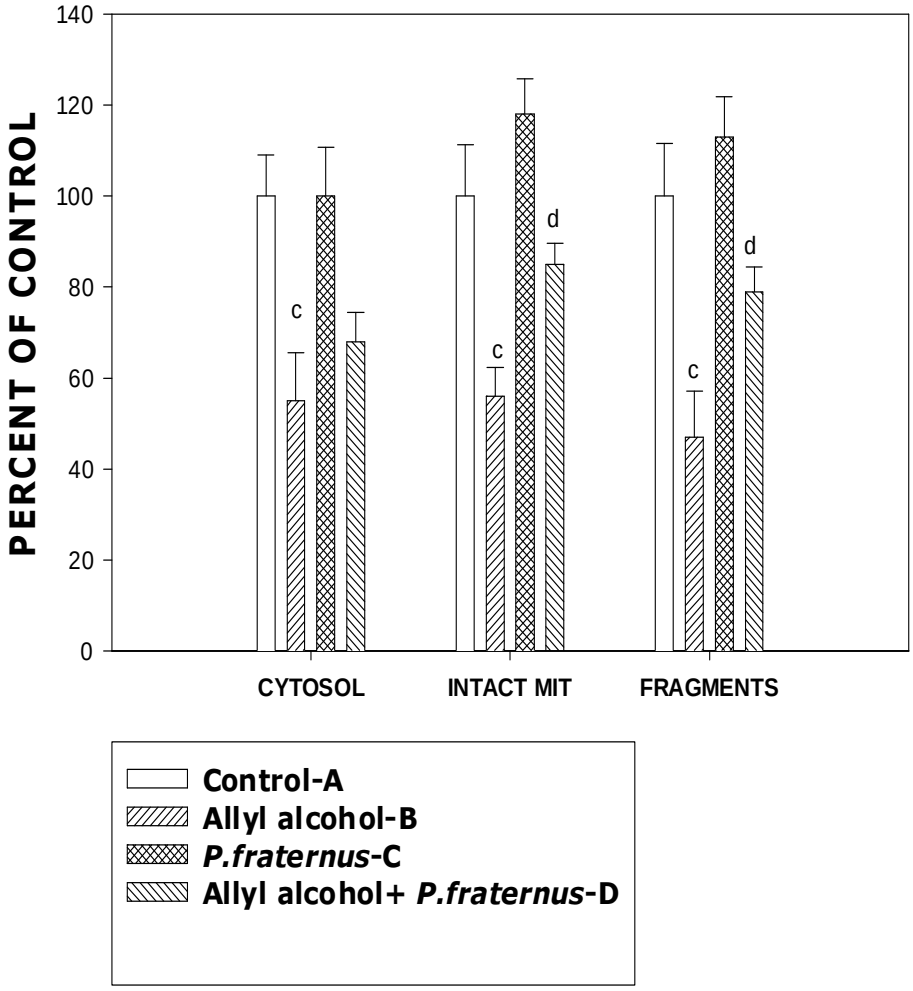
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on catalase activity



Values are given as percent control, and are mean ± SD of atleast four animals. 0.3mg of homogenate protein and 0.1mg of mitochondrial protein were used for each assay. Catalase activity is expressed as nmols of H_2O_2 consumed per min per mg protein. The control values in homogenate and mitochondria were 681 ± 39.05, 1816±108.17 respectively. $^{b}p<0.01$ vs. Group-A. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. Group-B.

FIGURE 11

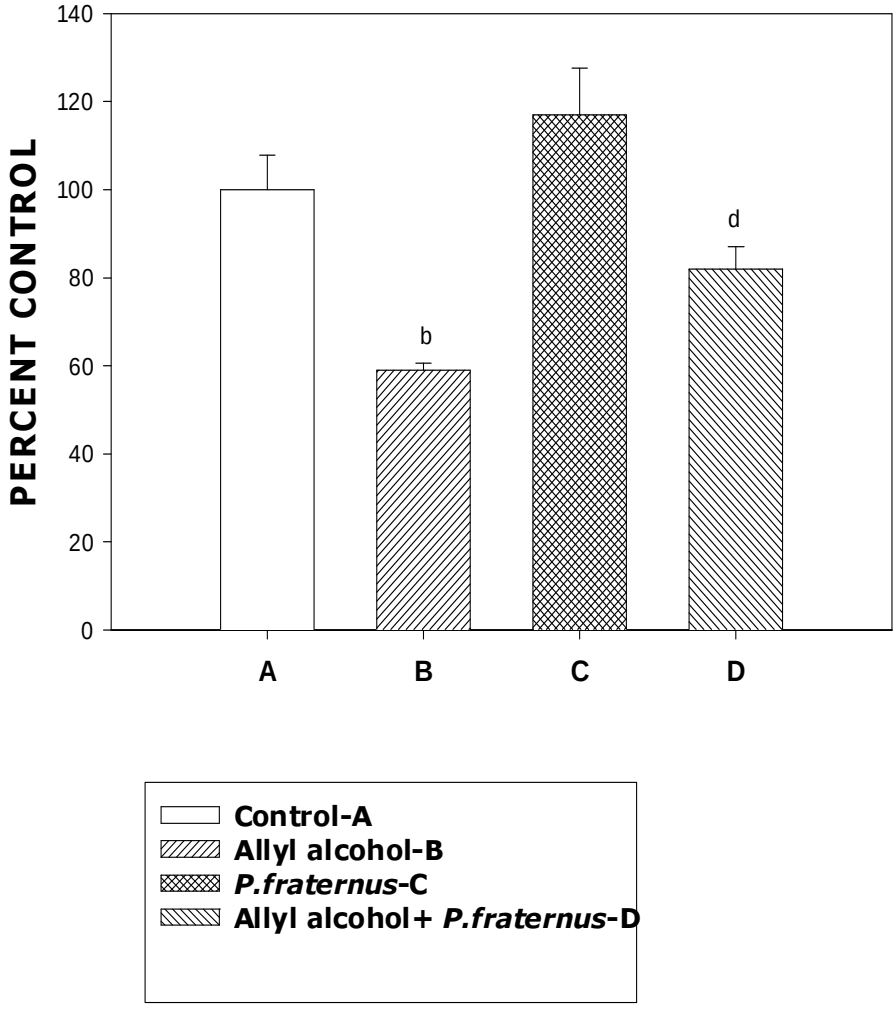
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on superoxide dismutase activity



Values are given as percent control, and are mean ± SD of atleast four animals. 0.1mg protein of sample (mitochondrial fragments or intact mitochondria or cytosol were used for each assay. Superoxide dismutase activity is expressed as units per milligram protein. The control values of superoxide dismutase of intact mitochondria, cytosol and membrane fragments were 4.53 ±0.513, 3.7±0.331, 2.84±0.328 respectively. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. Group-B.

FIGURE 12

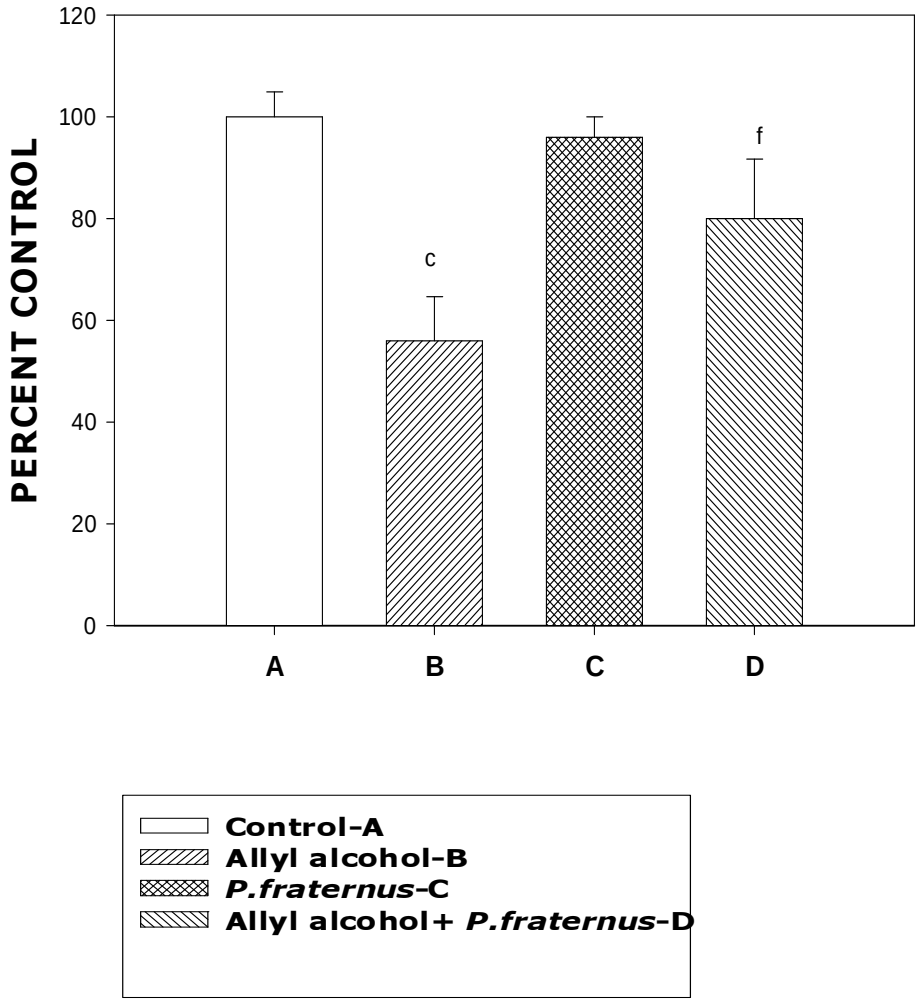
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on glutathione peroxidase activity



Values are given as percent control, and are mean ± SD of atleast four animals. 0.1mg of homogenate protein was used for each assay. Glutathione peroxidase activity is expressed as nmols of NADPH oxidized per min per milligram protein. The control value of glutathione peroxidase was 172.6±13.42. $^{b}p<0.01$ vs. Group-A. $^{d}p<0.01$ vs. Group-B.

FIGURE 13

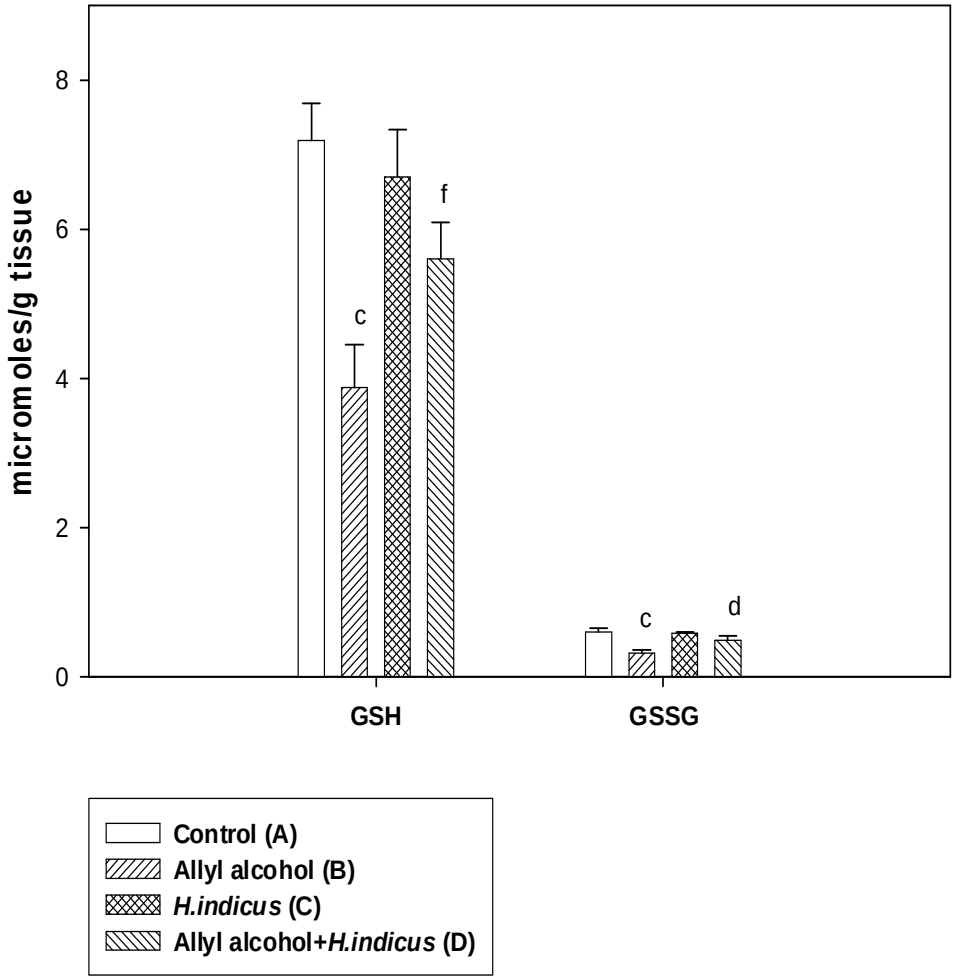
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on glutathione reductase activity



Values are given as percent control, and are mean ± SD of atleast four animals. 0.1mg of mitochondrial protein was used for each assay. Glutathione reductase activity is expressed as nmols of NADPH oxidized per min per milligram protein. The control value of glutathione reductase was 74.6±3.65. $^{c}p<0.001$ vs. Group-A. $^{f}p<0.05$ vs. Group-B.

FIGURE 14

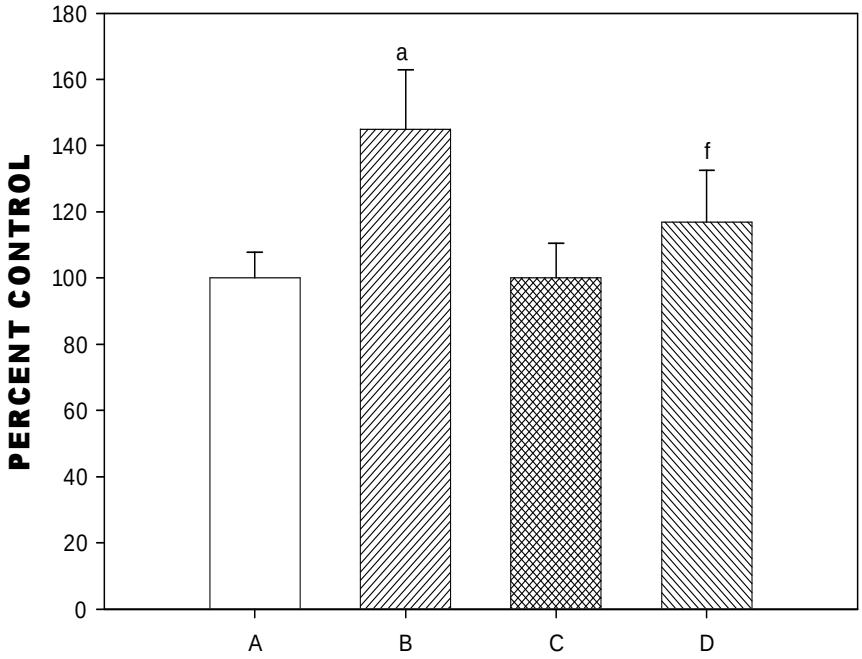
Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on levels of glutathione



Values are mean ± SD of atleast four animals. Glutathione levels are expressed as µmoles per g tissue. The control values of GSH, GSSG were 7.19±0.97, 0.603±0.048 respectively. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. Group-B. $^{f}p<0.05$ vs. Group-B.

FIGURE 15

Effect of administration of allyl alcohol with or without prior administration of *P.fraternus* on alcohol dehydrogenase activity

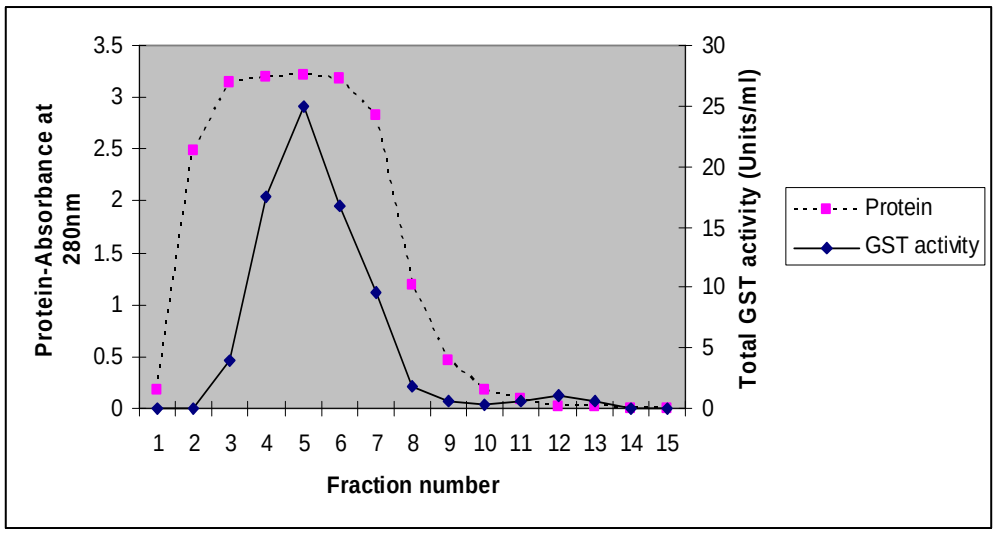


Control-A
Allyl alcohol-B
***P.fraternus*-C**
Allyl alcohol+ *P.fraternus*-D

Values are given as percent control, and are mean ± SD of atleast four animals. 0.6mg protein of cytosol was used for each assay. Alcohol dehydrogenase activity is expressed as µmoles of NADH formed/min/mg. The control value of alcohol dehydrogenase was 19±1.52. $^{a}p<0.05$ vs. Group-A. $^{f}p<0.05$ vs. Group-B.

FIGURE 16

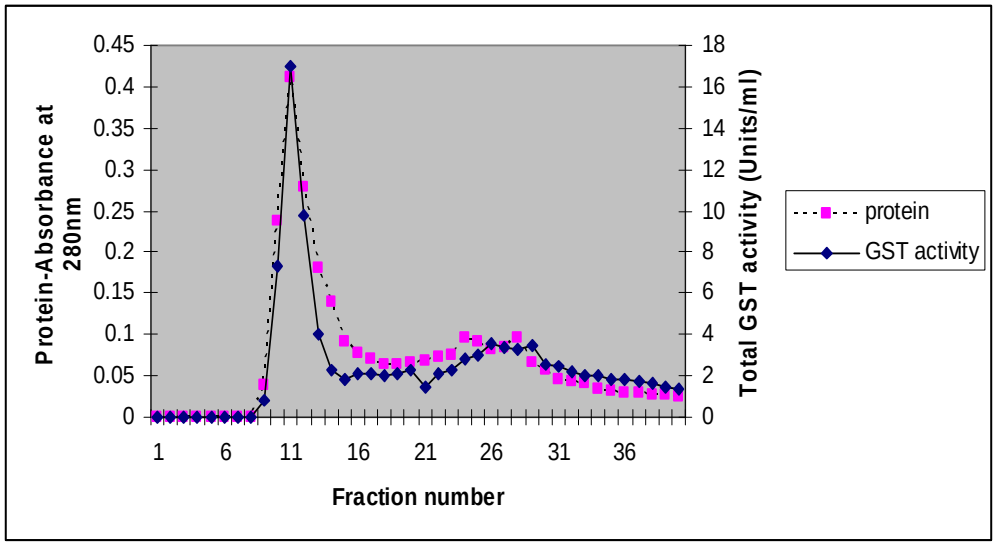
Gel filtration of cytosol from control rat liver on sephadex G-150 column



The column was previously equilibrated with 25mM Tris-Hcl, pH-8.0. Same buffer was used as eluent and 5.0ml fractions were collected at a flow rate of 1ml/min. GST activity was measured using CDNB as substrate and the protein was measured from absorption at 280nm.

FIGURE 17

GSH affinity elution pattern of control liver GST



Affinity chromatography of pooled fractions from gel filtration of cytosol from control rat liver on GSH linked epoxy activated sepharose 6B. The

column was washed with 10mM potassium phosphate buffer pH 7.0 containing 0.15M KCl. This was followed by elution with 50mM potassium phosphate buffer pH 7.5 containing 10mM GSH and fractions were collected at a flow rate of 1ml/min. GST activity was measured using CDNB as substrate and the protein was measured from absorption at 280nm.

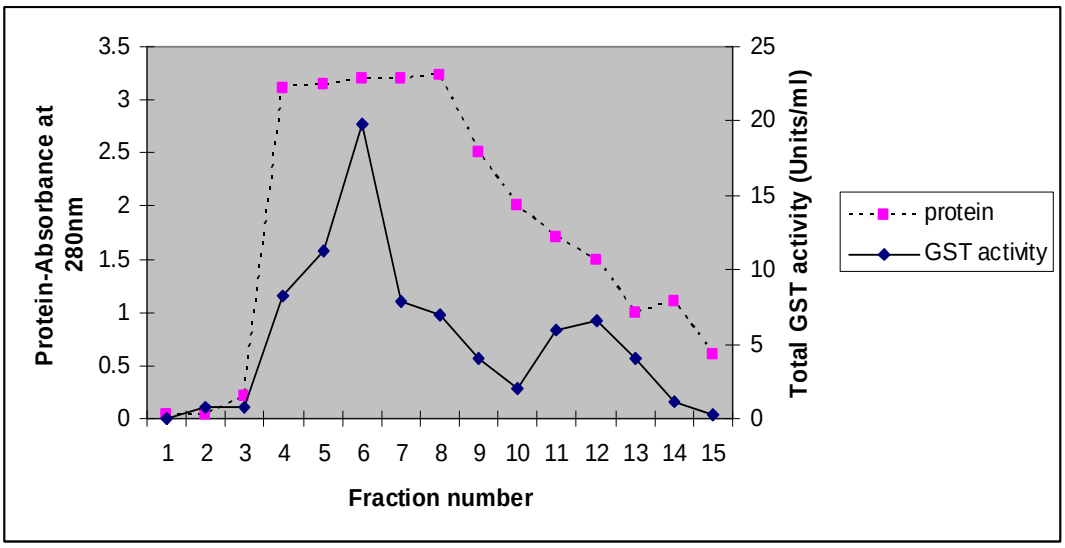
TABLE I

Typical purification profile of control rat liver cytosolic GST

	Total protein	**Total activity**	**Specific activity**	**Yield**	**Fold purification**
Crude extract	385	266	0.69	100	1
Gel filtration	184	238	1.29	89	1.87
Affinity column	8.5	180	21	68	30

FIGURE 18

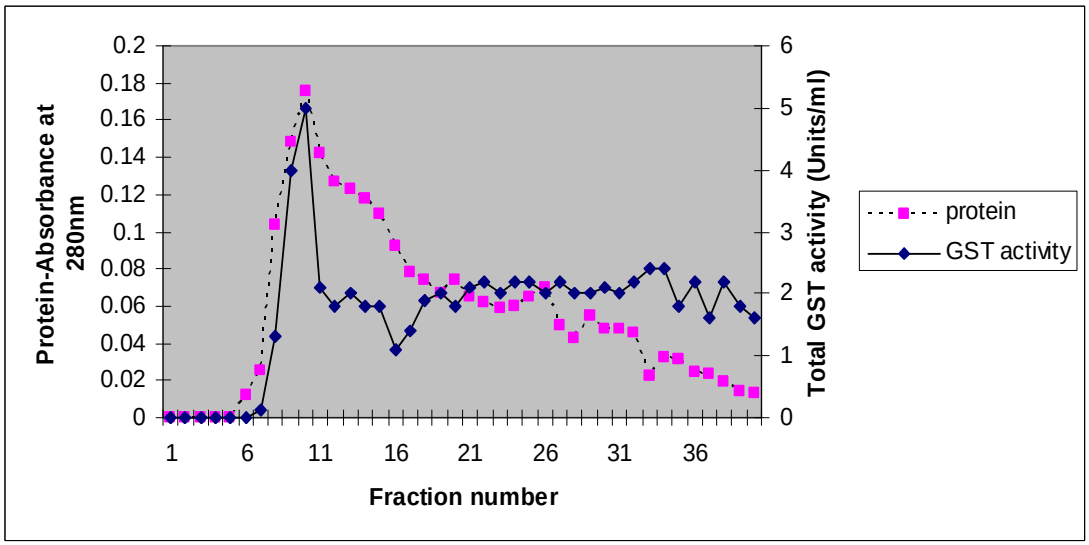
Gel filtration of cytosol from allyl alcohol administered rat liver on Sephadex G-150 column



The column was previously equilibrated with 25mM Tris-Hcl, pH-8.0. Same buffer was used as eluent and 5.0ml fractions were collected at a flow rate of 1ml/min. GST activity was measured using CDNB as substrate and the protein was measured from absorption at 280nm.

FIGURE 19

GSH affinity elution pattern of allyl alcohol administered liver GST



Affinity chromatography of pooled fractions from gel filtration of cytosol from allyl alcohol administered rat liver on GSH linked epoxy activated sepharose 6B. The column was washed with 10mM potassium phosphate

buffer pH 7.0 containing 0.15M KCl. This was followed by elution with 50mM potassium phosphate buffer pH 7.5 containing 10mM GSH and fractions were collected at a flow rate of 1ml/min. GST activity was measured using CDNB as substrate and the protein was measured from absorption at 280nm.

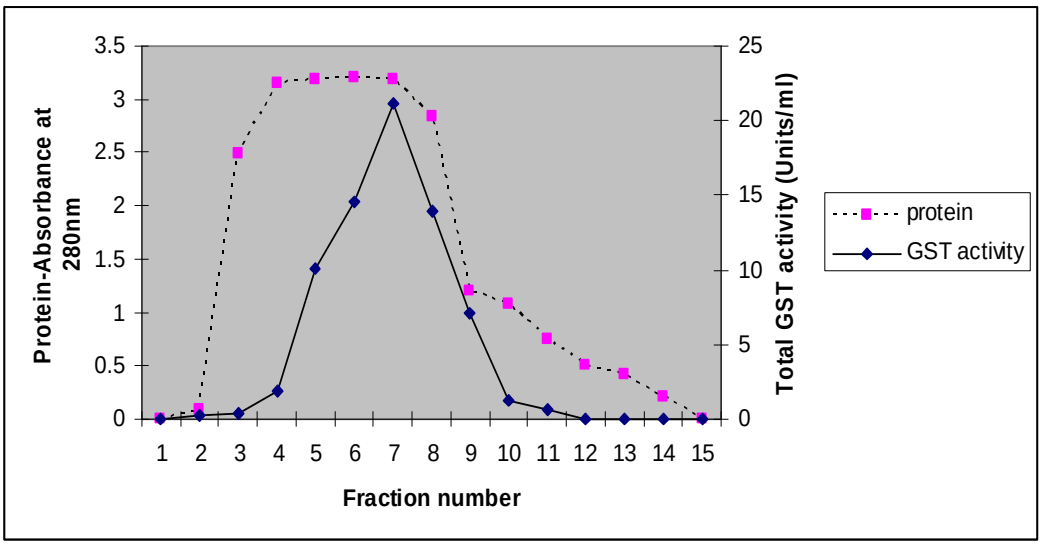
TABLE 2

Typical purification profile of cytosolic GST from allyl alcohol treated rat liver

	Total protein	**Total activity**	**Specific activity**	**Yield**	**Fold purification**
Crude extract	583	182	0.31	100	1
Gel filtration	338	155	0.57	85	1.8
Affinity column	12	112	8.7	62	28

FIGURE 20

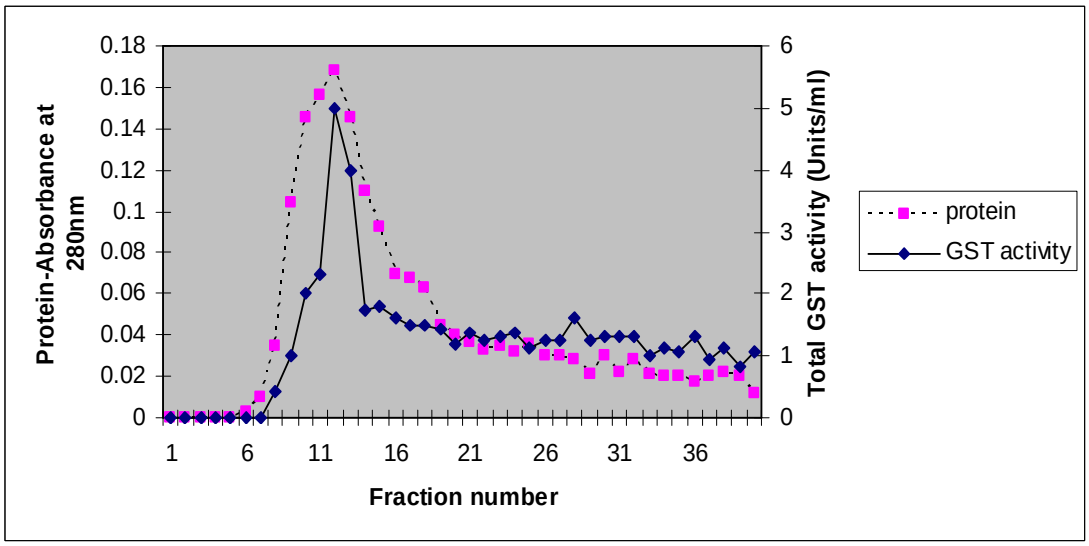
Gel filtration of cytosol from allyl alcohol+*P.fraternus* administered rat liver on sephadex G-150 column



The column was previously equilibrated with 25mM Tris-Hcl, pH-8.0. Same buffer was used as eluent and 5.0ml fractions were collected at a flow rate of 1ml/min. GST activity was measured using CDNB as substrate and the protein was measured from absorption at 280nm.

FIGURE 21

GSH affinity elution pattern of allyl alcohol+*P.fraternus* administered rat liver GST



Affinity chromatography of pooled fractions from gel filtration of liver cytosol from rats administered with both allyl alcohol and *P.fraternus* on

GSH linked epoxy activated sepharose 6B. The column was washed with 10mM potassium phosphate buffer pH 7.0 containing 0.15M KCl. This was followed by elution with 50mM potassium phosphate buffer pH 7.5 containing 10mM GSH and 1.0ml fractions were collected at a flow rate of 1ml/min. GST activity was measured using CDNB as substrate and the protein was measured from absorption at 280nm.

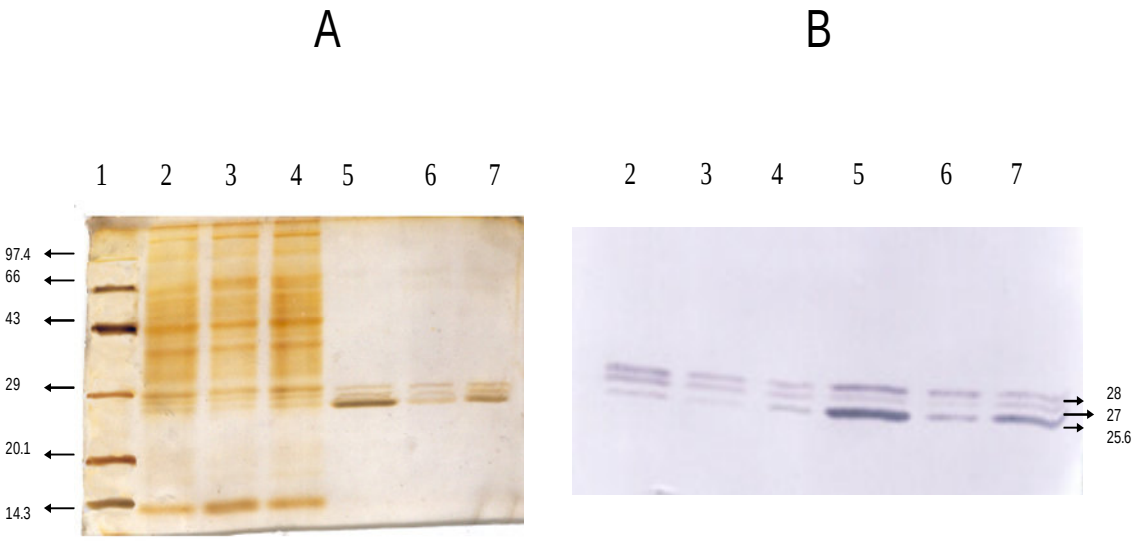
TABLE 3

Typical purification profile of cytosolic GST from allyl alcohol+*P.fraternus* treated rat liver

	Total protein	**Total activity**	**Specific activity**	**Yield**	**Fold purification**
Crude extract	450	198	0.44	100	1
Gel filtration	222	171	0.77	86	1.75
Affinity column	8	118	14.7	60	33

FIGURE 22

SDS-PAGE and western blot of hepatic glutathione transferases



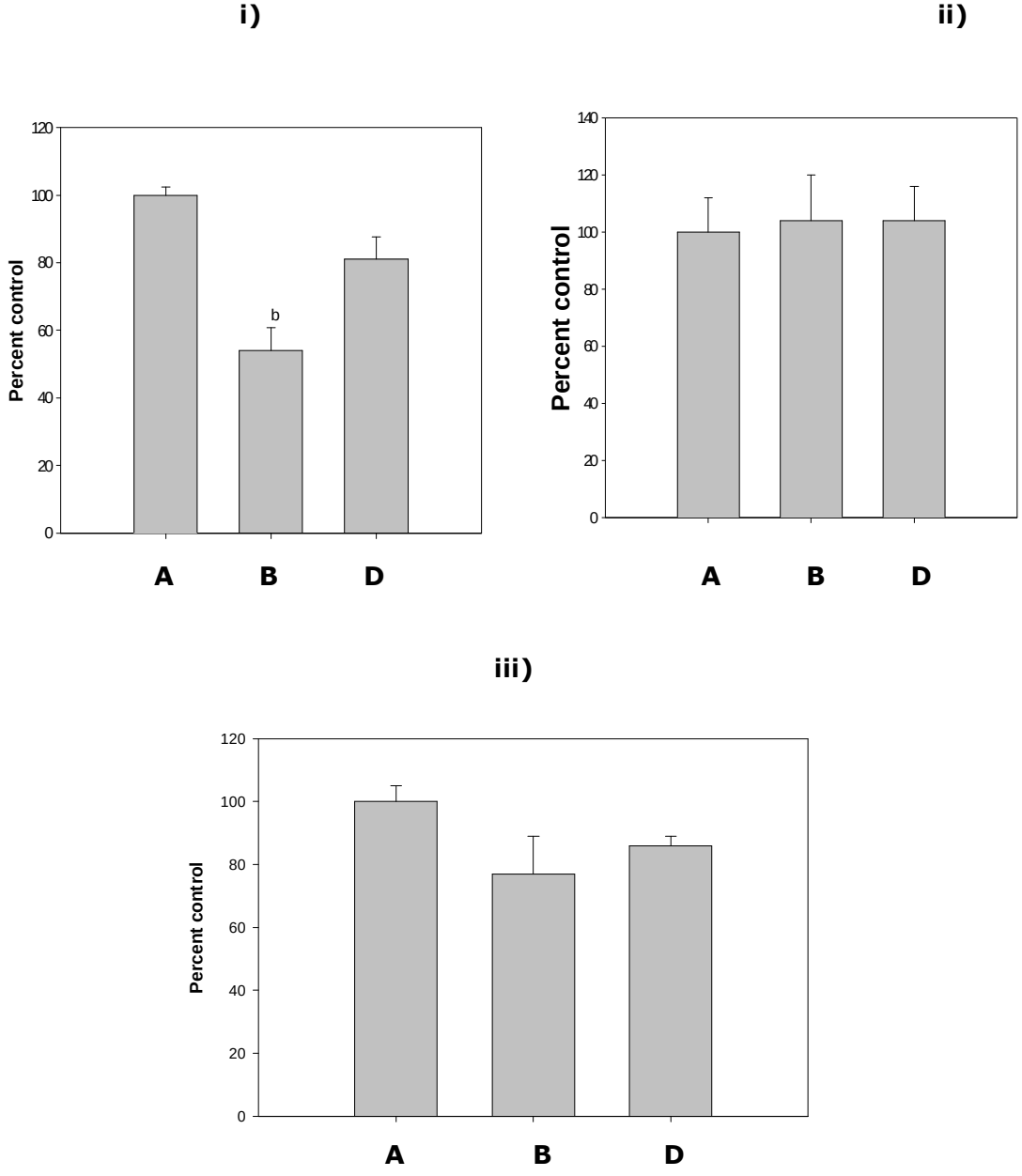
A- Shows SDS-PAGE of hepatic glutathione transferases. 10µg of crude extract or 1 µg of affinity purified cytosolic GSTs were loaded on 12% resolving gel.

B- Shows western blot of hepatic glutathione transferases

1. **Molecular weight marker**
2. **Crude extract from control rat liver**
3. **Crude extract from allyl alcohol treated rat liver**
4. **Crude extract from allyl alcohol + *p.fraternus* treated rat liver.**
5. **Affinity purified cytosolic GST from control rat liver**
6. **Affinity purified cytosolic GST from allyl alcohol treated rat liver**
7. **Affinity purified cytosolic GST from allyl alcohol+ *P.fraternus* treated rat liver**

FIGURE 23

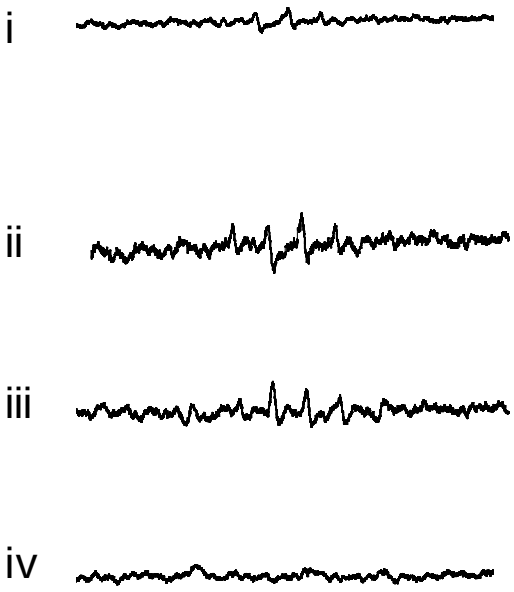
Densitometric scan of affinity purified GSTs



i, ii and iii shows densitometric quantification of Ya, Yb and Yc subunits of GST in arbitary units. $^{b}p<0.01$ vs. control group. Densitometer was used for quantification.
A, B and D are affinity purified GST subunits from control, allyl alcohol administered and allyl alcohol+*P.fraternus* administered rat liver cytosol.

FIGURE 24

Original traces of ESR signals of DMPO-OH adducts using glutamate + malate as substrate



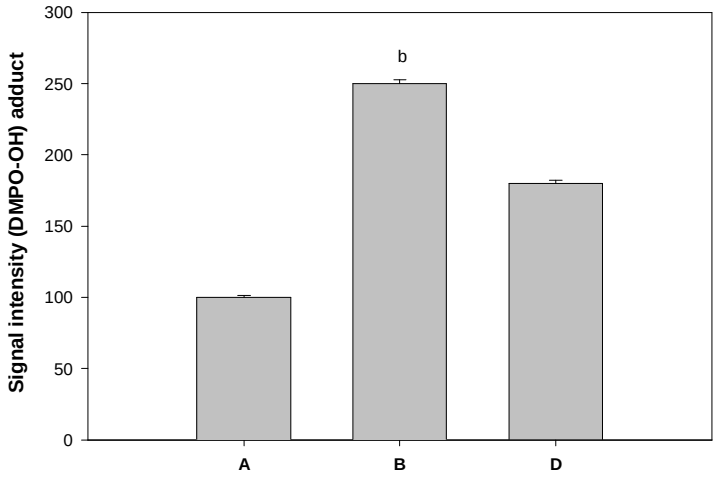
For ESR analysis mitoplasts (1 mg protein) were placed in 200µl of buffer (230mM mannitol, 70mM sucrose, 20mM Tris-HCl, pH adjusted to 7.4 with Mops) in the presence of respiratory substrates glutamate (7.5mM), malate (7.5mM) and inhibitors like antimycin (1µg/mg protein). DMPO (160mM) was added and the ESR spectra were recorded on a JES-FA 200 ESR spectrometer. Instrument settings were as follows: microwave power, 20mW; microwave frequency, 9.42 GHz; time constant, 0.03sec; scan time, 4min; scan width, 100G.

i, ii and iii shows original traces of ESR signals of rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*P.fraternus* administered) in the presence of glutamate and malate.

iv It is due to the addition of superoxide dismutase which completely abolished the ESR signal.

FIGURE 25

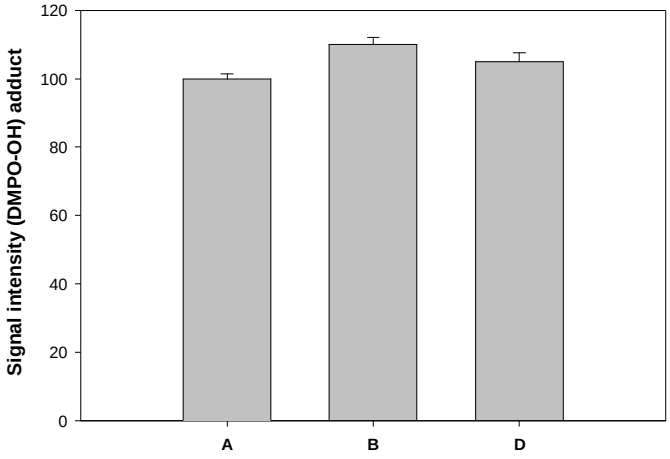
Signal intensities of DMPO-OH adducts in rat liver mitoplasts in the presence of glutamate and malate



It shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*P.fraternus* administered) in the presence of glutamate and malate. $^{b}p<0.01$ vs. control Group.

FIGURE 26

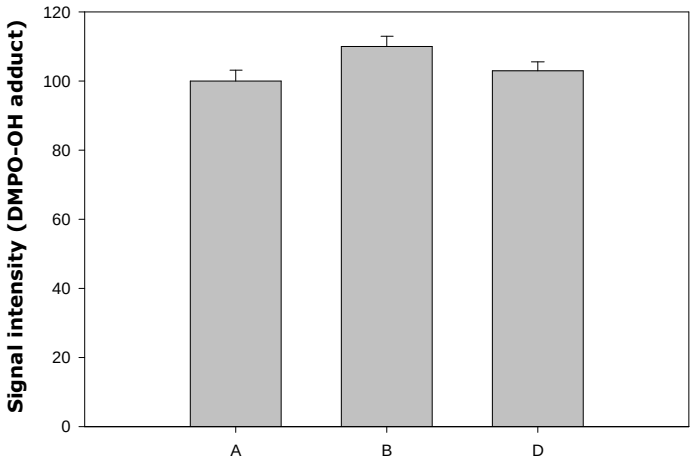
Signal intensities of DMPO-OH adducts in rat liver mitoplasts in the presence of glutamate, malate and antimycin



It shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*P.fraternus* administered) in the presence of antimycin (1µg/mg protein), glutamate and malate. There was no significant difference.

FIGURE 27

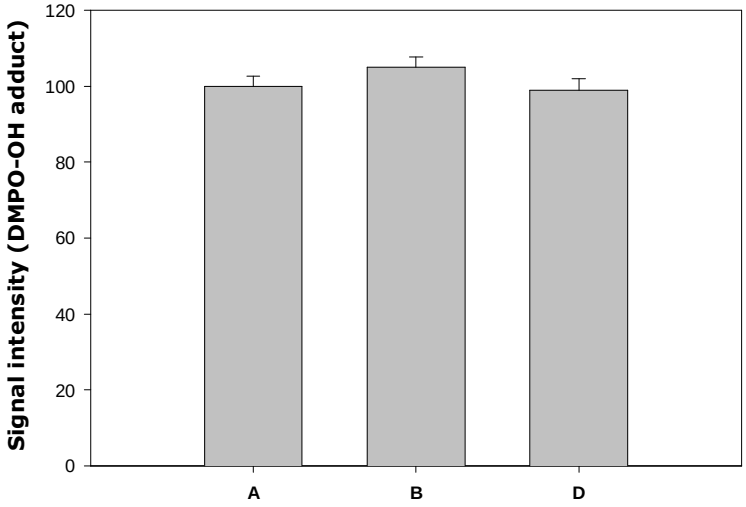
Signal intensities of DMPO-OH adducts in rat liver mitoplasts in the presence of succinate



It shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*P.fraternus* administered) in the presence of succinate (7.5mM). There was no significant difference.

FIGURE 28

Signal intensities of DMPO-OH adducts in rat liver mitoplasts in the presence of succinate and antimycin



It shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*P.fraternus* administered) in the presence of succinate (7.5mM) and antimycin (1µg/mg protein). There was no significant difference.

Hemidesmus indicus

In the present study the effect of administration of allyl alcohol on the oxidative stress and the protective effect of prior administration of aqueous extract of *Hemidesmus indicus* on allyl alcohol induced oxidative stress were studied.
The percent protective effect due to the prior administration of *Hemidesmus indicus* was calculated as follows.
[100/100 – (Value of group B)] X (Value of group D – Value of group B)
In other words a 100% protection (on a given parameter) means that the value is back to the control level which is normalized as 100.

NADH oxidase (through glutamate and malate)

Fig.29A is a original trace of oxygen concentration obtained by oxytherm respirometer. ADP stimulated respiration is taken as state 3 and ADP exhausted respiration as state 4. Figure 29B shows the effect of administration of allyl alcohol and the protective effect of prior administration of *H.indicus* on state 3 respiration, respiratory control ratio (RCR) and P/O ratio.

Externally added NADH cannot penetrate the tightly coupled mitochondria. So, glutamate and malate were used to reduce the internal NAD^+ pool and generate NADH in the matrix which gives electrons to the respiratory chian. Studies on this enzyme complex give information on the ability of transfer of electrons through all the three sites. The P/O ratio is the index of efficiency of the system to conserve energy in the form of ATP, while RCR gives an index of the integrity of the mitochondrial membrane.

State 3 respiration, RCR and P/O ratio were decreased by 32, 49, and 29% due to the administration of allyl alcohol when compared to controls (Figure 29) and this decrease is statistically significant. There was a significant increase on state 3 respiration, RCR and P/O in rats administered both allyl alcohol and *Hemidesmus indicus* when compared to rats administered only allyl alcohol. Administration of *Hemidesmus indicus* prior to allyl alcohol administration offered protection of 53, 47 and 62% on state 3 respiration, RCR and P/O ratios respectively. Administration of the plant extract alone did not show any change (Figure 29).

Studies on Succinate oxidase:

When succinate was used as substrate (electron transfer through site II + site III) there was a decrease of 33, 38 and 22% on state 3 respiration, RCR and P/O ratios respectively. There was a significant increase on state 3 respiration, RCR and P/O in rats administered allyl alcohol and *Hemidesmus indicus* when compared to rats administered only allyl alcohol. Administration of *Hemidesmus indicus* prior to allyl alcohol offered protection of 61, 79 and 68% on state 3 respiration, RCR and P/O ratios respectively (Figure-30). There was no significant effect on succinate oxidation in rats administered *Hemidesmus indicus* extract alone. These results shown that the administration of *Hemidesmus indicus* offered a significant amount of protection against the allyl alcohol induced toxicity.

Transaminases:

Aspartate amino transferase (AST):

Fig-31 and 32 shows the effect of allyl alcohol and *Hemidesmus indicus* on serum and liver aspartate aminotransferase (AST). Serum AST levels are significantly increased (625%) where as in liver homogenate AST levels are significantly decreased (49%) by the administration of allyl alcohol when compared to controls. These transaminases are known to decrease in liver and increase in serum during liver damage and are good index of the extent of the liver damage. Administration of *H.indicus* prior to allyl alcohol offered protection against the liver damage to an extent of 51, 46% in homogenate and serum. Thus the prior administration of aqueous extract of *Hemidesmus indicus* offered significant protection when compared to rats administered only allyl alcohol.

Alanine amino transferase (AlAT):

Fig-31 and 32 shows the activity of alanine amino transferase. The activity of AlAT decreased by 63% in liver homogenate where as it increased four folds (441%) in the serum by the administration of allyl alcohol. Administration of *H.indicus* prior to allyl alcohol offered protection of 55, 33% in homogenate and serum. Prior administration of aqueous extract of *Hemidesmus indicus* offered significant protection against the liver damage induced by allyl alcohol.

Membrane potential:

Fig-33A shows the original traces of fluorescence changes of ANS in submitochondrial particles. The addition of succinate energizes the membrane and builds the membrane potential which is followed by an increase in ANS fluorescence. The addition of CCCP, an uncoupler, deenergizes the membrane and membrane potential is dissipated which is followed by decrease in the fluorescence. The fluorescence changes were less due to allyl alcohol administration when compared to control. Fig-33B shows the effect of allyl alcohol with and without prior administration of *H.indicus* on membrane potential. Significant decrease in membrane potential was observed by the administration of only allyl alcohol. Prior administration of aqueous extract of *H.indicus* offered protection to an extent of 50%.

Lipid peroxide level:

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *Hemidesmus indicus* on lipid peroxide level is shown in Figure-34. In both homogenate and mitochondria the lipid peroxide level is significantly increased (84 and 109% respectively) due to the administration of only allyl alcohol compared to controls. There was a significant decrease in the lipid peroxide level of both homogenate and mitochondria in rats administered both *H.indicus* and allyl alcohol when compared to rats administered only allyl alcohol. Prior administration of aqueous extract of *Hemidesmus indicus* offered protection of 74 and 74% on lipid peroxides of both homogenate and mitochondria respectively.

Administration of *Hemidesmus indicus* alone decreased the lipid peroxide level (11%) in the homogenate and increased (10%) in mitochondria and these changes are not statistically significant.

Protein carbonyls:

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *Hemidesmus indicus* on protein carbonyls is shown in Figure-35. The carbonyl content is significantly increased (91%) due to the administration of allyl alcohol. The prior administration of aqueous extract of *Hemidesmus indicus* offered protection (44%) and prevented the raise in the carbonyl content due to the administration of allyl alcohol.

Total sulphydryl groups:

Figure-36 shows the effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *Hemidesmus indicus* on total sulphydryl groups. The total sulphydryl groups are significantly decreased (47%) due to the administration of allyl alcohol. The prior administration of aqueous extract of *Hemidesmus indicus* offered protection to an extent of 70%.

Superoxide radicals:

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *Hemidesmus indicus* on the generation of superoxide radicals is shown in Figure-37. The superoxide radicals are significantly increased (217%) due to the administration of allyl alcohol. Prior administration of aqueous extract of *Hemidesmus indicus* prevented the raise in superoxide level and protected to an extent of 51%. There was a significant difference when group of rats administered with both *H.indicus* and allyl alcohol (group-D) compared with both control group (group-A) or rats administered only allyl alcohol (group-B).

Catalase:

Figure 38 shows the effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *Hemidesmus indicus* on the generation of catalase activity. In both homogenate and mitochondria the catalase activity is significantly decreased (57 and 21% respectively) due to the administration of allyl alcohol. There was a significant increase in the catalase activity of both homogenate and mitochondria in group of rats administered both *H.indicus* and allyl alcohol (group-D) when compared to rats administered only allyl alcohol (group-B). Prior administration of aqueous extract of *Hemidesmus indicus* offered protection to an extent of 40 and 147% on catalase activity of both homogenate and mitochondria respectively.

Superoxide dismutase (SOD):

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *Hemidesmus indicus* on the generation of Superoxide dismutase activity is shown in Figure-39. SOD activity is significantly decreased in intact mitochondria, cytosol and membrane fragments (43, 43 and 57% respectively) due to the administration of allyl alcohol. There was a significant increase in the SOD activity of intact mitochondria and membrane fragments and cytosol in rats administered both *H.indicus* and allyl alcohol (group-D) when compared to rats administered only allyl alcohol (group-B). Prior administration of aqueous extract of *Hemidesmus indicus* offered protection of 60, 42 and 38% on SOD activity of intact mitochondria, cytosol and membrane fragments respectively.

Glutathione peroxidase (GSHPx):

Figure-40 shows the effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *Hemidesmus indicus* on glutathione peroxidase activity. The GSHPX activity is significantly decreased (57%) due to the administration of allyl alcohol. The prior administration of aqueous extract of *Hemidesmus indicus* offered protection (66%) and prevented the decrease in the GSHPx activity due to the administration of only allyl alcohol. There was a significant difference in rats administered both *H.indicus* and allyl alcohol (group-D) when compared to rats administered only allyl alcohol (group-B).

Glutathione reductase (GR):

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *Hemidesmus indicus* on glutathione reductase activity is shown in Figure-41. The GR activity is significantly decreased (44%) due to the administration of allyl alcohol. Prior administration of aqueous extract of *Hemidesmus indicus* offered protection of 39% on GR activity. There was a significant difference in rats administered both *H.indicus* and allyl alcohol (group-D) when compared to rats administered only allyl alcohol (group-B).

Alcohol dehydrogenase:

Fig-43 shows the effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *Hemidesmus indicus* on alcohol dehydrogenase activity The alcohol dehydrogenase activity is significantly increased (45%) due to the administration of allyl alcohol. Prior administration of aqueous extract of *Hemidesmus indicus* offered protection of 73% on alcohol dehydrogenase activity. There was a significant decrease in rats administered both *H.indicus* and allyl alcohol (group-D) when compared to rats administered only allyl alcohol (group-B).

Glutathione levels:

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *Hemidesmus indicus* on levels of glutathione is shown in Figure-42. The reduced glutathione (GSH) and oxidized glutathione (GSSG) are significantly decreased (45 and 47%) due to the administration of allyl alcohol. Prior administration of aqueous extract of *Hemidesmus indicus* offered protection of 49 and 64% on GSH and GSSG levels. There was a significant difference in rats administered both *H.indicus* and allyl alcohol (group-D) when compared rats administered only allyl alcohol (group-B).

Glutathione transferase (GST):

Gel filtration on sephadex G-150 column:

Gel filtration profiles of rat liver cytosol from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*H.indicus* administered) were given in figures 44, 46 and 48 respectively. The active fractions were pooled and loaded on affinity column.

Affinity purification of cytosolic GST from rat liver cytosol:

Table 1, 2 and 3 shows the purification profile of rat liver cytosolic GSTs from group-A (control), group-B (allyl alcohol administered) and group-D (*H.indicus* and allyl alcohol administered). The crude extract of control liver showed a CDNB specific activity of 0.69 $\mu mol.min^{-1}.mg\ protein^{-1}$. The affinity-purified GSTs had a 22 $\mu mol.min^{-1}.mg\ protein^{-1}$ specific activity giving a 30 fold purification with a yield of 65%. The cytosolic GSTs of allyl alcohol treated rat liver showed a specific activity of 0.31 $\mu mol.min^{-1}.mg\ protein^{-1}$. After purification by affinity chromatography, the specific activity obtained was 8.7$\mu mol.min^{-1}$ .mg $protein^{-1}$ giving a 28 fold purification with an overall yield of 62%. The cytosolic GSTs of *Hemidesmus indicus* along with allyl alcohol treated rat liver showed a specific activity of 0.44 $\mu mol.min^{-1}.mg\ protein^{-1}$. After purification by affinity chromatography, the specific activity obtained was 15.4 $\mu mol.min^{-1}.mg\ protein^{-1}$ resulting in a 35 fold purification with an overall yield of 66%. Affinity elution patterns of rat liver cytosolic GSTs from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*H.indicus* administered) were given in figures 45, 47 and 49 respectively.

SDS PAGE:

The affinity purified GSTs were resolved into three subunits on SDS-PAGE with molecular weights of 25.6, 27 and 28 kDa which were designated as Ya, Yb and Yc respectively. Calibration of relative molecular weight was done by including the molecular weight standards along with affinity purified GSTs Fig 50-A shows SDS-PAGE of affinity purified rat liver cytosolic GSTs from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*H.indicus* administered).

Western blotting:

The cytosolic proteins as well as affinity purified GSTs were analyzed by immuno blotting after electrophoresis using polyclonal anti bodies raised against affinity purified GSTs. Ya subunit was shown to be decreased significantly (fig 51i) upon allyl alcohol administration. Prior administration of aqueous extract of *Hemidesmus indicus* offered significant protection in Ya subunit. There was no significant difference in Yb and Yc subunits by the administration of allyl alcohol (fig. 51 ii and iii). Fig 50-B shows western blot and Fig. 51 shows densitometric scans of western blots.

Measurement of radicals using spin trap DMPO by ESR:

Figure-52 i, ii and iii shows original traces of ESR signals of DMPO-OH adduct of rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*H.indicus* administered) in the presence of glutamate and malate. Figure-52 iv shows addition of superoxide dismutase completely abolished the ESR signal. Figure-53 shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*H.indicus* administered) in the presence of glutamate and malate. ESR signal intensity of DMPO-OH adduct was statistically increased due to the administration of allyl alcohol. Prior administration of aqueous extract of *H.indicus* offered protection of 57%. Figure-54 shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*H.indicus* administered) in the presence of antimycin, glutamate and malate. The change in the signal intensities of DMPO-OH adduct of these 3 groups was not statistically significant in the presence of antimycin. Figure-55 shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*H.indicus* administered) in the presence of succinate. The change in the signal intensities of DMPO-OH adduct of these 3 groups was not statistically significant. Figure-56 shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*H.indicus* administered) in the presence of antimycin and succinate. The change in the signal intensities of DMPO-OH adduct of these 3 groups was not statistically significant in the presence of antimycin.

Histopathology:

Fig- 57i and ii shows histology of control rat liver and liver of rats administered with allyl alcohol. 58i and ii shows histology of liver of rats administered only *H.indicus* and rats administered both *H.indicus* + allyl alcohol. In rats administered with allyl alcohol the normal architecture of liver was lost with degenerative changes where as in control rats normal liver hepatic cells architecture was observed. Prior administration of rats with *H.indicus*, showed mild degenerative changes compared to rats administered only allyl alcohol. Fig 59i and ii shows histology of liver of rats administered only *P.fraternus* and rats administered both *P.fraternus* + allyl alcohol and. Prior administration of rats with *P.fraternus*, showed mild degenerative changes compared to rats administered only allyl alcohol.

FIGURE 29A

Effect of administration of allyl alcohol with or without prior administration of *H.indicus* on the rate of respiration using glutamate + malate as substrate

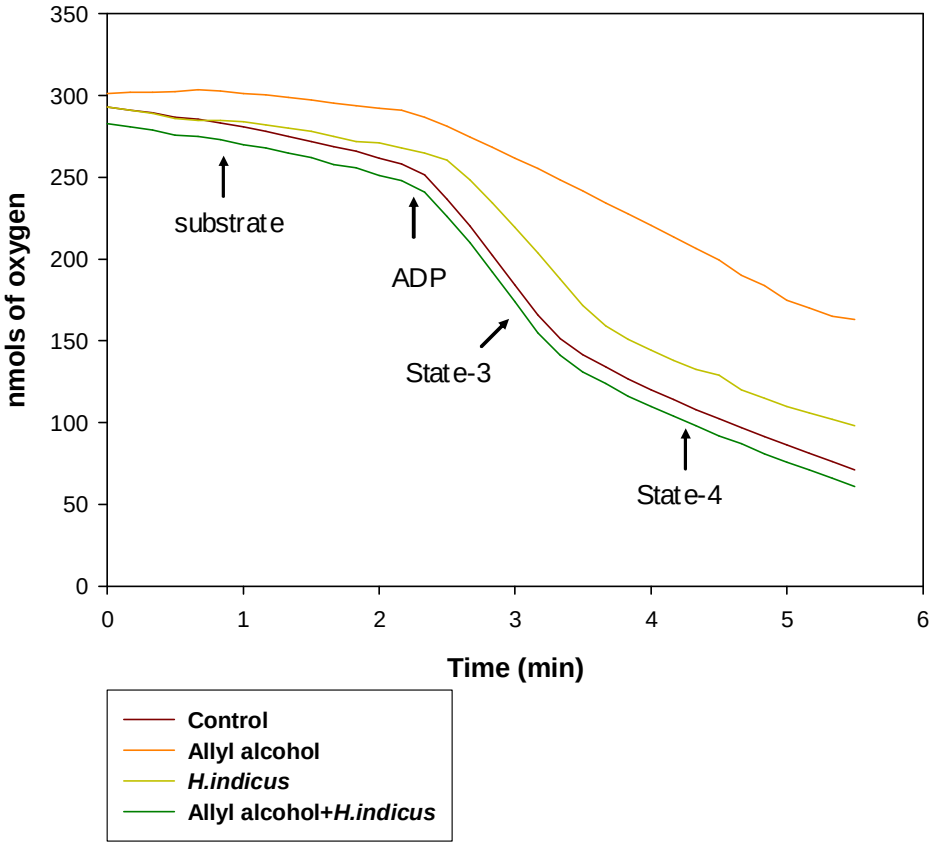
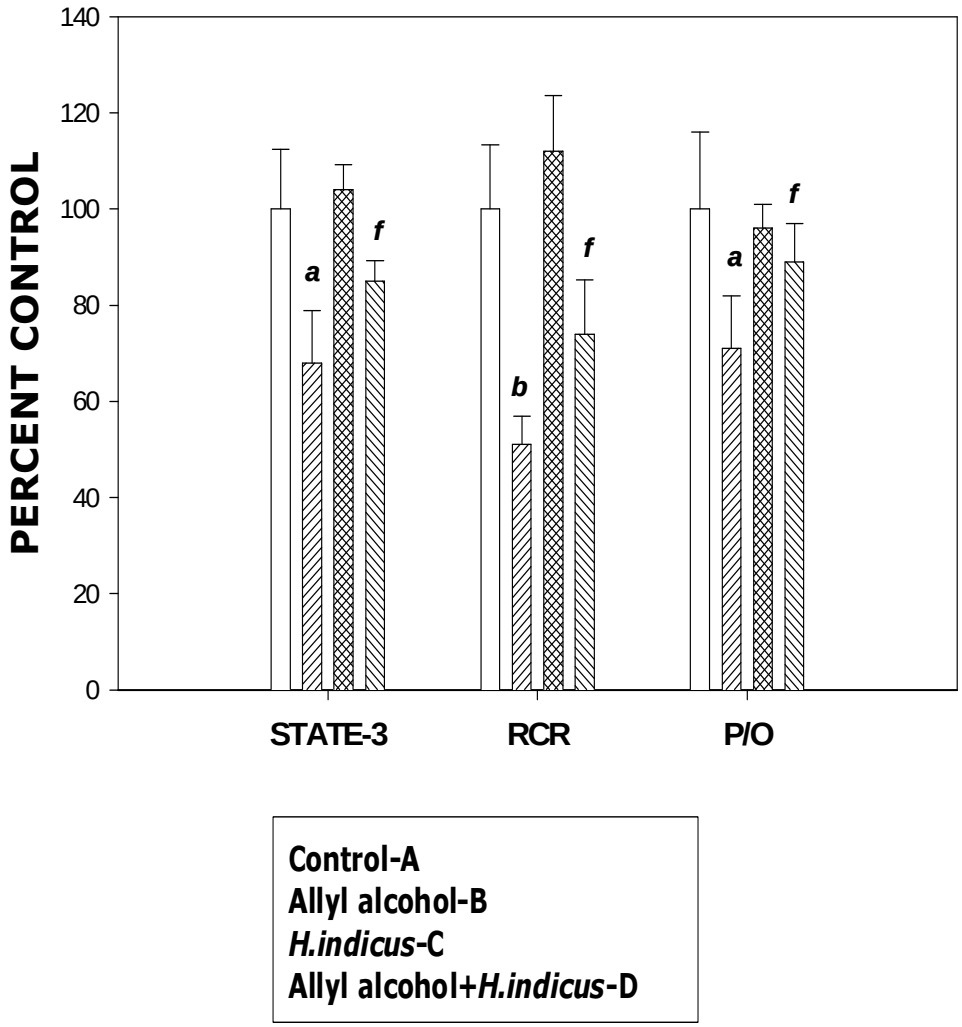


Figure 1A shows the original traces of oxygen concentration obtained by oxytherm respirometer. The arrows indicate the time point at which substrate/ADP is added. ADP stimulated respiration is taken as state 3 and ADP exhausted respiration as state 4. The experiment was done with 2mg of mitochondrial protein in 1.0ml of the buffer (50 mM sucrose, 50 mM Tris-Hcl, 20 mM potassium phosphate, 2 mM EDTA, 7 mM magnesium chloride, pH 7.4) in the air tight container. Additions were done with Hamilton syringe.

FIGURE 29B

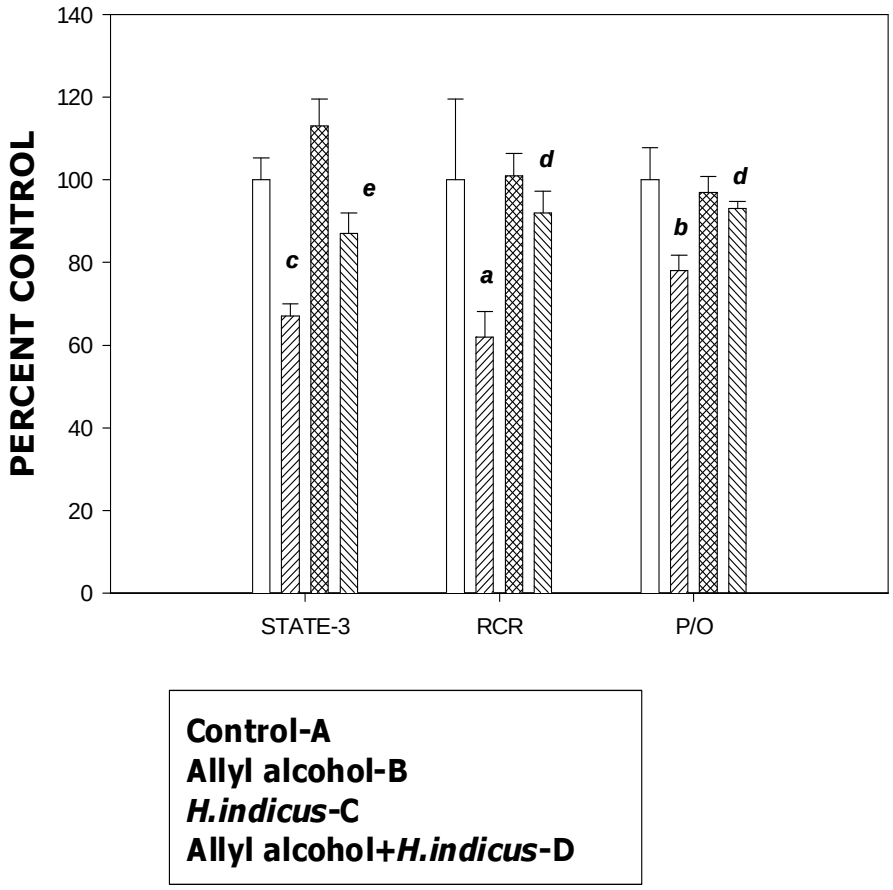
Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on the rate of respiration, RCR, P/O ratio using glutamate + malate as substrate



Values are mean± SD from at least four rats in each group. 2mg protein was used for each assay. Rate of respiration was measured using glutamate + malate as substrate by the addition of 400 nmols of ADP. It is expressed as nano moles of oxygen per minute per milligram protein. RCR is the ratio of ADP stimulated state-3 to ADP exhausted state 4 respiration and P/O=ADP/O ratio. All the values are expressed relative to controls which were taken as 100. Control values for the rate of respiration, RCR, P/O ratio using glutamate and malate as substrates were 40.0±4.9, 4.2±0.5, 3.1± 0.6respectively. $^{a}p<0.05$ vs. Group-A. $^{b}p<0.01$ vs. Group A. $^{f}p<0.05$ vs. Group-B.

FIGURE 30

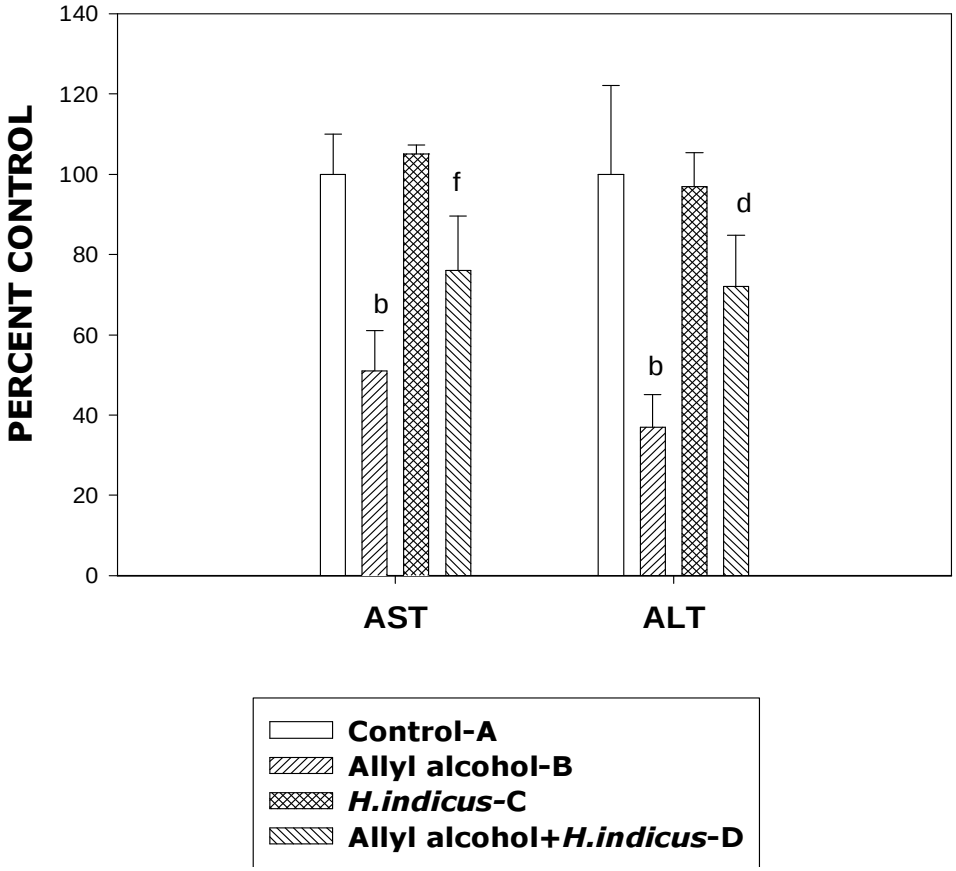
Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on the rate of respiration, RCR, P/O ratio using succinate as substrate



Values are mean± SD from at least four rats in each group. 2mg protein was used for each assay. Rate of respiration was measured using succinate as substrate by the addition of 200 nmols of ADP. It is expressed as nano moles of oxygen per minute per milligram protein. RCR is the ratio of ADP stimulated state-3 to ADP exhausted state 4 respiration and P/O=ADP/O ratio. All the values are expressed relative to control which were taken as 100. Control values for the rate of respiration, RCR, P/O ratio using succinate as substrate were 48.0±2.5, 4.1±0.5, 2.0±0.2 respectively. $^{a}p<0.05$ vs. Group-A. $^{b}p<0.01$ vs. Group-A. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. Group-B. $^{e}p<0.001$ vs. Group B.

FIGURE 31

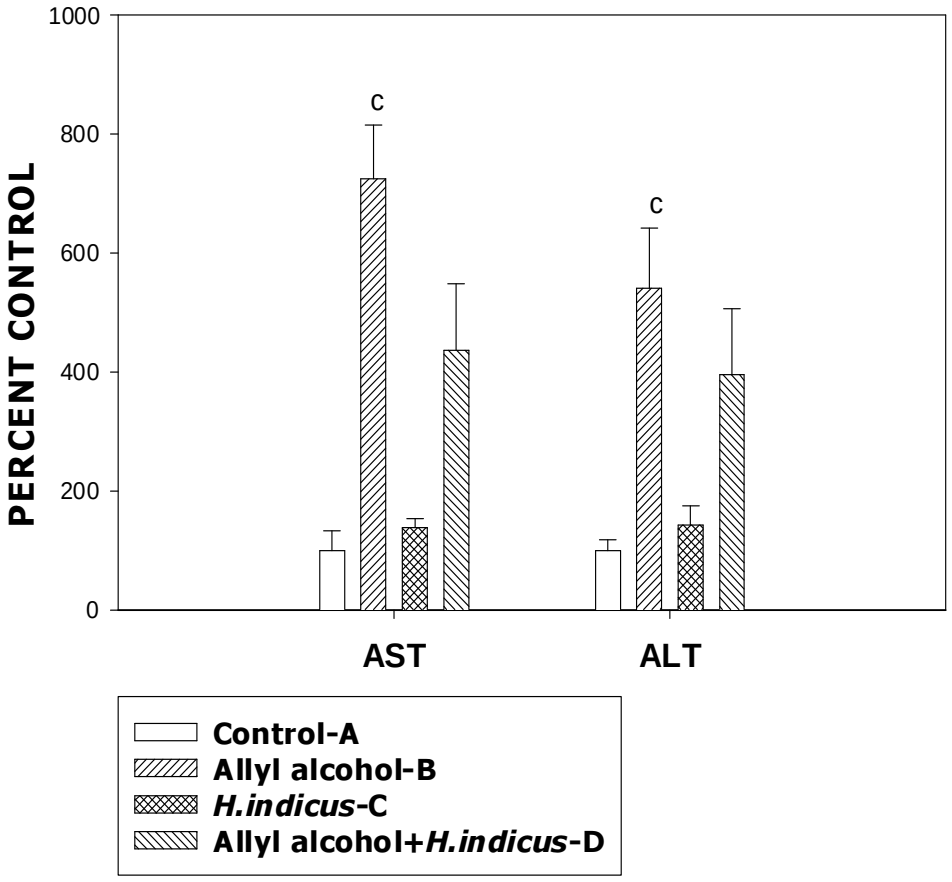
Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on Aspartate amino transferase (AST) and alanine amino transferase activity (AlAT) in liver homogenate



Values are given as percent control, and are mean ± SD of atleast four animals. 40µg protein was used for each assay. Aspartate amino transferase (AST) and alanine amino transferase (AlAT) activities are expressed as µmoles of NADH oxidized per hour per mg protein. The control values of AST and AlAT in liver homogenate were 51±5.13, 3.02±0.67 respectively. $^{b}p<0.01$ vs. Group-A. $^{d}p<0.01$ vs. Group-B. $^{f}p<0.05$ vs. Group-B.

FIGURE 32

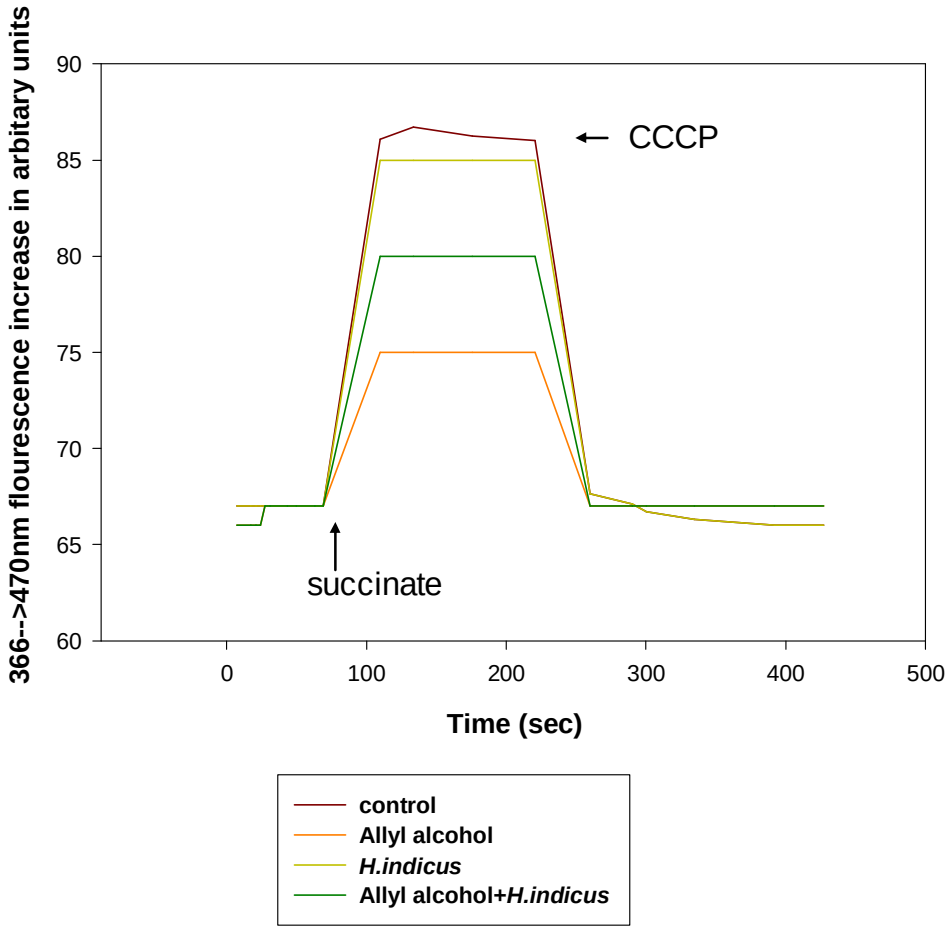
Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on Aspartate amino transferase (AST) and alanine amino transferase activity (AlAT) in serum



Values are given as percent control, and are mean ± SD of atleast four animals. Aspartate amino transferase (AST) and alanine amino transferase (AlAT) activities are expressed as µmoles of NADH oxidized per hour per mg protein. The control values of AST and AlAT in serum were 0.094±0.031, 0.015±0.002 respectively. $^{c}p<0.001$ vs. Group-A.

FIGURE 33A

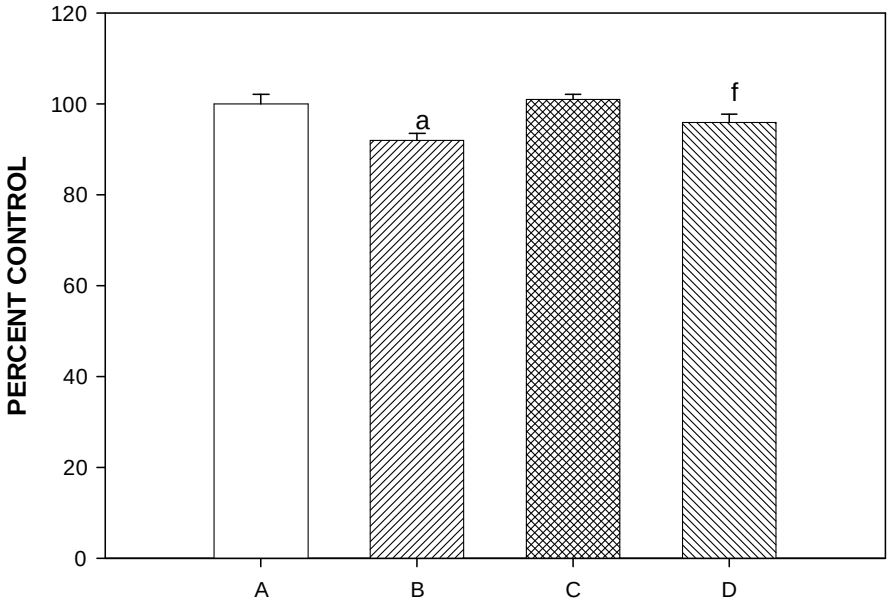
Effect of administration of allyl alcohol with or without the administration of *Hemidesmus indicus* on membrane potential in SMP



This figure shows original traces of fluorescence changes of ANS in submitochondrial particles**.** 1mM succinate was added to the reaction system [submitochondrial particles (380 µg protein), buffered medium (containing 250mM sucrose, 10mM Tris-HCl, 5mM $MgCl_2$, pH 7.5) and 10µM ANS in a total volume of 1.0 ml] to energise the membrane and the fluorescence change was recorded. The addition of succinate induced an increase in ANS fluorescence. After the fluorescence change reached to a plateau, CCCP (a potent uncoupler) was added (0.3µM) to bring down the membrane to deenergised state. The intensity of fluorescence was measured in a fluorescence spectrophotometer. The excitation and emission wave lengths used were 366nm and 470nm respectively.

FIGURE 33B

Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on membrane potential in SMP

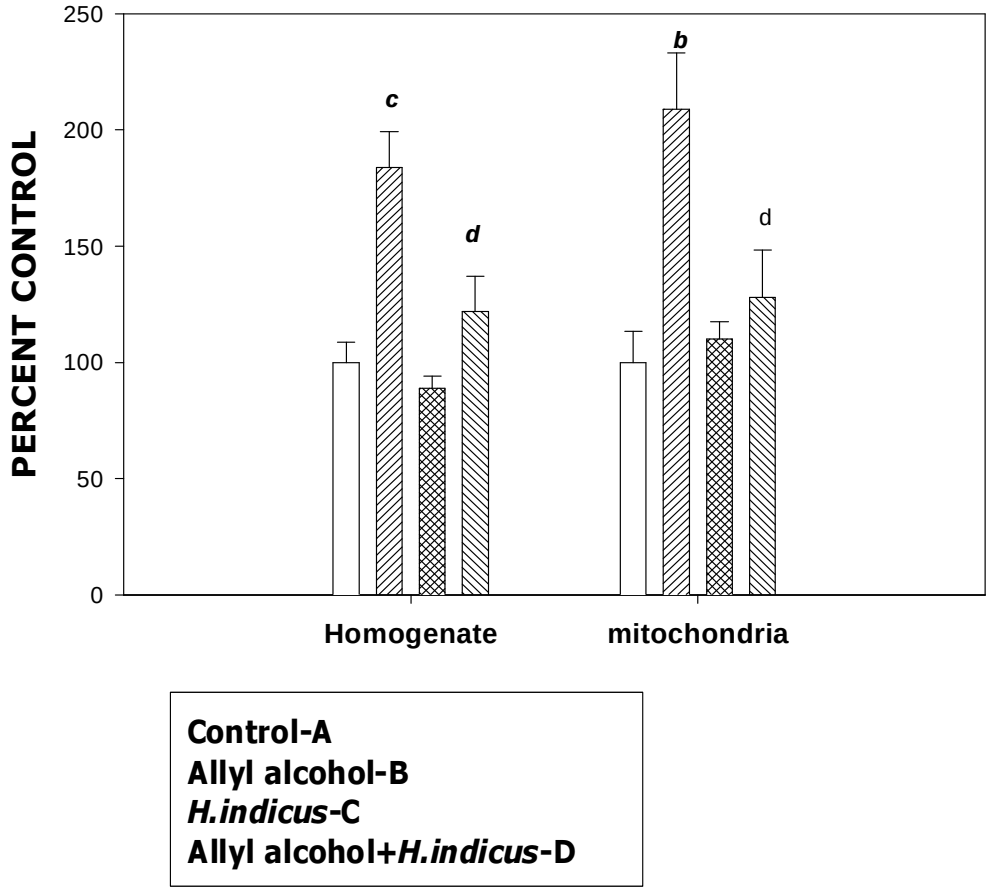


Control-A
Allyl alcohol-B
***H.indicus*-C**
Allyl alcohol+*H.indicus*-D

Values are given as percent control, and are mean ± SD of atleast four animals. 380µg SMP protein was used for each assay. Membrane potential is expressed as millivolts. The control value was 150±3.0. $^{a}p<0.05$ vs. Group-A. $^{f}p<0.05$ vs. Group-B.

FIGURE 34

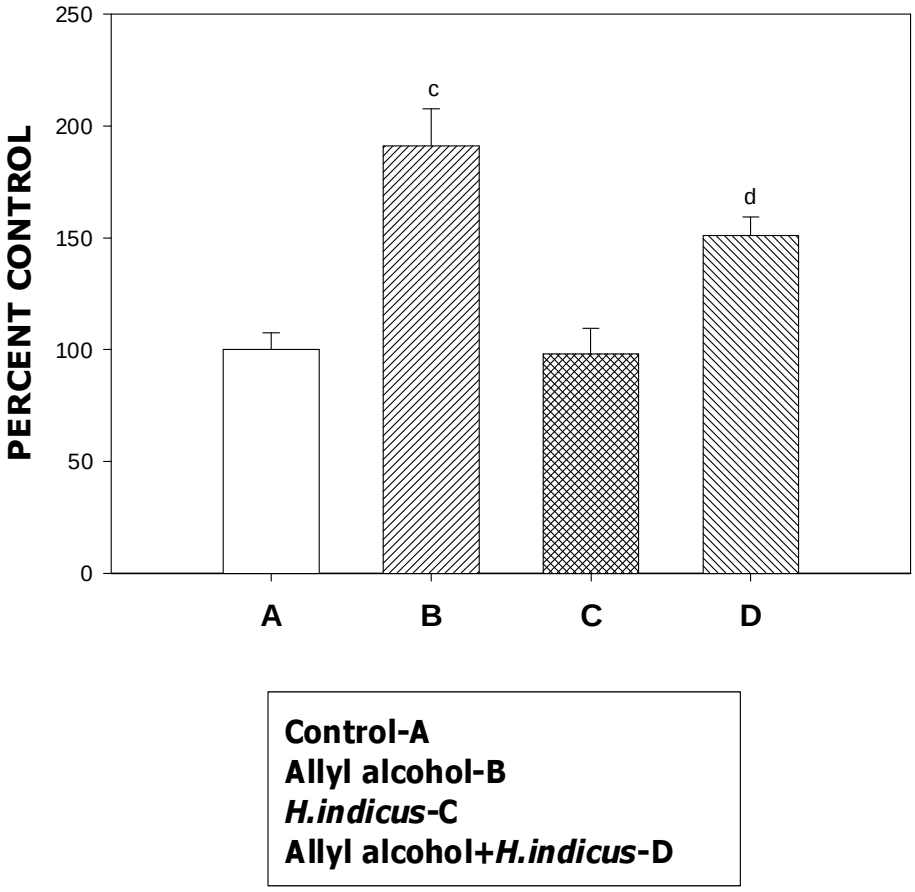
Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on lipid peroxide level in liver homogenate and mitochondria



Values are given as percent control, and are mean ± SD of at least four animals. 5mg protein was used for each assay. Lipid peroxide level is expressed as nmol MDA formed per 100mg protein. The control values in homogenate and mitochondria were 115±10.0, 108±14.4 respectively. $^{b}p<0.01$ vs. Group-A. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. Group-B.

FIGURE 35

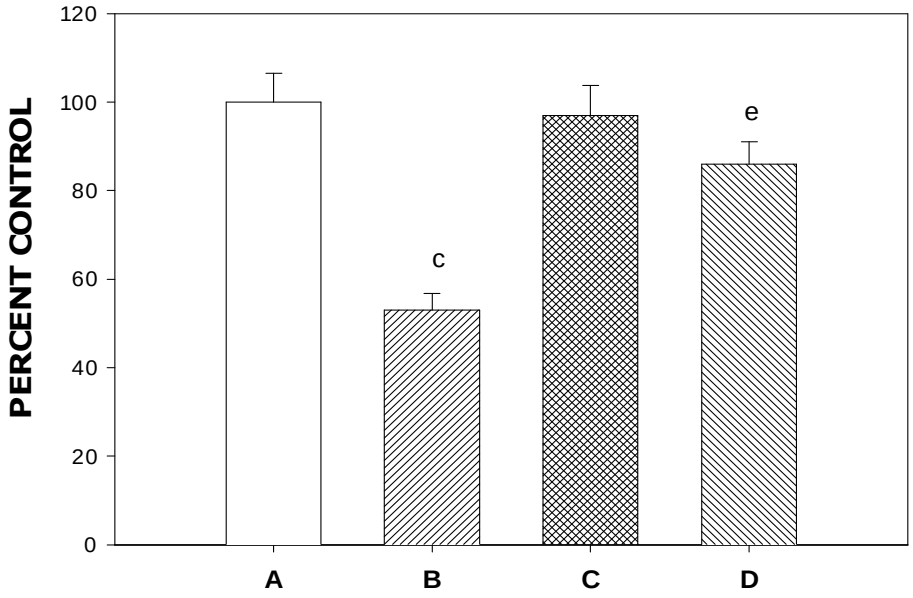
Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on protein carbonyl content



The values are mean ± SD of at least four animals. 4mg protein was used for each assay. Protein carbonyl content is expressed as nmol per mg protein. The control value for the protein carbonyl content was 0.8±0.06. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. Group-B.

FIGURE 36

Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on total sulphydryl groups in rat liver mitochondria

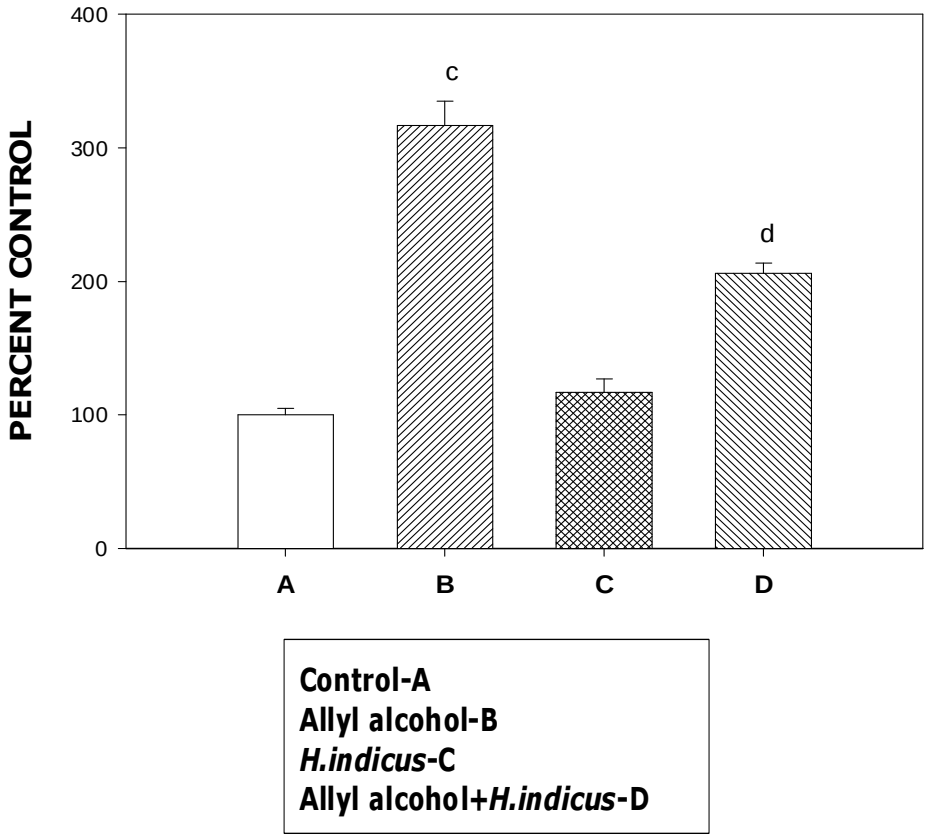


Control-A
Allyl alcohol-B
***H.indicus*-C**
Allyl alcohol+*H.indicus*-D

The values are mean ± SD of at least four animals. 1mg protein was used for each assay. Total sulphydryl groups are expressed as nmol per milligram protein. The control value for total sulphydryl groups were 8.7±0.5. $^{c}p<0.001$ vs. Group-A. $^{e}p<0.001$ vs. Group-B.

FIGURE 37

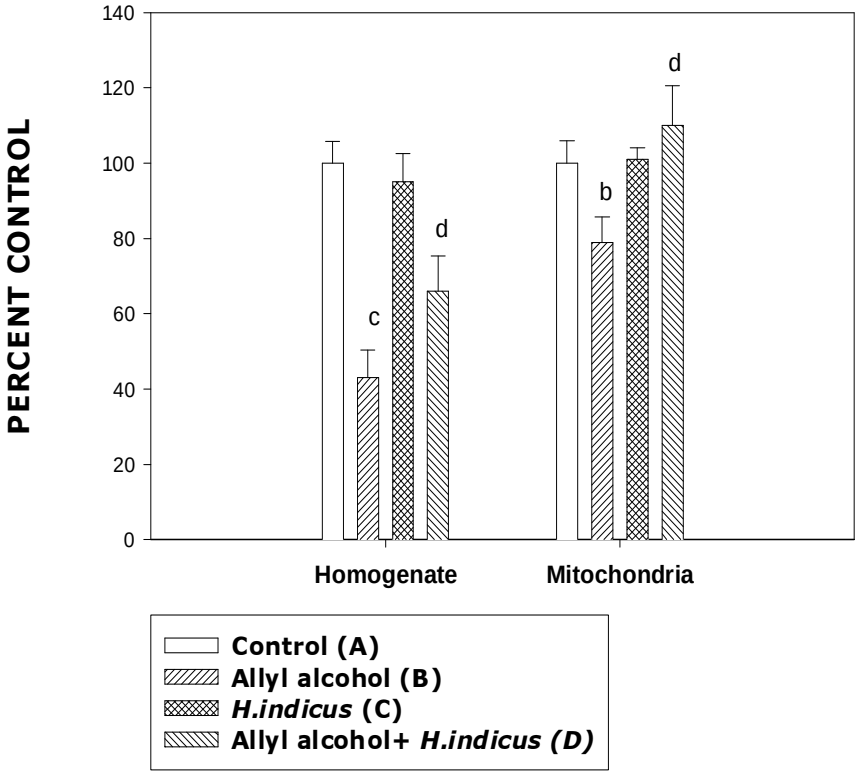
Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on generation of superoxide radicals in mitochondrial fragments



The values are mean ± SD of at least four animals. 0.1mg protein of mitochondrial fragments were used for each assay. Generation of superoxide radicals are expressed as nmol per minute per milligram protein. The control value for generation of superoxide radicals 14.5±0.7. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. Group-B.

FIGURE 38

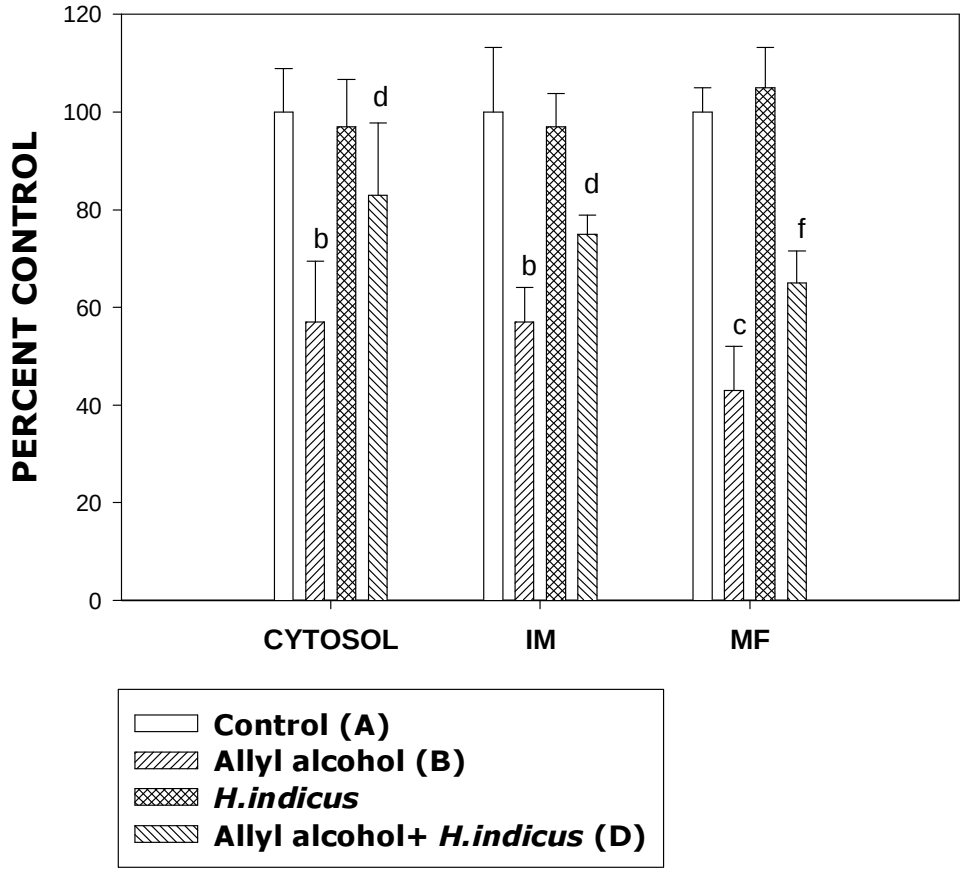
Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on catalase activity



Values are given as percent control, and are mean ± SD of atleast four animals. 0.3mg of homogenate protein and 0.1mg of mitochondrial protein were used for each assay. Catalase activity is expressed as nmols of H_2O_2 consumed per min per mg protein. The control values in homogenate and mitochondria were 681 ± 39.05, 1816±108.17 respectively. $^{b}p<0.01$ vs. Group-A. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. Group-B.

FIGURE 39

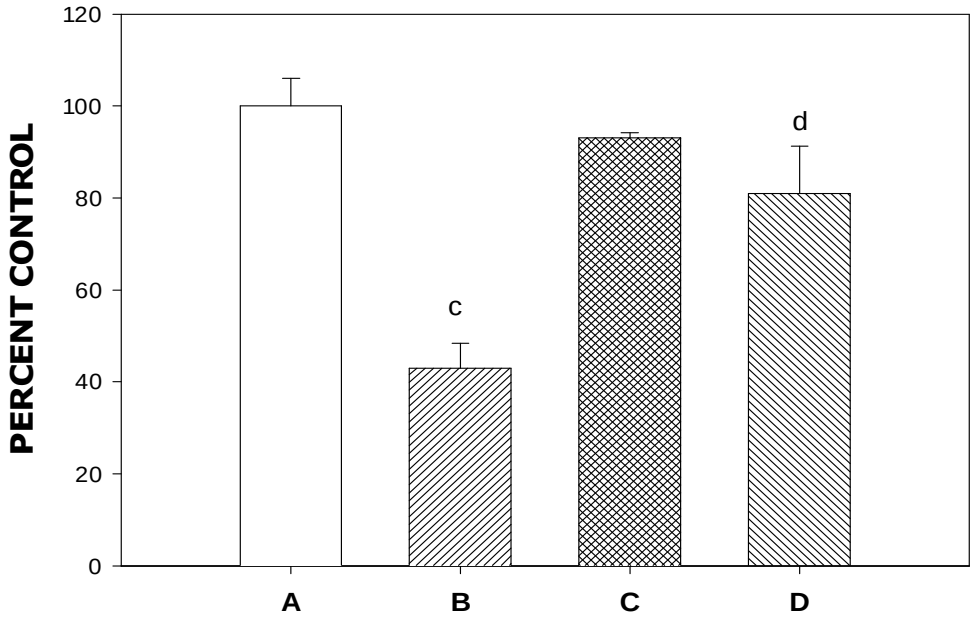
Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on superoxide dismutase activity



Values are given as percent control, and are mean ± SD of atleast four animals. 0.1mg protein of sample (mitochondrial fragments or intact mitochondria or cytosol were used for each assay. Superoxide dismutase activity is expressed as units per milligram protein. The control values of Superoxide dismutase of intact mitochondria, cytosol and membrane fragments were 4.45 ±0.591, 3.7± 0.331, 3.06±0.152 respectively. $^{b}p<0.01$ vs. Group-A. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. Group-B. $^{f}p<0.05$ vs. Group-B.

FIGURE 40

Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on glutathione peroxidase activity

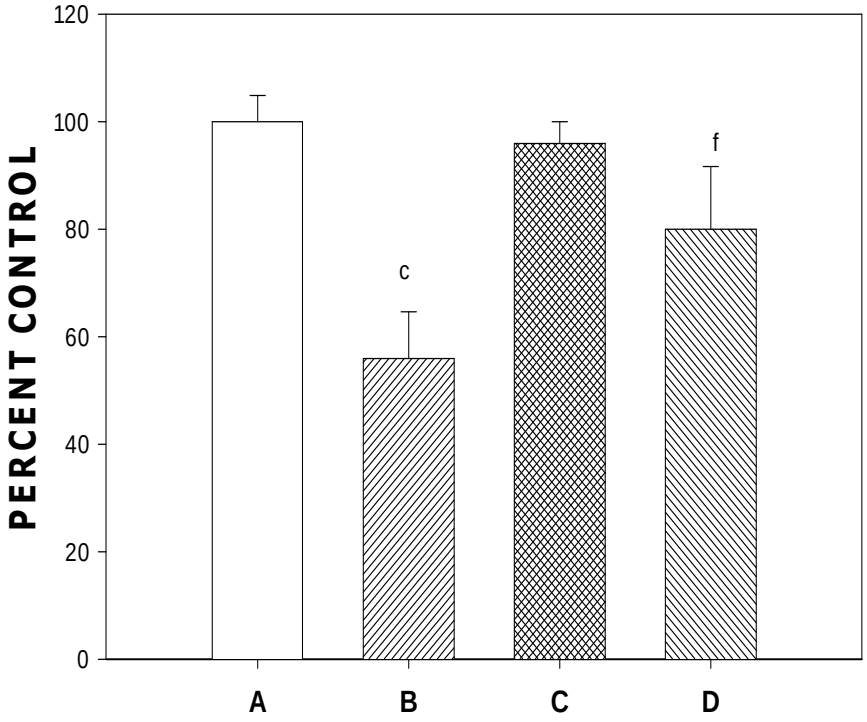


Control-A
Allyl alcohol-B
***H.indicus*-C**
Allyl alcohol+*H.indicus*-D

Values are given as percent control, and are mean ± SD of atleast four animals. 0.1mg of homogenate protein was used for each assay. Glutathione peroxidase activity is expressed as nmols of NADPH oxidized per min per milligram protein. The control value of glutathione peroxidase was 169±7.81. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. Group-B.

FIGURE 41

Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on glutathione reductase activity

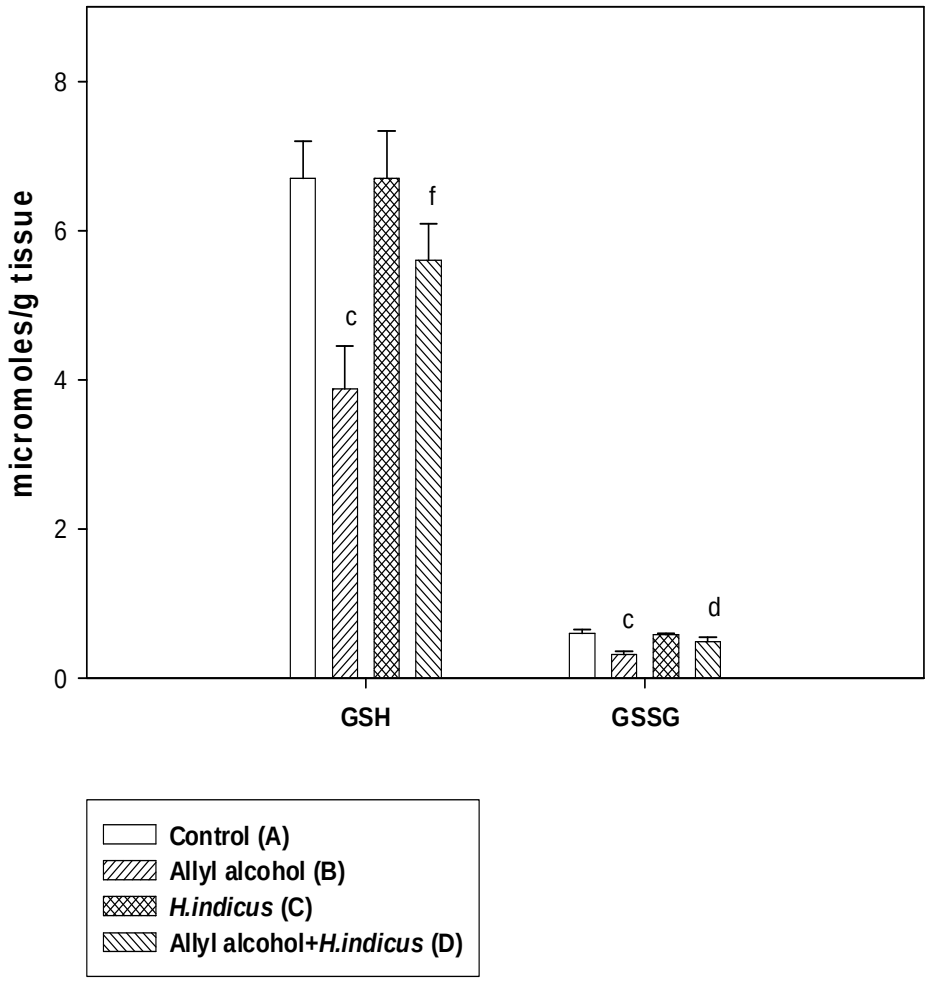


Control-A
Allyl alcohol-B
***H.indicus*-C**
Allyl alcohol+*H.indicus*-D

Values are given as percent control, and are mean ± SD of atleast four animals. 0.1mg of mitochondrial protein was used for each assay. Glutathione reductase activity is expressed as nmols of NADPH oxidized per min per milligram protein. The control value of glutathione reductase was 74.6±3.65. $^{b}p<0.01$ vs. Group-A. $^{c}p<0.001$ vs. Group-A. $^{f}p<0.05$ vs. Group-B.

FIGURE 42

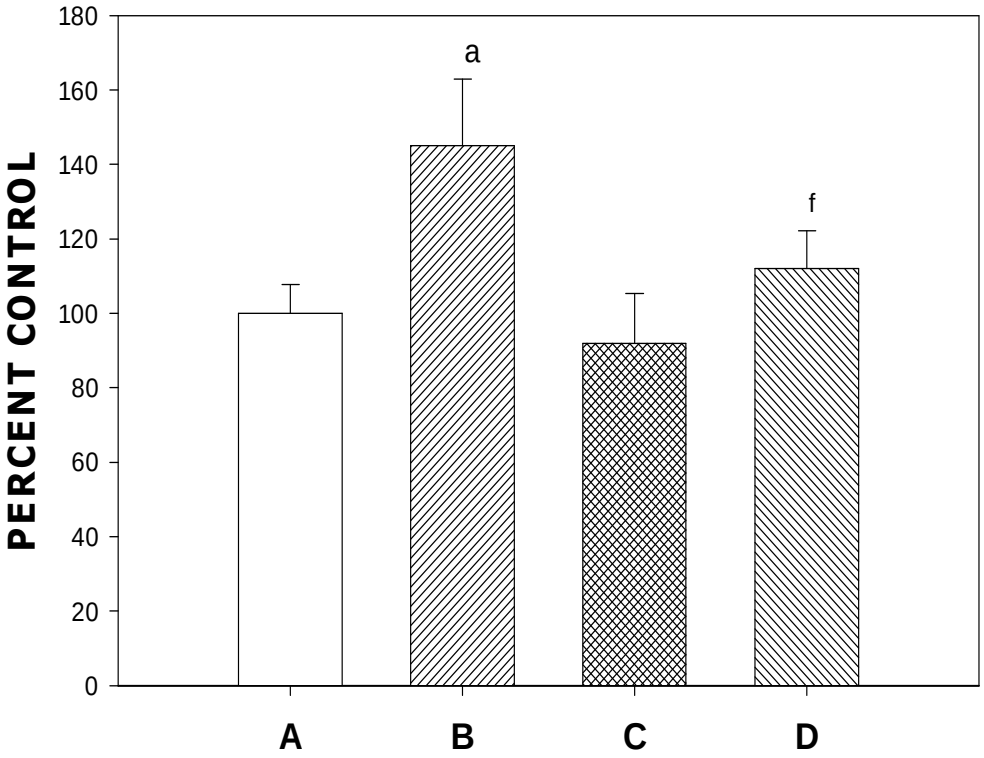
Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on levels of glutathione



Values are mean ± SD of atleast four animals. Glutathione levels are expressed as µmoles per g tissue. The control values of GSH, GSSG and were 6.77±0.66, 0.532±0.059 respectively. $^{c}p<0.001$ vs. Group-A. $^{d}p<0.01$ vs. Group-B. $^{f}p<0.05$ vs. Group-B.

FIGURE 43

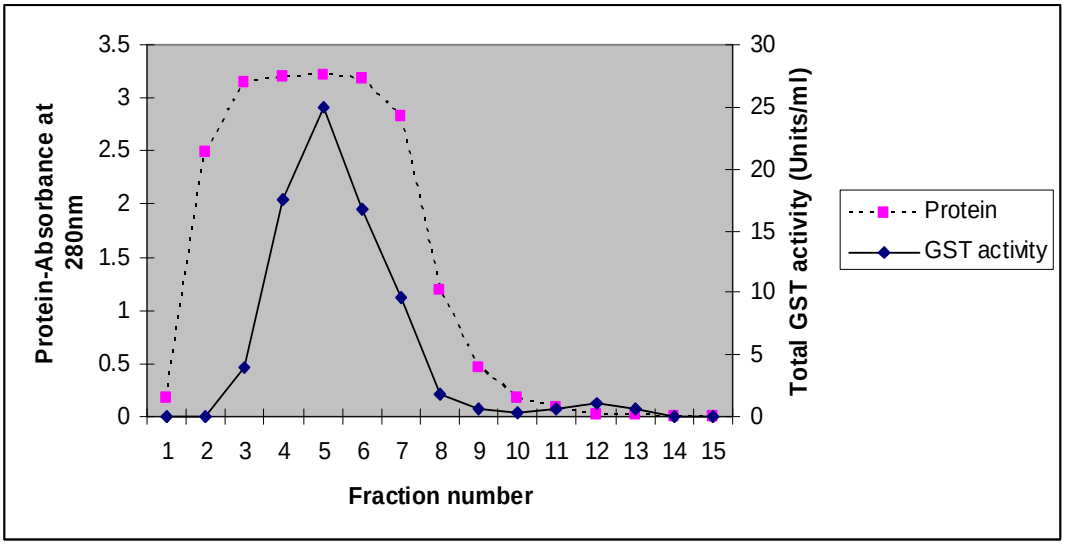
Effect of administration of allyl alcohol with or without prior administration of *Hemidesmus indicus* on alcohol dehydrogenase activity



Values are given as percent control, and are mean ± SD of atleast four animals. 0.6mg protein of cytosol was used for each assay. Alcohol dehydrogenase activity is expressed as µmoles of NADH formed/min/mg. The control value of alcohol dehydrogenase activity was 19.6±1.52. $^{a}p<0.05$ vs. Group-A. $^{f}p<0.05$ vs. Group-B.

FIGURE 44

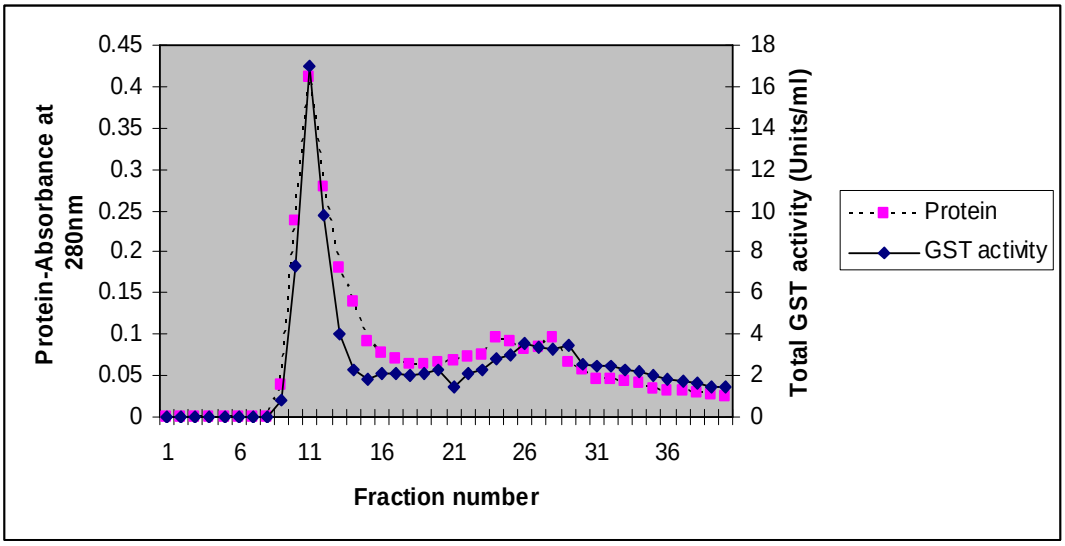
Gel filtration of cytosol from control rat liver on sephadex G-150 column



The column was previously equilibrated with 25mM Tris-Hcl, pH-8.0. Same buffer was used as eluent and 5.0ml fractions were collected at a flow rate of 1ml/min. GST activity was measured using CDNB as substrate and the protein was measured from absorption at 280nm.

FIGURE 45

GSH affinity elution pattern of control liver GST



Affinity chromatography of pooled fractions from gel filtration of cytosol from control rat liver on GSH linked epoxy activated sepharose 6B. The column was washed with 10mM potassium phosphate buffer pH 7.0

containing 0.15M KCl. This was followed by elution with 50mM potassium phosphate buffer pH 7.5 containing 10mM GSH and fractions were collected at a flow rate of 1ml/min. GST activity was measured using CDNB as substrate and the protein was measured from absorption at 280nm.

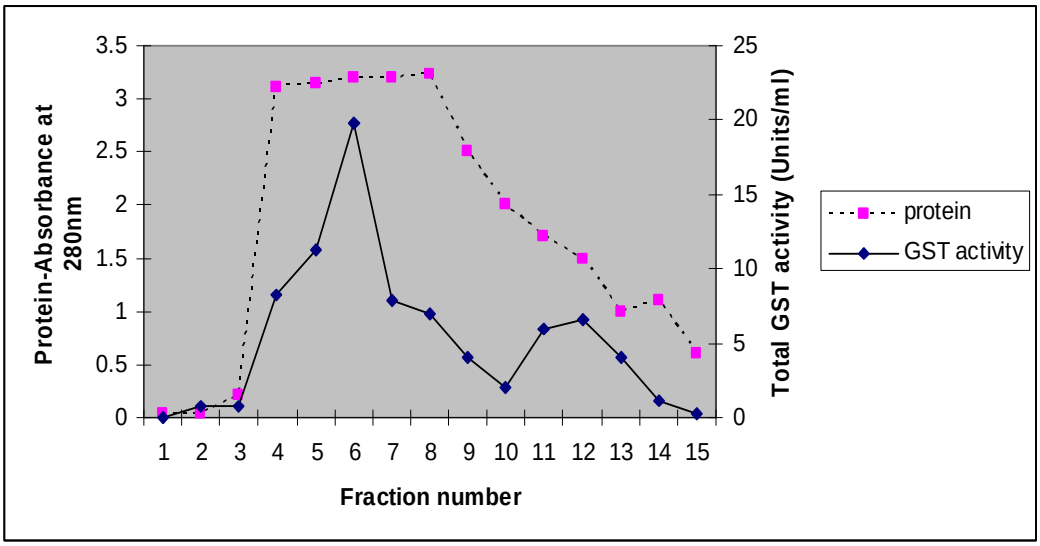
TABLE I

Typical purification profile of control rat liver cytosolic GST

	Total protein	**Total activity**	**Specific activity**	**Yield**	**Fold purification**
Crude extract	385	266	0.69	100	1
Gel filtration	230	230	1.00	86	1.4
Affinity column	7.8	172	22	65	30

FIGURE 46

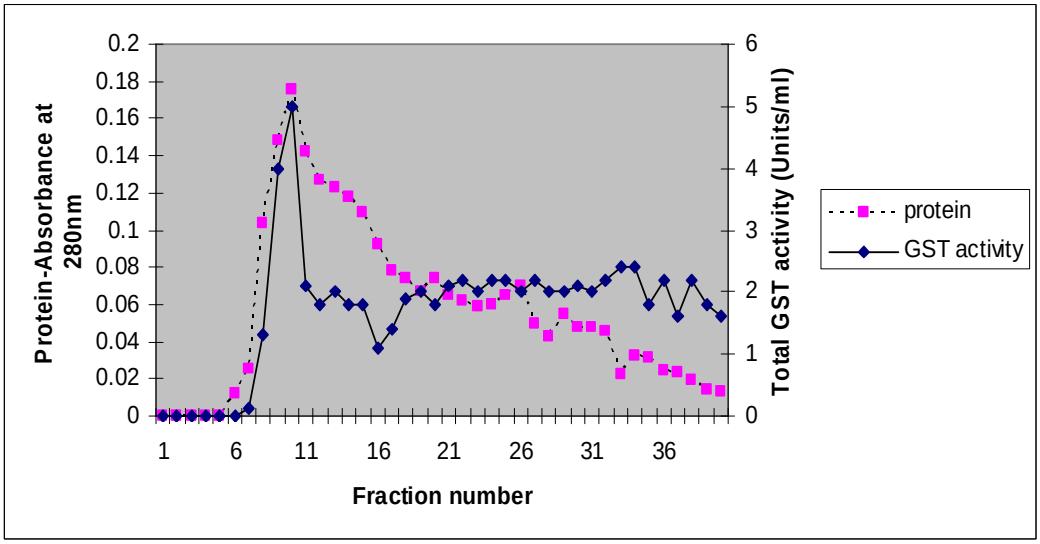
Gel filtration of cytosol from allyl alcohol administered rat liver on Sephadex G-150 column



The column was previously equilibrated with 25mM Tris-Hcl, pH-8.0. Same buffer was used as eluent and 5.0ml fractions were collected at a flow rate of 1ml/min. GST activity was measured using CDNB as substrate and the protein was measured from absorption at 280nm.

FIGURE 47

GSH affinity elution pattern of allyl alcohol administered liver GST



Affinity chromatography of pooled fractions from gel filtration of cytosol from allyl alcohol administered rat liver on GSH linked epoxy activated sepharose 6B. The column was washed with 10mM potassium phosphate buffer pH 7.0 containing 0.15M KCl. This was followed by elution with 50mM potassium phosphate buffer pH 7.5 containing 10mM GSH and

fractions were collected at a flow rate of 1ml/min. GST activity was measured using CDNB as substrate and the protein was measured from absorption at 280nm.

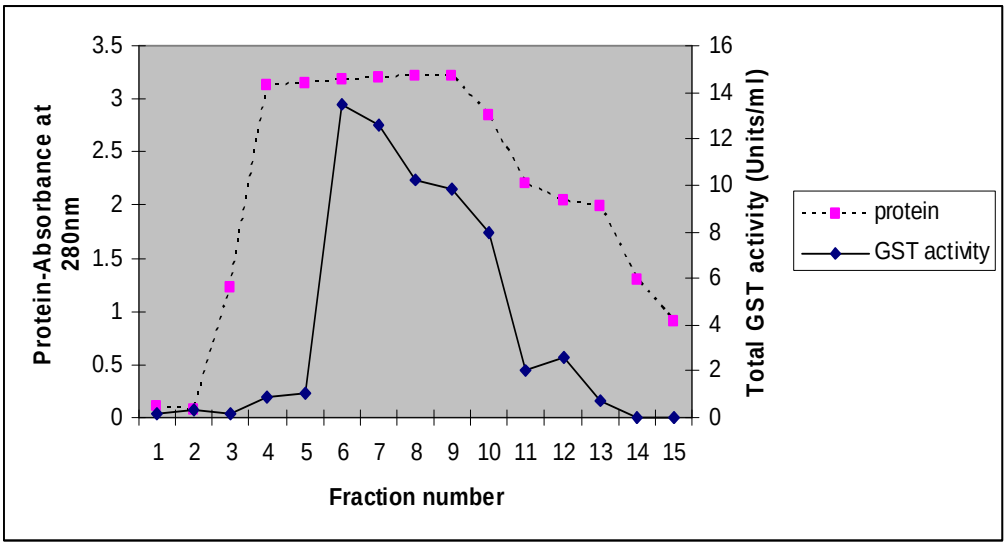
TABLE 2

Typical purification profile of allyl alcohol treated rat liver cytosolic GST

	Total protein	Total activity	Specific activity	Yield	Fold purification
Crude extract	583	182	0.31	100	1
Gel filtration	338	155	0.57	85	1.8
Affinity column	12	112	8.7	62	28

FIGURE 48

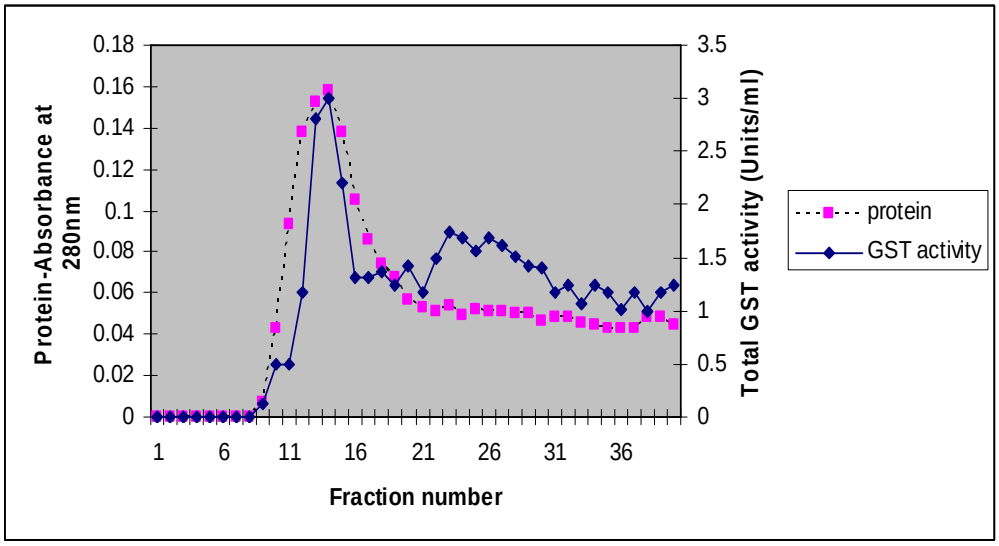
Gel filtration of cytosol from allyl alcohol+*H.indicus* administered rat liver on sephadex G-150 column



The column was previously equilibrated with 25mM Tris-Hcl, pH-8.0. Same buffer was used as eluent and 5.0ml fractions were collected at a flow rate of 1ml/min. GST activity was measured using CDNB as substrate and the protein was measured from absorption at 280nm.

FIGURE 49

GSH affinity elution pattern of allyl alcohol+*H.indicus* administered rat liver GST



Affinity chromatography of pooled fractions from gel filtration of liver cytosol from rats administered with both allyl alcohol and *H.indicus* on GSH linked epoxy activated sepharose 6B. The column was washed with 10mM potassium phosphate buffer pH 7.0 containing 0.15M KCl. This was

followed by elution with 50mM potassium phosphate buffer pH 7.5 containing 10mM GSH and 1.0ml fractions were collected at a flow rate of 1ml/min. GST activity was measured using CDNB as substrate and the protein was measured from absorption at 280nm.

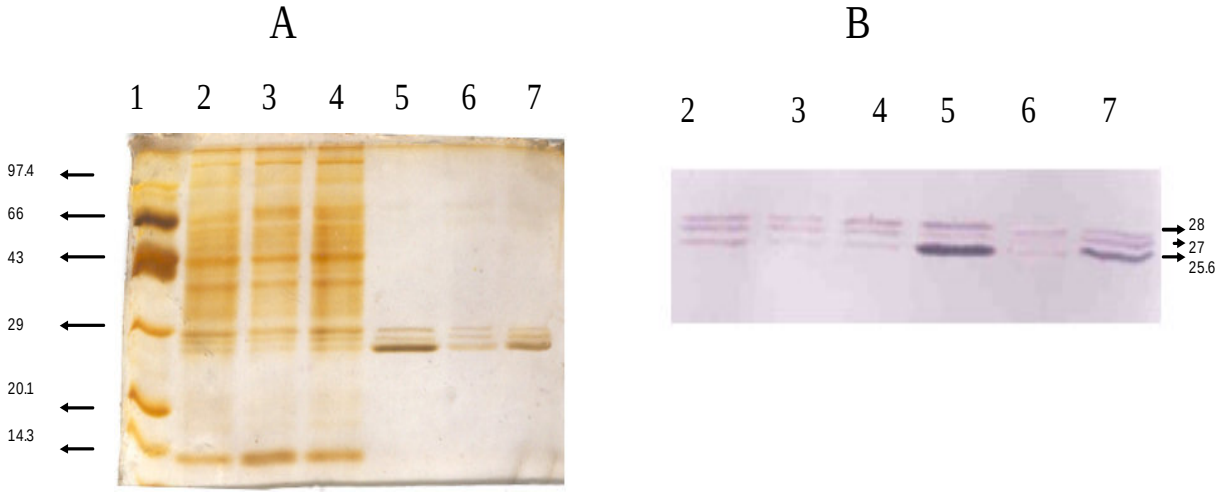
TABLE 3

Typical purification profile of allyl alcohol+*H.indicus* treated rat liver cytosolic GST

	Total protein	**Total activity**	**Specific activity**	**Yield**	**Fold purification**
Crude extract	616	272	0.44	100	1
Gel filtration	300	230	0.76	84	1.72
Affinity column	11	179	15.4	66	35

FIGURE 50

SDS-PAGE and westernblot of hepatic glutathione transferases

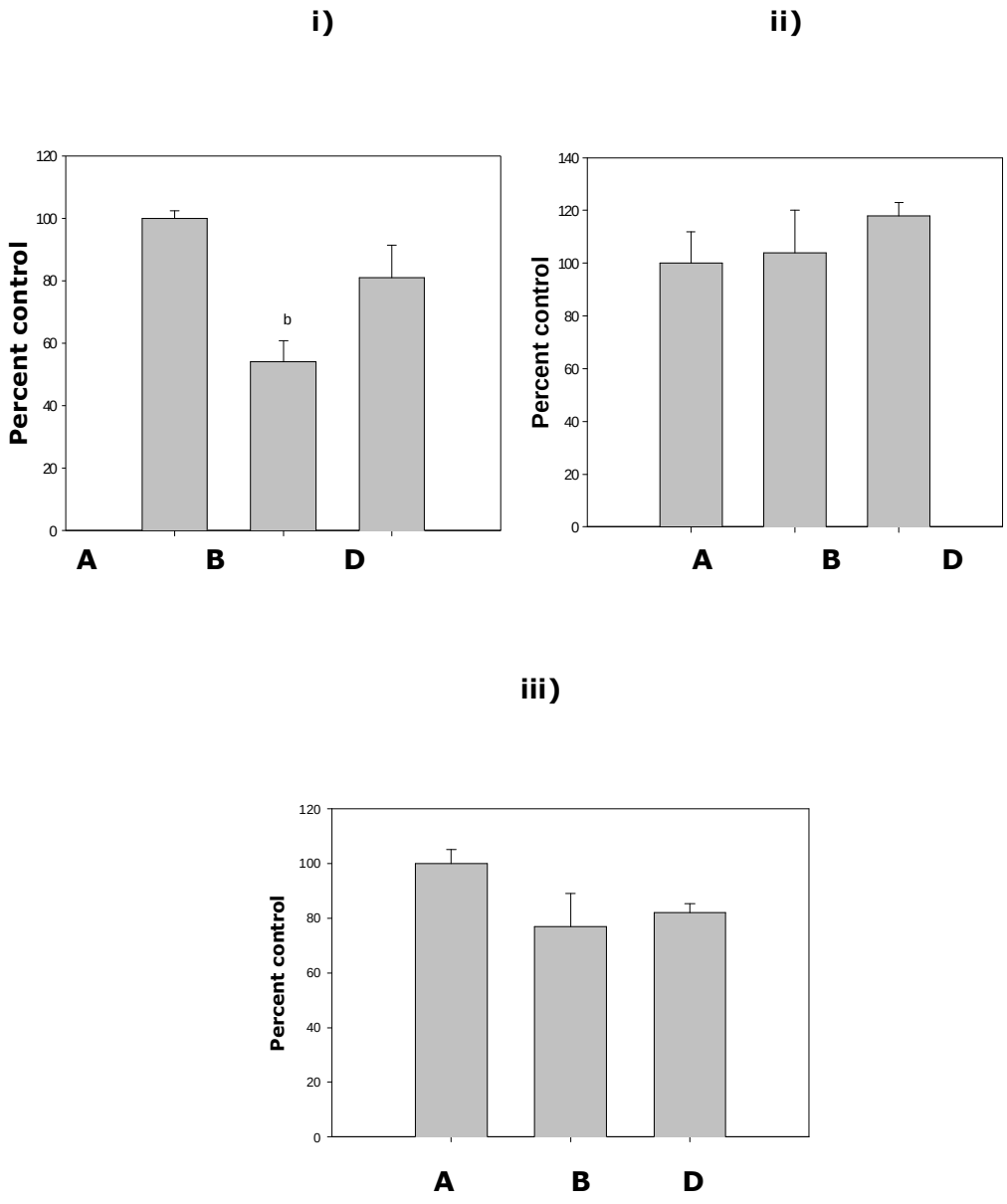


A- Shows SDS-PAGE of hepatic glutathione transferases. 10µg of crude extract or 1 µg of affinity purified cytosolic GSTs were loaded on 12% resolving gel.

B- Shows western blot of hepatic glutathione transferases

1. **Molecular weight marker**
2. **Crude extract from control rat liver**
3. **Crude extract from allyl alcohol treated rat liver**
4. **Crude extract from allyl alcohol + *H.indicus* treated rat liver.**
5. **Affinity purified cytosolic GST from control rat liver**
6. **Affinity purified cytosolic GST from allyl alcohol treated rat liver**
7. **Affinity purified cytosolic GST from allyl alcohol + *H.indicus* treated rat liver.**

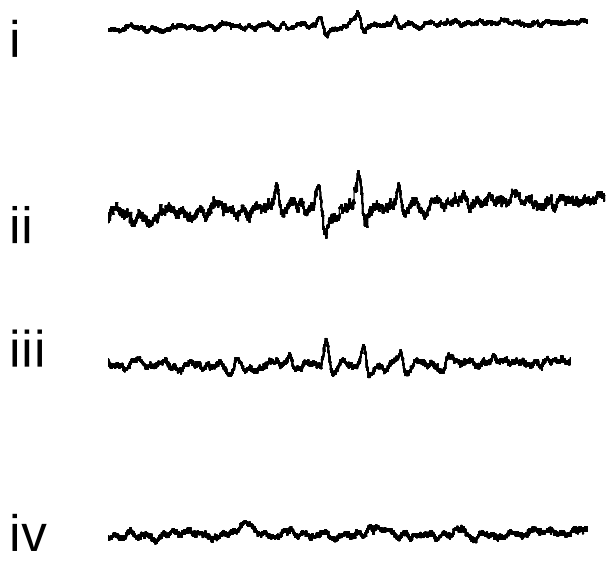
FIGURE 51



i, ii and iii shows densitometric quantification of Ya, Yb and Yc subunits of GST in arbitary units. $^{b}p<0.01$ vs. control group. Densitometer was used for quantification.
A, B and D are affinity purified GST subunits from control, allyl alcohol administered and allyl alcohol+*H.indicus* administered rat liver cytosol.

FIGURE 52

Original traces of ESR signals of DMPO-OH adducts using glutamate + malate as substrate

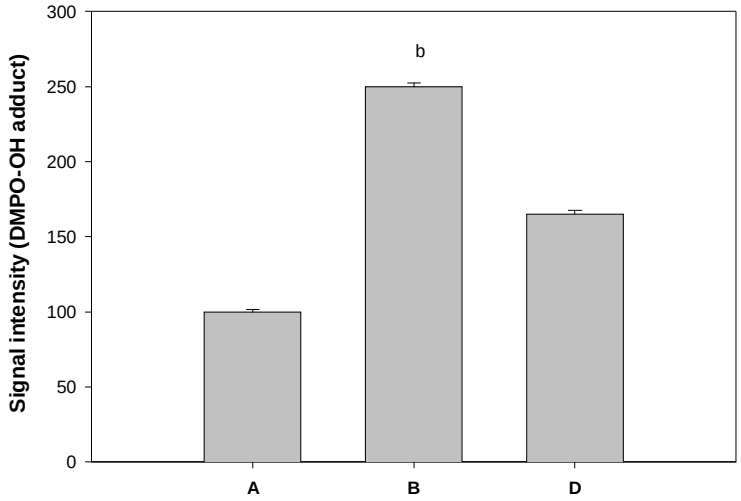


For ESR analysis mitoplasts (1 mg protein) were placed in 200µl of buffer (230mM mannitol, 70mM sucrose, 20mM Tris-HCl, pH adjusted to 7.4 with Mops) in the presence of respiratory substrates glutamate (7.5mM), malate (7.5mM) and inhibitors like antimycin (1µg/mg protein). DMPO (160mM) was added and the ESR spectra were recorded on a JES-FA 200 ESR spectrometer. Instrument settings were as follows: microwave power, 20mW; microwave frequency, 9.42 GHz; time constant, 0.03sec; scan time, 4min; scan width, 100G.
i, ii and iii shows original traces of ESR signals of rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*H.indicus* administered) in the presence of glutamate and malate.

iv It is due to the addition of superoxide dismutase which completely abolished the ESR signal.

FIGURE 53

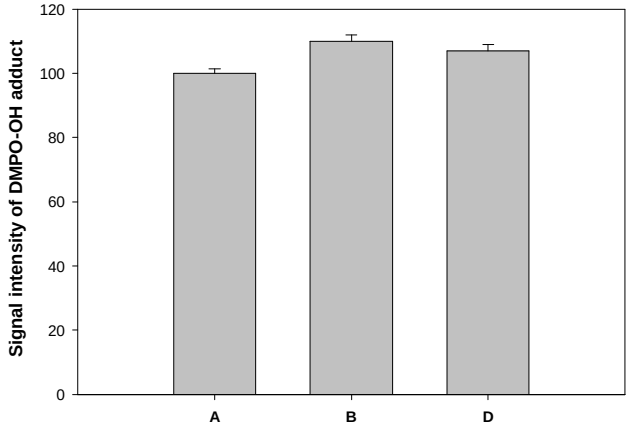
Signal intensities of DMPO-OH adducts in rat liver mitoplasts in the presence of glutamate and malate



It shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*H.indicus* administered) in the presence of glutamate and malate. $^{b}p<0.01$ vs. control Group.

FIGURE 54

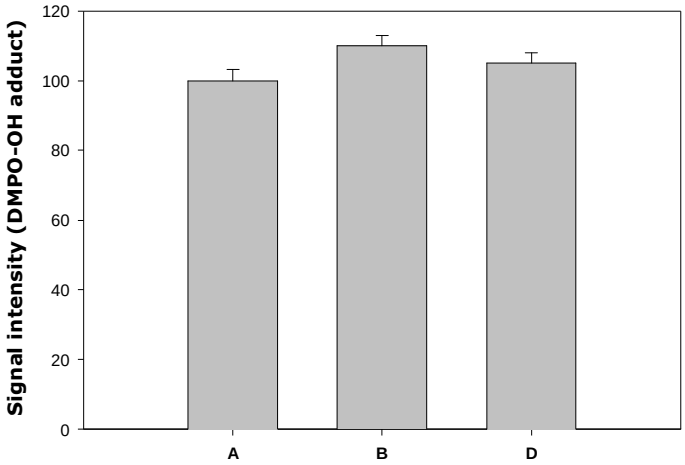
Signal intensity of DMPO-OH adduct in rat liver mitoplasts in the presence of glutamate, malate and antimycin



It shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*H.indicus* administered) in the presence of antimycin, glutamate and malate. There was no significant difference.

FIGURE 55

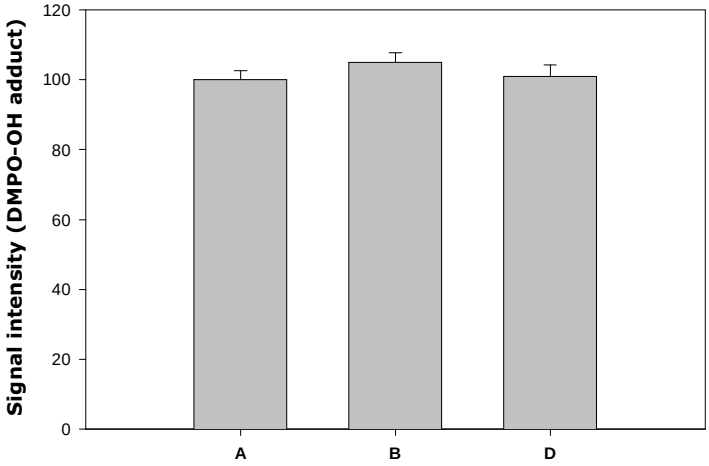
Signal intensities of DMPO-OH adducts in rat liver mitoplasts in the presence of succinate



It shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*H.indicus* administered) in the presence of succinate (7.5mM). There was no significant difference.

FIGURE 56

Signal intensity of DMPO-OH adduct in rat liver mitoplasts in the presence of succinate and antimycin



It shows signal intensities of DMPO-OH adducts in rat liver mitoplasts from group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+*H.indicus* administered) in the presence of succinate (7.5mM) and antimycin (1µg/mg protein). There was no significant difference.

Figure 57 i)

Histology of control rat liver. It shows normal hepatic cell architecture showing central vein.

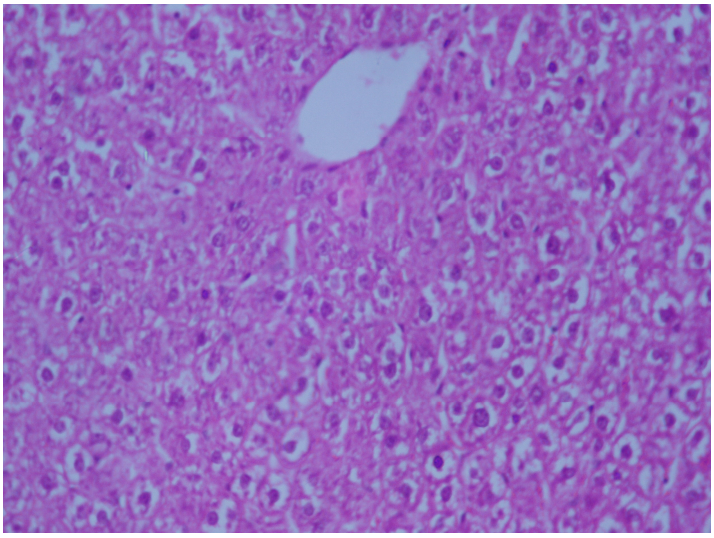
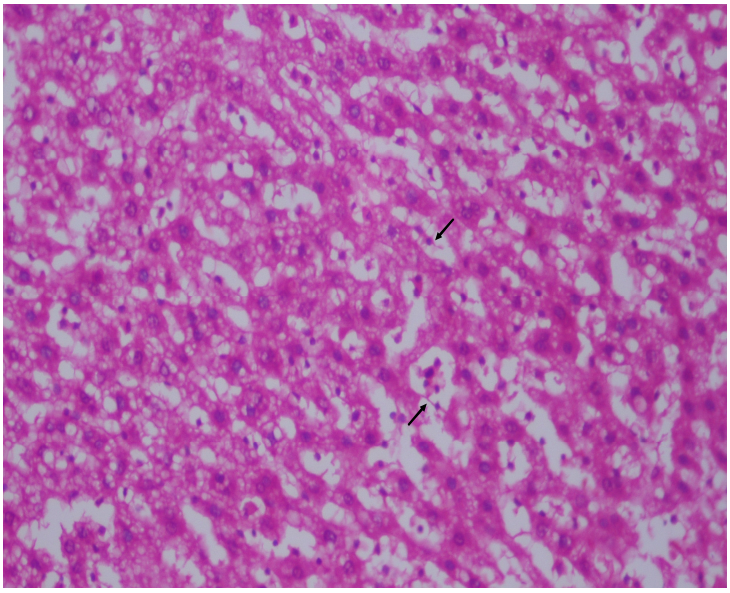


Figure 57 ii)

Histology of rat liver treated with allyl alcohol. It shows marked degenerative changes and mild fatty changes.



. **Figure 58 i)**

Histology of liver treated with only *Hemidesmus indicus* It shows normal hepatic cells architecture.

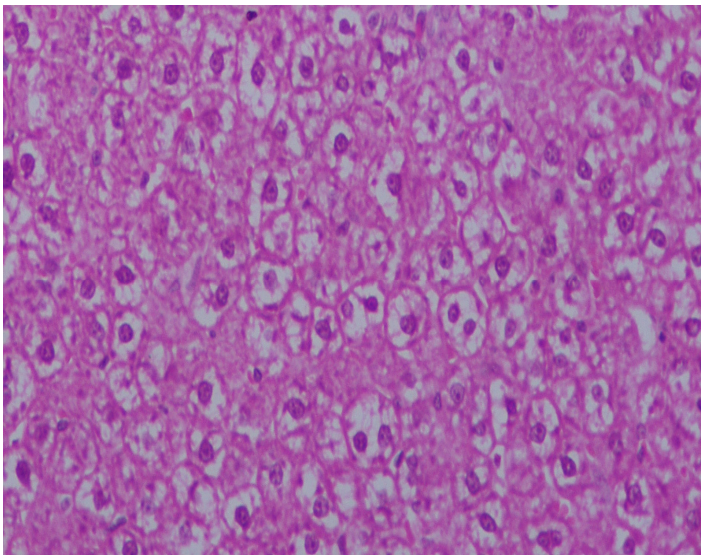


Figure 58 ii)

Histology of rat liver treated with both *Hemidesmus indicus* and allyl alcohol. It shows mild degenerative changes and mild dialation of sinusoidal spaces.

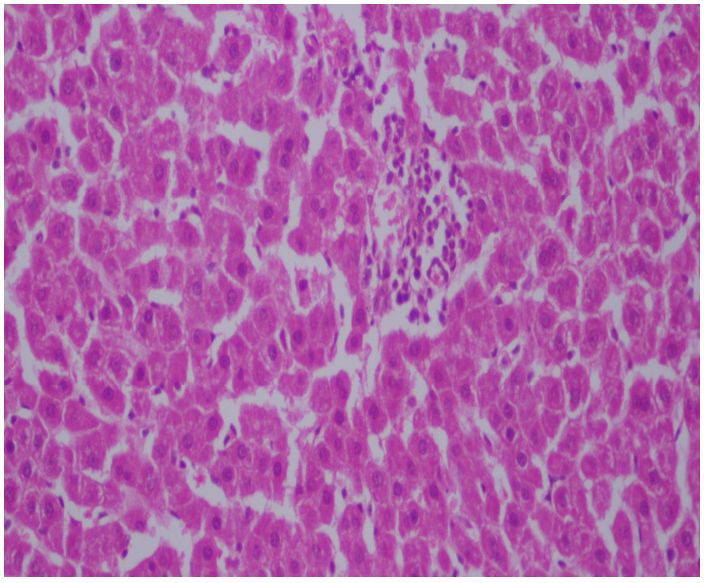


Figure 59 i)

Histology of rat liver treated with only *Phyllanthus fraternus.* It shows normal hepatic cells architecture.

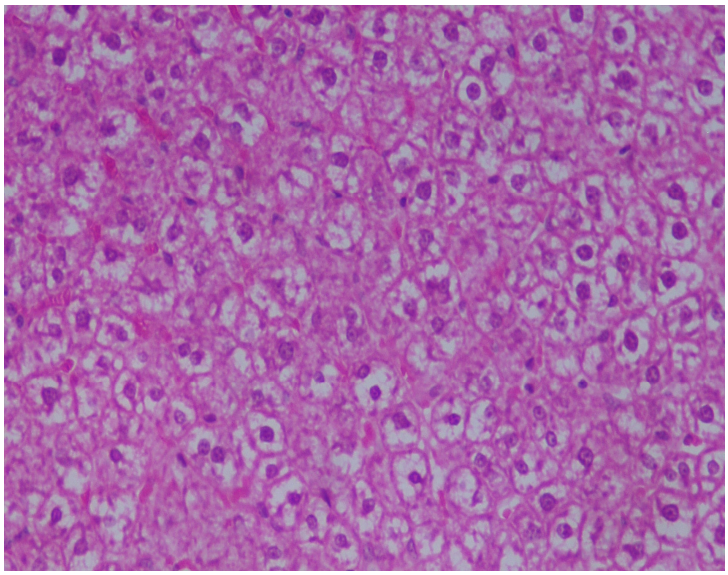
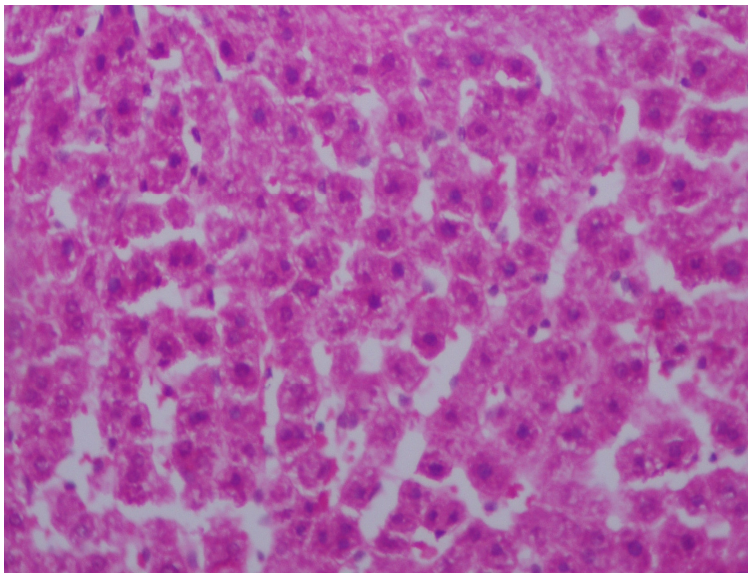


Figure 59 ii)

Histology of rat liver treated with both *Phyllanthus fraternus* and allyl alcohol. It shows mild degenerative changes.



DISCUSSION

In recent years, there has been a worldwide trend towards the use of the natural phytochemicals present in berry crops, teas, herbs, oilseeds, beans, fruits and vegetables (Kitts et al., 2000; Lee and Shibamoto, 2000). Free radicals, from both endogenous and exogenous sources, are implicated in the etiology of several degenerative diseases, such as coronary artery disease, stroke, rheumatoid arthritis, diabetes and cancer (Halliwell et al., 1992). High consumption of fruits and vegetables is associated with low risk for these diseases, which is attributed to the antioxidant vitamins and other phytochemicals (Ames et al., 1993; Prior, 2003; Weisburger, 1999). There is a great deal of interest in edible plants that contain antioxidants and health promoting phytochemicals.

It was reported earlier from our laboratory that mitochondrial dysfunction caused by the administration of alcohol (Sebastian and Setty, 1999) or thioacetamide (Padma and Setty, 1997) or carbon tetra chloride (Padma and Setty, 1999) could be prevented by prior administration of aqueous extract of *P.fraternus*. This study mainly involved on mitochondrial function and lipid peroxidation. Hepatotoxins like alcohol, carbon tetra chloride, thioacetamide and allyl alcohol induce toxicity and the mechanism by which they induce is different for each agent. For example: alcohol is known to induce ROS by decreasing the oxidation rates by electron transport chain and also it depletes SH groups. Carbon tetra chloride is converted to tri chloro methyl radical which is responsible for hepatotoxicity. Allyl alcohol used in this study is known to deplete GSH and induces oxidative stress. The mechanism by which the administration of plant extracts (*P.fraternus* or *H.indicus*) protect against oxidative stress is the important aspect of this study.

In mitochondria, the respiratory chain is stoichiometrically related to that of ATP synthesis (Pozzan et al., 1979). If the reducing substrate is glutamate or malate 3mol of ATP per pair of electrons are synthesized. If the substrate is succinate 2mol of ATP are synthesized. NADH oxidase which uses the electron carriers of all three sites of electron transport chain was inhibited (fig-1 and 29) in allyl alcohol administered rats. This indicates that there is an inhibition in the transfer of electrons through electron transport chain. RCR and P/O were also significantly decreased by the administration of allyl alcohol. Administration of the extract of *P.fraternus* or *H.indicus* prior to allyl alcohol relieved the inhibition significantly on NADH oxidase, RCR and P/O (fig-1 and 29). Succinate oxidase which uses the electron carriers of site 2 and site 3 of electron transport chain was significantly inhibited (fig-2 and 30) in allyl alcohol administered rats. RCR and P/O were also significantly decreased by the administration of allyl alcohol (fig-2 and 30) showing that mitochondria are uncoupled. Uncoupling of mitochondria normally stimulates respiration, but in this case (using glutamate+malate or succinate as substrates) an inhibition rather than stimulation is seen in the rate of respiration. This clearly indicated that there are blocks/inhibition in electron transport chain for the transfer of electrons due to the administration of allyl alcohol. The block/inhibition could be due to a limitation in the availability of functional electron carriers. Intraperitoneal injection of allyl alcohol to rats depressed state-3 respiration (Jacobs et al., 1987). Administration of *P.fraternus* or *H.indicus* prior to allyl alcohol provided significant protection of 72 and 53% respectively with glutamate+malate as substrate (fig-1 and 29) and

77 and 61% respectively with succinate as substrate (fig-2 and 30). The mitochondrial respiration is tightly coupled to oxidative phosphorylation in intact cells of normal tissues (Tzagoloff and Meyers, 1986). Tightly coupled mitochondria always have high RCR and P/O ratio. Decreased RCR and P/O ratio indicate the damage of mitochondrial membrane, there by rendering the membrane leaky to the ions and other biomolecules that leads to uncoupling of mitochondria. Increased permeability of the mitochondrial membrane leads to uncoupling of oxidative phosphorylation (Soussi et al., 1990). In isolated liver mitochondria fatty acid hydroperoxides, intermediate products of lipid peroxidation (LPO) cause permeability changes in the mitochondria leading to an uncoupling of oxidative phosphorylation (Masini et al., 1994).

Measurement of MDA (secondary product of lipid peroxidation), a useful method for determination of lipid peroxidation products (Sawicki et al., 1963; Placer et al., 1966) showed a significant increase in both liver homogenate and mitochondria by allyl alcohol administration. Lipid peroxidation is a free radical phenomenon and induces a series of alterations in the structure and function of cellular membranes. Prior administration of *P.fraternus* or *H.indicus* brought down the lipid peroxide level in rats administered with allyl alcohol (fig-6 and 34).

Membrane lipid peroxidation is an important pathophysiological event in a variety of diseases and stress conditions. Lipid peroxidation (LPO) results in a cascade of degenerative process from membrane denaturation to tissue damage. Biological membranes that are rich in poly unsaturated fatty acids are highly susceptible to free radical catalysed oxidation reactions. Lipid peroxidation has been demonstrated to occur in isolated mitochondria, lysosomes and microsomes (Tappel, 1972). In mitochondria about 90% of the fatty acids in the phospholipids are PUFAs, present mainly in phosphotidyl choline and Phosphotidyl ethanolamine, which account for approximately 80% of the total phospholipids and are susceptible to lipid peroxidation (Daum, 1985). The cell is equipped with an impressive set of system for protecting the integrity of the membrane by having antioxidants and antioxidant enzymes to scavenge free radicals. Vitamin E is the major natural lipophilic antioxidant in the membranes. However, under pathological conditions these systems can be overwhelmed and non-toxic drugs would be required for preventing the deleterious effects of lipid peroxidation. In the present study the two extracts (*P.fraternus* or *H.indicus*) showed that they have good potential to protect the membranes against lipid peroxidation (fig-6 and 34). Administration of the aqueous extract of the *P.fraternus* significantly decreased the thioacetamide (Padma and Setty, 1997) or carbon tetra chloride (Padma and Setty, 1999) induced lipid peroxidation *in vivo* and protected the liver from the hepatotoxin induced toxicity.

Transaminases are used as marker enzymes of liver damage. Aspartate aminotransferase mediates the reversible transfer of α-aminogroup from aspartate to α-ketoglutarate resulting in the production of glutamate and oxaloacetate. Alanine amino transferase mediates the transfer of α-amino group from alanine to α-ketoglutarate forming pyruvate and glutamate. Administration of allyl alcohol resulted in a significant decrease in the activities of these enzymes in the liver (fig-3 and 31). This decrease is

probably due to the leakage of the enzymes from liver into the extracellular compartment. If this extracellular compartment is in dynamic exchange with the blood, then the enzyme activities will increase in blood (Mukherjee, 1990). It was observed that on administration of allyl alcohol the levels of these enzymes were increased in the serum (fig-4 and 32). This is probably due to the leakage of enzymes from the liver cell due to the toxicity induced by the administration of allyl alcohol. Prior administration of the plant extracts (*P.fraternus* or *H.indicus*) along with allyl alcohol exerted significant protection against the liver toxicity induced by allyl alcohol (fig-3, 4, 31 and 32).

Membrane potential is the main component of the proton motive force (Mitchell and Moyle, 1969) and has significant control over mitochondrial respiration (Brand, 1990a, 1990b; Murphy, 1990). It is a sensitive index of the integrity of mitochondrial membranes and altered by changes in protein and lipid composition (Hoch, 1988; Hafner et al., 1988; Murphy, 1990). Membrane potential was decreased in allyl alcohol administered rats (fig-5 and 33). Under normal conditions, the transfer of electrons through the respiratory chain leads to the vectorial translocation of protons from the matrix to the cytosolic side. Any changes in the generation of proton gradient will have adverse effects on mitochondrial enegy production, in particular on ATP synthesis, because this proton gradient is the driving force for the formation of ATP from ADP. The present study shows that the driving force for ATP synthesis decreases significantly by the administration of allyl alcohol and prior administration of *P.fraternus* or H*.indicus* could protect the membranes and raised the membrane potential significantly (fig-5 and 33). This data also suggest that allyl alcohol disrupts the integrity of mitochondrial membranes and prior administration of plant extracts could protect the integrity of these membranes. The decreased P/O ratio due to allyl alcohol administration (fig-1, 2, 29 and 30) could be partly due to decreased driving force i.e membrane potential.

Allyl alcohol is metabolized by cytosolic alcohol dehydrogenase to acrolein, an unsaturated aldehyde (Rees and Tarlow, 1967). Alcohol dehydrogenase activity is increased in alllyl alcohol administered rats which indicates the formation of more amount of acrolein. Acrolein is a powerful electrophile and reacts with nucleophiles, such as sulfhydryl groups. The thiol group of glutathione is a favored target and so glutathione is primarily involved in the reaction which leads to GSH depletion. The reaction is markedly accelerated by the activity of cytosolic glutathione S-transferase. The nucleophilic glutathione is an important protective factor of hepatic cells in the detoxification of acrolein. That the alkylation of nucleophilic groups of cellular macromolecules affected by acrolein after glutathione depletion is the event that actually leads to cell injury (Ohno et al., 1985). Increased activity of alcohol dehydrogenase that occurs due to the administration of allyl alcohol (fig-15 and 43) leads to the generation of more acrolein and thus depletes GSH at faster rate and leads to oxidative stress. The administration of the plant extracts (*P.fraternus* or *H.indicus*) significantly decreased (fig-15 and 43) the alcohol dehydrogenase activity which leads to less acrolein and depletes GSH slowly and induce less oxidative stress. This partly explain the protective effect of plant extracts against the oxidative stress induced by allyl alcohol.

The carbonyl content is significantly increased (118%) due to the administration of allyl alcohol (fig-7 and 35). Typically, cellular nucleophiles target acrolein's β-carbon, generating carbonyl-retaining adducts (Esterbauer *et al*., 1991; Uchida *et al*., 1998b; Burcham and Fontaine, 2001). The reactive carbonyl may then react with neighboring nucleophiles to form inter or intramolecular cross-links (Esterbauer *et al*., 1991; Permana and Snapka, 1994; Kurtz and Lloyd, 2003). During reactions with protein, acrolein readily carbonylates lysine, cysteine and histidine side chains. It is shown that lysine modification involves sequential addition of two acrolein molecules to a given residue, followed by ring fusion and dehydration to form a six-membered heterocycle, *N*-(3-formyl-3,4-dehydropiperidino) lysine (FDP-lysine). There is increasing evidence for an involvement of toxic carbonyls in number of human diseases Calingasan *et al*., 1999; Tanaka *et al*., 2001). The prior administration of aqueous extract of *P. fraternus* or *H.indicus* offered protection of 99 and 44% on carbonyl content respectively (fig-7 and 35) and prevented the significant increase due to the administration of allyl alcohol.

Oxidants, inhibitors of enzymes containing an iron-sulfur center, free radicals and other reactive species cause the oxidation of biomolecules (e.g., protein, amino acids, lipid, and DNA), which leads to cell injury and death (McCord, 2000; Freidovich, 1999). Following initiation of lipid peroxidation, protein thiols of the membrane can be attacked by lipid derived radicals or reactive, lipid soluble aldehydes like 4-hydroxy nonenol and other hydroxyl alkenals originated within the lipid core of the cell membranes This results in a membrane protein thiol loss which in turn is associated with the development of hepato cellular injury (Pompella et al., 1991). Significant decrease on the total sulphydryl groups due to the administration of allyl alcohol was protected to an extent of 59 and 70% (fig-8 and 36) by prior administration of aqueous extract of *P. fraternus* or *H.indicus* respectively.

Oxidative stress is associated with increased formation of ROS that modifies phospholipids and proteins leading to peroxidation and oxidation of thiol groups (Molavi & Mehta, 2004). The assaults by ROS lead to changes in membrane permeability, membrane lipid bilayer disruption and functional modification of various cellular proteins.

Superoxide is generated from O_2 by multiple pathways (Gilbert, 2000; Freidovich, 1999; Wu and Morris, 1998; Evans and Halliwell, 2001). Under physiological conditions, there is a well-managed balance between formation and neutralization of ROS. Oxidative stress can occur when ROS production is accelerated or when the mechanisms involved in scavenging ROS are impaired. Increased production of ROS is thought to occur more frequently than dimnished antioxidant defence, and is postulated to play a role in the pathogenesis of several diseases (Halliwell and Gutteridge, 1999). Epinephrine is converted to adrenochrome by superoxide and using this principle, the adrenochrome can be followed spectrophotometrically and thus superoxide level can be estimated. The prior administration of aqueous extract of *P. fraternus* or *H.indicus* offered protection of 53 and 51% respectively on superoxide level (fig-9 and 37) and prevented the significant increase due to the administration of allyl alcohol.

ESR spin trapping offer sensitive method for superoxide determination. Spin trap is a compound which forms a stable free radical by reacting covalently with an unstable radical. Thus the radical trapped in a long-lived form which can be observed at room temperature using ESR. The hyperfine splitting of the adduct provides information which can aid in the identification of the radical. The addition of glutamate and malate resulted in a low intensity ESR signal characterstic of the DMPO-OH spin adduct (quartet signal with intensity ratios of 1:2:2:1). The DMPO-OH signal is formed by spontaneous decay of the DMPO-superoxide adduct (DMPO-OOH) in mitoplasts. Healthy mitochondria under normal conditions will not generate detectable free radicals coupled to substrate oxidation. The major sites in the electron transport chain for the generation of free radicals are complex I and complex III. The electron transport chain is to be inhibited with suitable inhibitors to see the maximum capacity of electron transport chain to generate free radicals. Inhibition in the flow of electrons will force them to go on to oxygen to generate superoxide. This process is minimum when electrons are flowing freely through the electron transport chain. When electron transport chain is inhibited by an inhibitor of complex 3 (eg:- antimycin), maximum amount of free radicals are generated and now one cannot see the difference between the normal mitochondria and mitochondria that have blocks in the electron transport chain due to experimental treatment. Fig 25, 26, 53 and 54 shows the ESR signal intensities of group-A (control), group-B (allyl alcohol administered) and group-D (allyl alcohol+plant extract administered). The change in the signal intensities of these 3 groups is not statistically significant in the presence of antimycin. When the same experiment was done in the absence of antimycin, the ESR signal intensity was statistically increased due to the administration of allyl alcohol (fig 25 and 53) and there was a significant protection (47 and 57%) due to the prior administration of *P.fraternus* or *H.indicus*. This shows that there is a significant block in the electron transport chain in group-B (allyl alcohol administered) which was responsible for generation of high amount of superoxide compared to group-A (control). When succinate was used as a substrate there was no statistically significant difference in ESR signal intensities due to the administration of allyl alcohol when compared with control, even in the presence (fig 54) or absence of antimycin (Fig-55). This demonstrates that there is no significant block in electron transport chain from succinate to oxygen for the generation of excess of free radicals. These results clearly indicate that the block is at site 1 and the significant amount of superoxide that is generated due to the administration of allyl alcohol is from complex I. Addition of SOD, abolishes the ESR signal completely, showing that the superoxide is the source for hydroxyl radical (fig 24 iv and 52 iv). In congestive heart failure mitochondria produce more superoxide than normal mitochondria in the presence of NADH but not succinate showing that and that complex I is the predominant source of such superoxide production (Idle et al., 1999).

The protective roles of glutathione against oxidative stress are (Masella et al., 2005): (i) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GSHPx), glutathione transferase etc. (ii) GSH participates in amino acid transport through the plasma membrane; (iii) GSH scavenges hydroxyl radical and singlet

oxygen directly, detoxifies hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase; (iv) glutathione regenerates vitamins C and E, back to their active forms (glutathione can reduce the tocopherol radical of Vitamin E directly, or indirectly, *via* reduction of semidehydroascorbate to ascorbate).

Under conditions of enhanced oxidative stress, GSH content decreases, this in turn increases the content of protein mixed disulphides. A significant number of proteins involved in signaling have critical thiols, such as receptors, protein kinases and some transcription factors can be altered in their function by formation of mixed disulphides. Acrolein formed from allyl alcohol (Miccadei et al., 1988) produces an abrupt depletion of GSH which leads to lipid peroxidation and cell death. In the present study GSH levels were significantly decreased due to the administration of allyl alcohol (fig 14 and 42). Prior administration of plant extracts (*P.fraternus* or *H.indicus*) significantly increased the GSH levels.

Administration of *P.fraternus* or *H.indicus* promoted the conversion of GSSG (oxidised glutathione) into GSH (fig 14 and 42) by the reactivation of hepatic glutathione reductase enzyme in allyl alcohol-administered rats. The availability of sufficient amount of GSH thus increased the detoxification of active metabolites of allyl alcohol through the involvement of glutathione peroxidase. The restoration of GSH level after the enduration of the plant extracts to such allyl alcohol-treated rats account for the protective efficacy of the extract.

Antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase are important as part of an organism's defence mechanisms against free radical production and the damage by these molecules (Wispe et al., 1992; Rothstein et al., 1994; Keyer and Imlay et al, 1996). Due to the defensive roles of these enzymes, the potential for antioxidant enzyme therapy is becoming more and more important in number of diseases.

Presumably, a decrease in catalase activity could be attributed to cross-linking and inactivation of the enzyme protein in the lipid peroxides. Decreased catalase activity is linked up to exhaustion of the enzyme as a result of oxidative stress caused by allyl alcohol. In the present study catalase activity was significantly decreased due to the administration of allyl alcohol (fig 10 and 38). Prior administration of plant extracts (*P.fraternus* or *H.indicus*) provided significant protection (fig 10 and 38). The catalase activity was restored to normal after treatment with extracts evidently shows the antioxidant property of the extracts against oxygen free radicals. *H. indicus* reversed cumene hydroperoxide-mediated inhibition of the activities of antioxidant enzymes such as catalase, glutathione peroxidase and glutathione reductase. Thus *H.indicus* diminishes cumene hydroperoxide-induced cutaneous oxidative stress, which plays an important role in tumor promotion (Sultana et al., 2003). The exact mechanism by which *H.indicus* exhibits antioxidative activity in allyl alcohol induced toxicity is not well known, although this evidence suggests its action and efficacy in interception of the free radicals and protection of cellular macromolecule from oxidant damage. GSH may be depleted either by a conjugation reaction with electrophiles or by inhibition of GSH regeneration from the oxidized glutathione and/or its biosynthesis.

The increased GSH (allyl alcohol+plant extract)) associated with glutathione peroxidase might help in reducing the formation of peroxidative stress as observed by the decrease in MDA due to the administration of these plant extracts. Treatment with allyl alcohol depletes selenium-dependent glutathione peroxidase, an enzyme responsible for detoxifying oxygen derived toxic species. Glutathione peroxidase catalyzes the reduction of hydrogen peroxide and organic hydroperoxides by using GSH. GSSG derived from glutathione peroxidase activity is then reduced to GSH by the reaction catalyzed by glutathione reductase. Glutathione peroxidase activity was significantly decreased due to the administration of allyl alcohol (fig 12 and 40). Gutathione reductase activity was also significantly decreased due to the administration of allyl alcohol (fig 13 and 41). In the present study, decreased glutathione peroxidase and glutathione reductase activities may be due to the low level of GSH in allyl alcohol treated rats. Prior administration of plant extracts (*P.fraternus* or *H.indicus*) provided significant protection on glutathione peroxidase activity (fig 12 and 40) and glutathione reductase activity (fig 13 and 41). Although glutathione peroxidase is a relatively stable enzyme, it has been reported that glutathione peroxidase may be inactivated under conditions of severe oxidative stress [Condell and Tappel, 1983].

In the present study superoxide dismutase activity was significantly decreased due to the administration of allyl alcohol. Prior administration of plant extracts (*P.fraternus* or *H.indicus*) provided significant increase in SOD activity when compared to rats administered only allyl alcohol (fig 11 and 39).

Studies on the overexpression of Cu,Zn SOD which occurs as a result of the trisomy in Down's syndrome suggest a role of this enzyme in the neuropathology of the disease (Iannello et al., 1999). Studies on end stage heart failure have shown that upregulation of myocardial catalase appears to be a compensatory response to the increased oxidative stress, although the other key antioxidant enzymes, SOD and glutathione peroxidase are not upregulated (Dieterich et al., 2000). Various stress factors may alter the levels of antioxidant enzyme expression in tissues (Sen and Packer, 1996; Allen and Tresini, 2000; Otieno et al., 2000).Coordinated expression of antioxidant enzymes does not necessarily occur in response to stimuli (Ma and Johnson, 1999; Rohrdanz et al., 2000; Wilson and Johnson, 2000). Transcriptional regulation appears to be the most common form of regulation over antioxidant enzyme expression (Harris., 1992; Nanji et al., 1995), and there is extensive investigation on genetic elements and mechanisms which are responsible for transcriptional regulation of antioxidant enzyme gene expression. The redox state probably plays a significant role in the expression of Mn-SOD activity in mitochondria (Wright and Reichenbecker., 1999).

It has beeen reported that oxidative stress modifies Mn SOD, Cu,Zn SOD and glutathione reductase gene expression at the transcription, post-transcriptional translational and post-traslational levels(Cryne et al., 2003). It is reported that menadione-induced oxidative stress not only decreases translational efficiency for Cu,Zn SOD but also increases the proteolysis rate(Cryne et al., 2003). Cellular injury from ROS has been implicated in the development and progression of several diseases. Increases in the

levels of ROS during periods of oxidative stress, are detected by redox-sensitive regulatory molecules in the cell and trigger a homeostatic response to prevent cellular injury called the oxidative stress response (Camhi et al., 1995). Included in the cellular oxidative stress response is the regulation of antioxidant enzymes gene expression, leading to increased antioxidant enzymes activities and, therefore, to a faster removal of the oxidants by the cell, protecting the cell against oxidative stress (Shull et., 1991; Ho et al., 1996; Rohardanz and Kahl, 1998; Franco et al., 1999). However, the cell response has a complex regulation and an increase in antioxidant enzymes mRNAs during oxidative stress do not always correlate with increased activities or protein content of these enzymes in mammalian cells and tissues (Ho et al., 1996; Rohardanz and Kahl, 1998; Franco et al., 1999, Clerch et al., 1998; Jackson et al., 1998).

Some studies on the effect of oxidative stress in mammalian cells have found increases in the activities of antioxidant enzymes and protein level that follow increases in mRNA level (Shull et., 1991; Ho et al., 1996; Rohardanz and Kahl, 1998; Franco et al., 1999; Clerch et al., 1998; Jackson et al., 1998; Yoshioka et al., 1994). However there are other studies with mammalian cells and tissues that show no correlation between the increase found in antioxidant enzyme mRNA and activities or protein content of these under oxidative stress. It is suggested that there is a translational block for the synthesis of these enzymes during oxidative stress (Ho et al., 1996; Rohardanz and Kahl, 1998; Franco et al., 1999; Clerch et al., 1998; Jackson et al., 1998). The increase in mRNA levels found during oxidative stress may occur to compensate for the decrease of translational efficiency and to maintain the protein levels and enzyme activities.

Thus, allyl alcohol not only increases free radical production in the liver, but also decreases its ability to detoxify reactive oxygen species. Possible reasons for the decreased SOD and glutathione peroxidase activities might be due to the inhibition of enzyme protein synthesis, inhibition of enzymes by allyl alcohol or some lipid peroxidation products. Recently, it was shown that depletion of mitochondrial SOD by about 50% results in a functional decline of oxidative phosphorylation, an increase in oxidative stress, an increase in rates of apoptosis in an age-dependent manner (Kokoszka et al., 2001), and depletion of mitochondrial GSH (Williams et al., 1998), suggesting that Mn-SOD is important to maintain the balance of mitochondrial redox state. Moreover, these data suggest that minor changes in Mn-SOD may have a significant impact on the antioxidant status of mitochondria and support the hypothesis that overexpression of Mn-SOD may be protective mechanism against mitochondrial oxidative stress.

GSTs represent one of the major cellular dafence mechanisms against electrophilic xenobiotics and their metabolites. It was shown that GSTs use 4-hydroxy nonenol and malonaldehyde (Awasthi et al., 1995) as substrates and that among all GST classes alpha-class shows high selenium independent glutathione peroxidase activity (Awasthi et al., 1975). Since GSTs play an important role in detoxification and physiological functions, effect of allyl alcohol administration on cytosolic GSTs, where majority of allyl alcohol is metabolized was studied. In the current study, the overall

GST activity was measured using CDNB, as substrate show significant decrease in the allyl alcohol treated rat liver cytosol compared to control. Prior administration of plant extracts (*P.fraternus* or *H.indicus*) increased the GST activity which indicates the induction of a detoxifying system to enhance the conjugational capacity of GST for inactivation of electrophiles. The GST enzyme system consists of several isozymes. In rat liver, the GST isozymes are mainly made up of three major subunits, the Ya (Mwt-25600), Yb (Mwt-27000) and Yc (Mwt-28000). Each subunit has a specific function. Ya and Yc catalyse the reduction of hydro peroxides, isomerisation of prostaglandin. Yb subunit involved in the formation of leukotrienes. In the present study SDS-PAGE shows Ya, Yb and Yc subunits of GST (fig 22A and 50A). Significant decrease in the Ya subunit was observed by the administration of allyl alcohol (fig 23i and 51i). Prior administration of plant extracts (*P.fraternus* or *H.indicus*) provided significant protection (fig 23i and 51i). Alpha class GST isoenzymes, hGSTA1-1 and hGSTA2-2 are involved in the protection mechanisms against lipid peroxidation (Yang et al., 2001).

The histological changes induced by allyl alcohol is evidenced by degenerative changes of liver (fig-57ii). Histopathological findings also support the protective effect of the extract (*H.indicus* or *P.fratenus*) as evidenced by the liver with mild degenerative changes (fig-58ii and 59ii).

Petrol extract of whole plant (Bhatnagar et al., 1961) and the leaf extract (Bhowmick and Chaudhary, 1982) of *P.fraternus* reported to have antifungal activity. Ethanolic extract of whole plant showed anticancer activity in the mouse and antispasmodic activity on isolated ginea pig ileum (Dhar et al., 1968). The aqueous extract from the leaves of it reported to have hypoglycaemic effect in normal as well as alloxan-diabetic rabbits (Ramakrishnan et al., 1982). The hydroalcoholic extract (HE) given intraperitoneally, produced significant inhibition of acetic acid-induced abdominal constrictions (Adair et al., 2000). Ahmed et al., 2002 have reported that the methanolic fraction was most active among all fractions studied in protecting liver against carbon tetra chloride induced toxicity.

Chemical studies of this plant show that the leaves of *P. fraternus* contain a number of lignans and alkaloids. Phyllanthin (a bitter constituent) and hypophyllanthin (a non-bitter compound) were isolated from the leaves of *P. fraternus* and identified as lignans. The hexane extract of the leaves gave three additional lignans, niranthin, nirtetralin and phyltetralin. Among phyllanthin, hypophyllanthin, triacontanal and tricontanol isolated from a hexane extract of *P. fraternus*, phyllanthin and hypophyllanthin showed a protective effect against carbon tetra chloride and galactosamine induced cytotoxicity in primary cultured rat hepatocytes, while triacontanal was protective only against galactosamine induced toxicity (Syamasunder et al., 1985). Two alkamides (E, E-2,4-octadienamide and E,Z-2,4-decadienamide) have been isolated from *P. fraternus*, a plant that is used in Ghanaian traditional medicine to treat malaria. The compounds possess an alpha, beta, gamma, delta-unsaturated conjugated amide, a feature believed to enhance antiplasmodial activity. In vitro assay of the two alkamides showed to possess moderate antiplasmodial activity (Sittia et al., 1998).

A diet rich in brussels sprouts (300 g/d) markedly decreases the urinary excretion of 8-hydroxydeoxyguanosine in humans, indicating a reduction of DNA oxidation (Verhagen et al., 1995). Similarly, dietary supplementation of cabbage and broccoli extracts to rats decreases free radical-induced tissue damage brought about by irradiation (Fang et al., 1987). Moreover, phytic acid has a high chelation potential and can be supplemented to diets for suppressing iron-catalyzed oxidative reactions and potentially for reducing the incidence of colonic cancer and inflammatory bowel disease (Graf and Eaton, 1990). Collectively, these studies suggest that phytochemicals may be used as effective antioxidants for improving human health.

Hemidesmus indicus is employed in traditional medicine for gastric ailments. It mainly consists of essential oils and phytosterols like hemidesmol, hemidesterol, and saponins. Phytochemical investigations have shown the presence of coumarino-lignoids, flavonoids and triterpenoids (Mandal *et al*., 1991; Rastogi and Mehrotra, 1995). It was reported earlier that nine pregnane glycosides viz. Desinine (Oberai, 1985), Indicine, Hemidine (Prakash *et al*., 1991), Indi-cusin (Deepak *et al*., 1995), Hemidescine, Emidine (Chandra *et al*., 1994), medidesmine, Hemisine and Demicine (Deepak *et al*., 1997b) were isolated from *Hemidesmus indicus*. Phytochemical studies on the roots of *Hemidesmus indicus* resulted in the isolation of six new pentacyclic triterpenes including two oleanenes, three ursenes and a lupene formulated us lup-1, 12-dien-3-on-21-ol including a known compound, beta-amyrin acetate, on the basis of spectroscopic techniques and chemical means (Roy *et al*., 2001).

Ethanolic extract of *Hemidesmus indicus* at a dose level of 1.5 and 3.0 mg/kg body weight in acetone prior to that of cumene hydroperoxide treatment reported to inhibit cumene hydroperoxide-induced cutaneous oxidative stress (Sultana *et al*., 2003). It was reported that oral treatment with the ethanol extract of *Hemidesmus indicus* roots at dose of 100 mg/kg, for 15 days significantly prevented rifampicin and isoniazid-induced hepatotoxicity in rats (Prabakan *et al*., 2000).

Animal studies have shown that dietary phytochemical antioxidants are capable of removing free radicals. Among them, phenolic and polyphenolic compounds, such as flavonoids and catechin in edible plants, exhibit potent antioxidant activities (Decker, 1995). A large body of the literature has documented the beneficial effects of tea polyphenolic compounds on scavenging superoxide and hydroxyl radicals (Fang et al., 1998), and on their role in the prevention and therapy of disease. These polyphenols also enhance Cu,Zn-SOD activity and decrease malondialdehyde concentrations (Cui et al., 2000).

From all these studies it appears that the oxidative stress induced by allyl alcohol is mainly attributed to the free radical production due to GSH depletion which is responsible for oxidation of membrane proteins and increased lipid peroxidation which in turn responsible for the observed mitochondrial dysfunction. The mode of action of both these plant extracts in exerting the hepatoprotective activity against allyl alcohol may be due to the increased mitochondrial membrane integrity, increased glutathione (reduced) levels and activation of antioxidative enzymes such as glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase which inturn scavenge free radicals.

SUMMARY

The important findings of the present study are

1. On administration of allyl alcohol the activities of aspartate and alanine amino transferases increased significantly in serum and decreased in liver indicating liver damage. Administration of *P.fraternus* prior to allyl alcohol offered protection against the liver damage to an extent of 60 and 38% on AST activity in liver and serum respectively, where as 48 and 27% on AlAT activity respectively. Administration of *H.indicus* prior to allyl alcohol offered protection against the liver damage to an extent of 51 and 46% on AST activity in liver and serum respectively where as 55 and 33% on AlAT activity respectively.

2. NADH oxidase which gives information on the ability of transfer of electrons through all three sites of electron transport chain was decreased significantly in rats administered with allyl alcohol. Administration of *P. fraternus* or *H.indicus* prior to allyl alcohol administration offered protection of 72% and 53% respectively against allyl alcohol induced inhibition on respiration.

3. Succinate oxidase which gives information on the electron transfer through site II and III of electron transport chain was also decreased significantly on administration of allyl alcohol. Administration of *P.fraternus* or *H.indicus* prior to allyl alcohol administration offered protection of 77% and 61% respectively against allyl alcohol induced inhibition on respiration.

4. RCR which is an index of membrane integrity was decreased by the administration of allyl alcohol showing that the integrity of mitochondria was damaged. P/O ratio, an index of the efficiency of the system to conserve energy in the form of ATP, was decreased significantly with allyl alcohol. Administration of *P. fraternus* or *H.indicus* prior to allyl alcohol administration offered protection of 40 and 47% respectively on RCR, 80 and 62% respectively on P/O ratio against allyl alcohol induced mitochondrial dysfunction. Similar protection was observed when succinate was used as substrate.

5. Lipid peroxide level was increased significantly in liver homogenate and mitochondria of allyl alcohol administered rats. Administration of *P.fraternus* or *H.indicus* prior to allyl alcohol administration offered protection of 88 and 74% respectively on homogenate, 91 and 74% respectively on mitochondria against allyl alcohol induced increase in lipid peroxidation.

6. Protein carbonyl level was increased significantly in mitochondria of allyl alcohol treated rats. Administration of *P. fraternus* or *H.indicus* prior to allyl alcohol administration offered protection of 100 and 44% respectively against allyl alcohol induced increase in protein carbonyls.

7. Total sulphydryl groups were decreased significantly in mitochondria by the administration of allyl alcohol. Administration of *P.fraternus* or *H.indicus* prior to allyl alcohol administration offered protection of 59 and 47% respectively against allyl alcohol induced decrease on sulphydryl groups.

8. Glutathione (GSH) which is essential for maintanance of intracellular redox state was decreased significantly by the administration of allyl alcohol. Administration of *P. fraternus* or *H.indicus* prior to allyl alcohol administration offered protection of 41 and 49% respectively against allyl alcohol induced decrease in GSH level.

9. Superoxide radical levels were increased significantly by the administration of allyl alcohol. Administration of *P.fraternus* or *H.indicus* prior to allyl alcohol administration offered protection of 53 and 51% respectively against allyl alcohol induced increase in superoxide level.

10. Antioxidant enzymes (scavenger of free radicals) such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase activities decreased significantly in allyl alcohol administered rats. Administration of *P.fraternus* or *H.indicus* prior to allyl alcohol administration offered significant protection and increased the activities of these enzymes.

11. Studies on purified glutathione transferase (detoxify xenobiotic compounds) from allyl alcohol treated rats showed that Ya subunit of the enzyme was decreased significantly. Administration of *P.fraternus* or *H.indicus* prior to allyl alcohol administration offered significant protection against allyl alcohol induced decrease of Ya subunit.

12. Alcohol dehydrogenase activity was increased significantly by the administration of allyl alcohol. Administration of *P.fraternus* or *H.indicus* prior to allyl alcohol administration offered protection of 62 and 73% against allyl alcohol induced increase in alcohol dehydrogenase activity.

In conclusion the administration of allyl alcohol damages the membrane integrity, decreases the rate of respiration and ATP synthesis. Depletion of GSH, decreased antioxidant enzyme activities and increased production of free radicals lead to lipid peroxidation, protein oxidation and membrane damage. In this study a significant protection against these damages was observed due to the prior administration of *P.fraternus* or *H.indicus*.

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Protective effect of *Phyllanthus fraternus* against allyl alcohol-induced oxidative stress in liver mitochondria

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Abstract

The effect of administration of allyl alcohol on the oxidative stress and the protective effect due to administration of an aqueous extract of *Phyllanthus fraternus* against allyl alcohol-induced damage in liver mitochondria were studied. When rats were treated with allyl alcohol, the rate of mitochondrial respiration was decreased significantly with both NAD^+- and FAD-linked substrates. The respiratory control ratio, an index of membrane integrity and the P/O ratio, a measure of phosphorylation efficiency also decreased significantly. There was a significant increase in the lipid peroxide level and the protein carbonyl content. A significant decrease was observed in the total sulphydryl groups and a significant increase in the generation of superoxide radicals. Administration of rats with an aqueous extract of *Phyllanthus fraternus* (100 mg/kg) prior to allyl alcohol administration showed protection of 72, 40 and 80% using glutamate + malate (NADH oxidation) and 77, 54 and 20% using succinate as substrate on state 3, RCR and P/O ratio, respectively. The protection on lipid peroxide level was 88 and 91% in homogenate and mitochondria, respectively. In case of protein carbonyls, total sulphydryl groups and on the generation of superoxide radicals the protection was 99, 59 and 53%, respectively.

Keywords: Allyl alcohol; Oxidative stress; Plant extract; Lipid peroxidation; Reactive oxygen species; Acrolein; Mitochondria; Protein carbonyl; Superoxide radicals; Sulphydryl groups; Euphorbiaceae; *Phyllanthus fraternus*

1. Introduction

Plants and plant products are part of the vegetarian diet and a number of them exhibit medicinal properties. Several Indian plants are also being used in Ayurvedic and Siddha medicines. The medicinal properties of several herbal plants have been documented in ancient Indian literature and the preparations have been found to be effective in the treatment of diseases (Handa et al., 1996). The reports indicate that there is an inverse relationship between the dietary intake of antioxidant-rich foods and the incidence of human diseases (Sies, 1993; Halliwell, 1997). Hence, search for new synthetic and natural antioxidants is essentially important. Although initial research on antioxidants was mostly on isolated pure compounds, recent focus is more on natural formulations (Hagerman et al., 1998; Haramaki and Packer, 1995). It has been found that compounds in their natural formulations are more active than their isolated form (Khopde et al., 2001). *Phyllanthus fraternus* (Euphorbiaceae) is an annual herb, whose stem is non-erect and 30 cm long, the leafy shoots are 5–10 cm long, oblong and joined to the brachlets of the stem, six sepals in the flower, distributed in India, Pakistan and introduced into Saudi Arabia, Africa and West Indies (Abedin et al., 2001). It is widely distributed in the northern region of India and is used as a folklore remedy for the treatment of various diseases of liver by traditional healers and tribals (Kirtikar and Basu, 1975).

Allyl alcohol (AA) is a chemical used in manufacturing processes for food flavoring and in agriculture as a weed killer (Beauchamp et al., 1985; Atzori et al., 1989). It irritates mucous membranes and is especially harmful for the liver as it produces cell necrosis selectively in the periportal zone. It is metabolized by cytosolic alcohol dehydrogenase to acrolein, an unsaturated aldehyde (Rees and Tarlow, 1967). Acrolein is a powerful electrophile and reacts with nucleophiles, such as sulfhydryl groups.

Abbreviations: ADP, adenosine diphosphate; BSA, bovine serum albumin; DNPH, 2,4-dinitro phenyl hydrazine; DTNB, 5,5'-dithio bis-(2-nitro benzoic acid); EDTA, ethylene diamine tetraaceticacid; FAD, flavin adenine dinucleotide; GSH, glutathione (reduced); MDA, malonaldehyde bis dimethyl acetal; NADH, nicotinamide adenine dinucleotide (reduced); P/O, phosphate to oxygen ratio; RCR, respiratory control ratio; SOD, superoxide dismutase; TCA, trichloro acetic acid

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The thiol group of glutathione is a favored target and so glutathione is primarily involved in the reaction, which leads to GSH depletion. The reaction is markedly accelerated by the activity of cytosolic glutathione *S*-transferase. The nucleophilic glutathione is an important protective factor of hepatic cells in the detoxification of acrolein.

Free radicals are defined as molecules having an unpaired electron in the outer orbit. They are generally unstable and very reactive. There is a lot of evidence revealing the role of reactive oxygen species (ROS) in several diseases. ROS are generated as byproducts of cellular metabolism, primarily in the mitochondria (Szocs, 2004). Small, physiological amounts of ROS are a cellular requirement (Hensley et al., 2000). However, ROS have the potential to cause damage and, hence, cells possess many antioxidant systems for scavenging them. In addition to antioxidant enzymes, several small-molecule antioxidants play an important role in the antioxidant defence systems.

The mitochondrial electron transport system is a source for superoxide generation. An increase in dietary energy intake enhances mitochondrial free radical production, which results in oxidative stress (Gilbert, 2000; Balaban et al., 2005). The acrolein produced would cause decreased protein synthesis and inhibition of respiration (Serafini-cessi, 1972). The toxicity leads to alterations in membrane integrity mainly due to protein thiol modification and lipid peroxidation. Thiol groups of cysteine residues in proteins react readily with acrolein and it lead to changes in the enzyme activities.

Mitochondrial function was shown to be disrupted in patients with cirrhosis liver. It was reported earlier from our laboratory that mitochondrial dysfunction caused by the administration of alcohol (Sebastian and Setty, 1999) or thioacetamide (Padma and Setty, 1997) or carbon tetrachloride (Padma and Setty, 1999) could be prevented by prior administration of an aqueous extract of *Phyllanthus fraternus* and the protective effect was attributed mainly to the antioxidant property of the extract. The main objective of the present study is to show the protective effect of the prior administration of *Phyllanthus fraternus* on the oxidative stress that is induced by the administration of allyl alcohol, which is known to deplete GSH. Lipid peroxidation, protein oxidation, ROS (superoxide) levels and mitochondrial functions are used as markers of oxidative stress.

2. Methods

2.1. Chemicals

Sodium salts of succinate, glutamate, malate and ADP, xanthine, xanthine oxidase, epinephrine and guanidine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and were obtained from local firms.

2.2. Preparation of the aqueous extract of Phyllanthus fraternus

The whole plant of *Phyllanthus fraternus* including roots was homogenized at room temperature in water (5 g/12.5 ml) using motor and pestle. The homogenate was then filtered through a cheese cloth. The dry weight of the extract was determined by drying the extract in an oven. The water extract equivalent to 100 mg/kg was administered daily to each rat.

2.3. Treatment of experimental animals

Research on animals was carried out with approval from the Institutional Animal Ethics Committee (IEAC) of University of Hyderabad. Male Albino Wistar rats weighing 120 ± 20 g were taken from the animal house facility of University of Hyderabad and checked for proper growth for at least 8–10 days. They were fed with commercial pellet diet and tap water ad libitum. The animals were divided into four groups of four rats in each group.

Group A: Control rats, which received 0.5 ml of saline intraperitoneally.
Group B: Received allyl alcohol 4–5 mg in 0.5 ml volume of saline (0.7 mmol per kg body weight) intraperitoneally and sacrificed after 4 h.
Group C: Received orally aqueous extract of *Phyllanthus fraternus* (equivalent to 100 mg/kg) for a period of 4 days and then 0.5 ml of saline was given intraperitoneally and were sacrificed after 4 h.
Group D: Received orally aqueous extract of *Phyllanthus fraternus* (equivalent to 100 mg/kg) for 4 days and then allyl alcohol 4–5 mg in 0.5 ml volume of saline (0.7 mmol per kg body weight) was given and sacrificed after 4 h.

All the rats were fasted for 17 h before and 4 h after allyl alcohol administration.

2.4. Isolation of mitochondria

A slightly modified method of Laurence and Davies (1986) was used for the preparation of mitochondria. Liver was homogenized followed by differential centrifugation in ice cold medium containing 220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES, 0.2 mM EDTA and 0.36 mg/ml of BSA and adjusted to pH 7.4. The final pellet containing mitochondria was suspended in 3 ml of 0.25 M sucrose and the protein content was determined by Biuret method using BSA as a standard (Gornall et al., 1949). Mitochondria were used immediately for the measurement of oxidative phosphorylation and then were kept at $-80\,^{\circ}C$ until used for other studies.

2.5. Measurement of oxidative phosphorylation

The rate of respiration was determined using Hansatech Oxytherm Respirometer according to the method of Estabrook (1949). Respiration rates were measured at 25 °C in a buffer (containing 50 mM sucrose, 50 mM Tris–HCl, 20 mM potassium phosphate, 2 mM EDTA, 7 mM magnesium chloride, pH 7.4) and 1–2 mg of freshly isolated mitochondrial protein using an oxygen electrode disc in an airtight chamber of 1 ml volume. Malate (6.25 mM) and glutamate (3.125 mM) or succinate (0.02 M) was used as the substrates. Respiratory control

ratio (RCR) was obtained from the ratio of ADP stimulated state-3 respiration to ADP exhausted state-4 respiration and ADP/O = P/O ratio which was calculated according to Estabrook (1949). Respiration was initiated by the addition of 20 mM sodium succinate or 3.125 mM glutamate plus 6.25 mM malate for succinate oxidase and NADH oxidase, respectively. State-3 respiration was measured by the addition of 200 and 400 nmol of ADP for succinate oxidase and NADH oxidase, respectively.

2.6. Assay of lipid peroxides by thiobarbituric acid reaction

In liver homogenate and mitochondria, lipid peroxide level was determined according to the method of Ohkawa et al. (1979). A 10% liver homogenate was prepared in 1.15% KCl and mitochondria were isolated by differential centrifugation. Mitochondria were washed with 1.15% KCl and suspended in the same medium. To 5 mg of protein, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.67% (w/v) aqueous solution of thiobarbituric acid were added. The total volume was made up to 4 ml with water and the tubes were heated in a water bath at 95 °C for 60 min. A blank was also run simultaneously and tetramethoxy propane was used as an external standard. After cooling, 1.0 ml of water and 5 ml of *n*-butanol were added and the tubes were vortexed and then centrifuged at 2000 × *g* for 10 min at room temperature. The absorbance of the organic layer was measured at 535 nm. The extent of lipid peroxidation was expressed as nmol of MDA formed per 100 mg of protein.

2.7. Measurement of protein carbonyls

The concentration of protein carbonyls in mitochondria was determined using 2,4-dinitrophenylhydrazine (DNPH) assay according to the method of Reznick and Packer (1994) and Bailey et al. (2001). The mitochondria were divided into two portions containing 2–4 mg of protein in each. To one portion, 4 ml of 2N HCl was added and incubated at room temperature shaking intermittently for 1 h. The other portion was treated with 4 ml of 10 mM DNPH in 2N HCl and incubated by shaking intermittently for 1 h at room temperature. After incubation the mixture was precipitated with 10% TCA and centrifuged. The precipitate was washed thrice with 4 ml of ethanol:ethyl acetate (1:1). The final protein precipitate was dissolved in 6 M guanidine hydrochloride and the absorption at 370 nm (DNPH-treated sample minus sample blank) was determined. Carbonyl content was calculated using the molar extinction coefficient of 22,000 and expressed as nmol carbonyls per mg protein.

2.8. Determination of total sulphydryl groups

Mitochondrial suspension containing 1 mg of protein was mixed with 1.5 ml of 0.2 M Tris buffer and 0.1 ml of 0.01 M 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) by the method of Sedlak and Lindsay (1968). The mixture was made to 10 ml by adding 7.9 ml of absolute methanol. A reagent blank (without sample) and sample blank (without reagent) were prepared in a similar manner and color developed for 15 min and centrifuged at 3000 × *g* at room temperature for 15 min. The absorbance of the supernatant was measured at 412 nm and total sulphydryl groups were expressed as nmols per mg protein using DTNB molar extinction coefficient of 13,100.

2.9. Generation of superoxide radicals

Generation of superoxide radicals was measured spectrophotometrically by the method of Dionisi et al. (1975). The reaction mixture contains 0.25 M sucrose, 50 mM HEPES (pH 8.4), 1 mM epinephrine, 3 mM succinate and 0.5 mM EDTA and 0.1–0.2 mg mitochondrial fragments. The reaction of radical formation was initiated by the addition of 2.5 μg antimycin per milliliter. Generation of superoxide radicals was measured by the oxidation of epinephrine to adrenochrome which was quantified spectrophotometrically at 480 nm and was expressed as nmoles of adrenochrome formed per min per mg protein using molar extinction coefficient of adrenochrome as 4020.

2.10. Statistical analysis

All values were expressed as mean ± S.D. Statistical significance was performed by analysis of variance followed by Bonferror's test. $P < 0.05$ was considered to be significant.

3. Results

The effect of administration of allyl alcohol on the oxidative stress and the protective effect of an aqueous extract of *Phyllanthus fraternus* on allyl alcohol-induced toxicity were studied. Results of all the parameters in this study were expressed relative to control, which was taken as 100. The actual values for control group are given in the corresponding figures.

The percent protective effect due to the prior administration of *Phyllanthus fraternus* was calculated as follows.

$$\left[\frac{100}{100 - \text{value of group B}}\right] \times (\text{value of group D} - \text{value of group B})$$

In other words a 100% protection (on a given parameter) means that the value is back to the control level which is normalized as 100.

Externally added NADH cannot penetrate the tightly coupled mitochondria. So, glutamate and malate were used to reduce the NAD^+ pool in the matrix, which is then oxidized by the respiratory chain. This NADH oxidase gives information on the ability of transfer of electrons through all three sites of the electron transport chain. State 3 respiration, RCR and P/O ratio were decreased by 43, 55 and 29% compared to controls (Fig. 1) and this decrease is statistically significant. There was a significant decrease of RCR in group-D (allyl alcohol + *Phyllanthus fraternus*) when compared to group-A (control). Administration of *Phyllanthus fraternus* prior to allyl alcohol administration offered protection of 72, 40 and 80% on state 3 respiration, RCR and P/O ratios, respectively. Administration of the plant extract alone did not show any significant change. When succinate was used as substrate (electron transfer through site II + site III) there

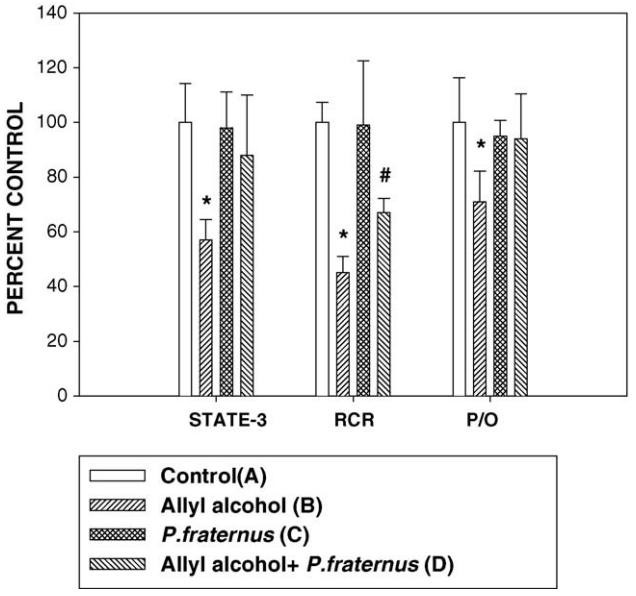

Fig. 1. Effect of administration of allyl alcohol with or without the administration of *Phyllanthus fraternus* on the rate of respiration, RCR, P/O ratio using glutamate + malate as substrate.Values are mean ± S.D. from at least four rats in each group. Rate of respiration was measured using glutamate + malate as substrate by the addition of 400 nmols of ADP. It is expressed as nanomoles of oxygen per minute per milligram protein. RCR is the ratio of ADP stimulated state-3 to ADP exhausted state 4 respiration and P/O = ADP/O ratio. All the values are expressed relative to controls, which were taken as 100. Control values for the rate of respiration, RCR, P/O ratio using glutamate and malate as substrates were 32.3 ± 4.6, 4.1 ± 0.3, 3.0 ± 0.7, respectively. $^{*}P < 0.05$ vs. Group-A. $^{\#}P < 0.05$ vs. Group-A.

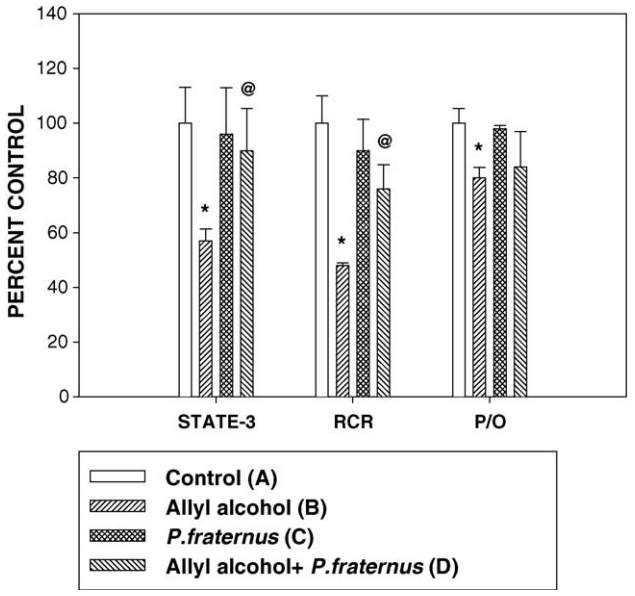

Fig. 2. Effect of administration of allyl alcohol with or without the administration of *Phyllanthus fraternus* on the rate of respiration, RCR and P/O ratio using succinate as substrate.Values are mean ± S.D. from at least four rats in each group. Rate of respiration was measured using succinate as substrate by the addition of 200 nmols of ADP. It is expressed as nanomoles of oxygen per minute per milligram protein. RCR is the ratio of ADP stimulated state-3 to ADP exhausted state 4 respiration and P/O = ADP/O ratio. All the values are expressed relative to control, which were taken as 100. Control values for the rate of respiration, RCR and P/O ratio using succinate as substrate were 53.3 ± 6.9, 4.0 ± 0.4, 2.0 ± 0.1, respectively. $^{*}P < 0.05$ vs. Group-A. $^{@}P < 0.05$ vs. Group-B.

was a decrease of 43, 52 and 20% on state 3 respiration, RCR and P/O ratios, respectively. There was a significant decrease on state 3 respiration and RCR in group-D when compared to group-B (allyl alcohol). This shows that there is a significant difference due to the administration of *Phyllanthus fraternus*. Administration of *Phyllanthus fraternus* prior to allyl alcohol offered protection of 77, 54 and 20% on state 3 respiration, RCR and P/O ratios, respectively (Fig. 2). There was no significant effect on succinate oxidation in rats administered with *Phyllanthus fraternus* extract alone. These results show that the administration of *Phyllanthus fraternus* offered a significant protection.

Effect of the administration of allyl alcohol with and without the prior administration of the aqueous extract of *Phyllanthus fraternus* on lipid peroxide level is shown in Fig. 3. In both homogenate and mitochondria the lipid peroxide level is significantly increased (65 and 109%, respectively) due to the administration of allyl alcohol. There was a significant decrease in both homogenate and mitochondria in group-D compared to group-B. Prior administration of the aqueous extract of *Phyllanthus fraternus* offered protection of 88 and 91% on lipid peroxides of both homogenate and mitochondria, respectively. Administration of *Phyllanthus fraternus* alone decreased the lipid peroxide level (28%) in the homogenate and increased (15%) in mitochondria. This change is not statistically significant.

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *Phyllanthus fraternus* on protein carbonyls is shown in Fig. 4. The carbonyl

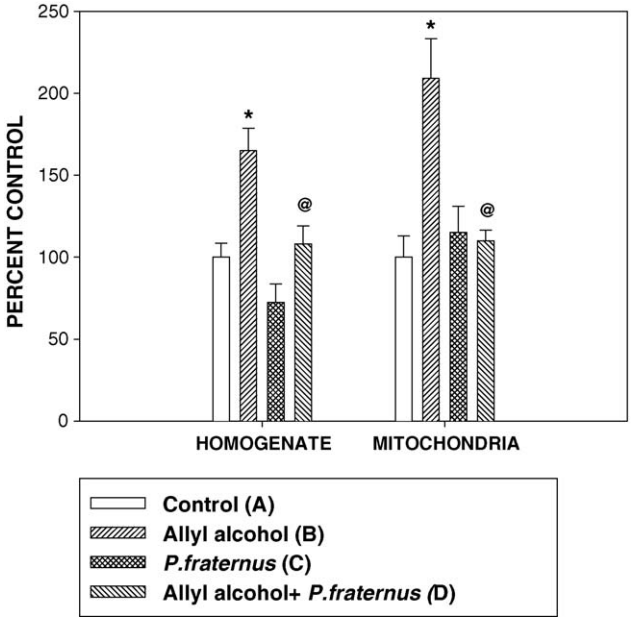

Fig. 3. Effect of administration of allyl alcohol with or without the administration of *Phyllanthus fraternus* on lipid peroxide level in liver homogenate and mitochondria.Values are given as percent control, and are mean ± S.D. of at least four animals. Lipid peroxide level is expressed as nmol MDA formed per 100 mg protein. The control values in homogenate and mitochondria are 115.0 ± 10.0, 108.3 ± 14.4, respectively. $^{*}P < 0.05$ vs. Group-A. $^{@}P < 0.05$ vs. Group-B.

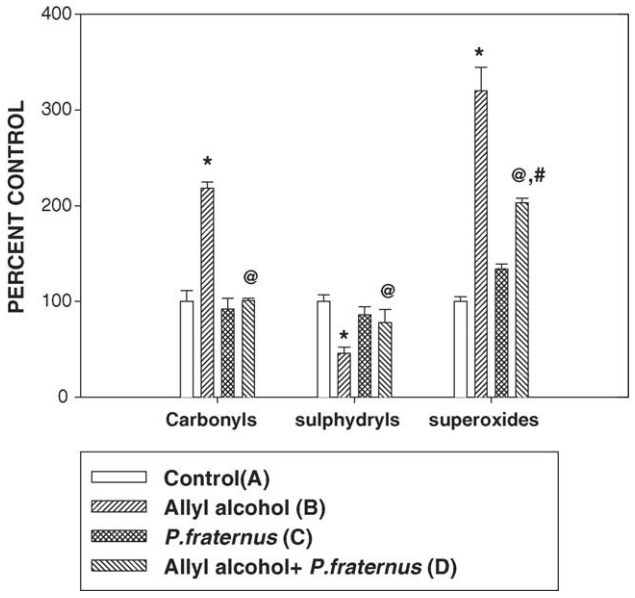

Fig. 4. Effect of administration of allyl alcohol with or without the administration of *Phyllanthus fraternus* on protein carbonyl content, total sulphydryl groups in rat liver mitochondria and generation of superoxide radicals in mitochondrial fragments.The values are mean ± S.D. of at least four animals. Protein carbonyl content, total sulphydryl groups are expressed as nanomole per milligram protein and generation of superoxide radicals are expressed as nanomole per minute per milligram protein. The control values for the protein carbonyl content, total sulphydryl groups and generation of superoxide radicals were 1.0 ± 0.1, 8.9 ± 0.6, 14.5 ± 0.7, respectively. $^{*}P < 0.05$ vs. Group-A. $^{\#}P < 0.05$ vs. Group-A. $^{@}P < 0.05$ vs. Group-B.

content is significantly increased (118%) due to the administration of allyl alcohol. Prior administration of aqueous extract of *Phyllanthus fraternus* offered complete protection (99%) and prevented the rise in the carbonyl content due to the administration of allyl alcohol in liver mitochondria. There was a significant difference between group-D and -B.

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *Phyllanthus fraternus* on total sulphydryl groups is shown in Fig. 4. The total sulphydryl groups are significantly decreased (54%) due to the administration of allyl alcohol. The prior administration of aqueous extract of *Phyllanthus fraternus* offered protection to an extent of 59%. The increase in group-D is statistically significant when compared with group-B.

Effect of the administration of allyl alcohol with and without the prior administration of aqueous extract of *Phyllanthus fraternus* on the generation of superoxide radicals is shown in Fig. 4. The superoxide radicals significantly increased (220%) due to the administration of allyl alcohol. Administration of aqueous extract of *Phyllanthus fraternus* prevented the rise and protected the superoxide level to an extent of 53%. Group-D is statistically significant when compared with group-A or -B.

4. Discussion

In the present study, the protective effect of *Phyllanthus fraternus* against oxidative stress induced by the administration of allyl alcohol was studied. In recent years, there has been a worldwide trend toward the use of natural phytochemicals present in berry crops, teas, herbs, oilseeds, beans, fruits and vegetables (Kitts et al., 2000; Lee and Shibamoto, 2000). Free radicals, from both endogenous and exogenous sources, are implicated in the etiology of several degenerative diseases, such as coronary artery diseases, stroke, rheumatoid arthritis, diabetes and cancer (Halliwell et al., 1992). High consumption of fruits and vegetables is associated with low risk for these diseases, which is attributed to the antioxidant vitamins and other phytochemicals (Ames et al., 1993; Prior, 2003; Weisburger, 1999). There is a great deal of interest in edible plants that contain antioxidants and health promoting phytochemicals.

Acrolein is a highly toxic aldehyde involved in a number of diseases as well as drug-induced toxicities. Acrolein also forms via hepatic biotransformation of the chemotherapeutic drugs cyclophosphamide and Ifosfamide (Ludeman, 1999). In addition, there is growing recognition that endogenous acrolein, formed via lipid peroxidation, mediates cell damage in various diseases of old age, including Alzheimer's disease (Uchida et al., 1998a; Uchida, 1999; Lovell et al., 2001). The 88–91% protective effect on lipid peroxidarion, (Fig. 3) demonstrated in this study by the prior administration of *Phyllanthus fraternus* is expected to have beneficial effect in a number of diseases like chronic heart disease, stroke, rheumatoid arthritis, diabetes and cancer for which the etiology is implicated to oxidative stress (Halliwell et al., 1992).

Under physiological conditions, approximately 1–3% of the O_2 consumed by the body is converted into superoxide and other ROS. Thus, although molecular oxygen is absolutely essential for aerobic life, it can be toxic under certain conditions. This phenomenon has been termed as the oxygen paradox (Gilbert, 2000). There is a growing link among mitochondrial metabolism rate and elimination of ROS and age related changes in mitochondrial function (Balaban et al., 2005).

Prior administration of *Phyllanthus fraternus* offered protection of 72, 40 and 80% for NADH oxidation and 77, 54 and 20% for succinate oxidation on state 3 respiration, RCR and P/O ratios, respectively (Figs. 1 and 2). Effect of allyl alcohol and the protective effect by *Phyllanthus fraternus* on mitochondrial respiration were different for the two different substrates used. Recent evidence suggests strongly that free radicals appear to be generated from complex I through oxidation of NADH on the matrix side of the inner mitochondrial membrane (St-Pierre et al., 2002).

Use of succinate as a main respiratory substrate can lead to radical generation by reverse electron flow from Complex II to complex I (St-Pierre et al., 2002). This mechanism is poorly understood and highly controversial because the methods available to measure rates of radical production from cells are insecure and prone to artifact (Duchen, 2004).

Administration of the aqueous extract of the *Phyllanthus fraternus* significantly decreased the thioacetamide (Padma and Setty, 1997) or carbon tetrachloride (Padma and Setty, 1999) induced lipid peroxidation in vivo and protected the liver from the hepatotoxin-induced toxicity. Co-administration of amla (*Emblica officinalis*) with dimethyl hydrazine significantly reduced the cytotoxic products such as MDA and conjugated

dienes (Anila kumar et al., 2004). Recently the extracts of *Phyllanthus niruri* is shown to have antioxidant and hepato-protective potential against carbon tetrachloride induced toxicity (Harish and Shivanandappa, 2006). The oral administration of different levels of the extract of *Nymphaea stellata* Willd flowers to rats for 10 days protected liver against carbon tetrachloride induced elevation in liver lipid peroxidation and reduction in liver glutathione (Bhandaekar and Khan, 2004). Green tea protects phospholipids from peroxidation and other deleterious effects due to ethanol consumption (Ostrowskaa et al., 2004).

Typically, cellular nucleophiles target acrolein's β-carbon, generating carbonyl retaining adducts (Esterbauer et al., 1991; Uchida et al., 1998b; Burcham and Fontaine, 2001). The reactive carbonyl may then react with neighboring nucleophiles to form inter or intramolecular cross-links (Esterbauer et al., 1991; Permana and Snapka, 1994; Kurtz and Lloyd, 2003). During reactions with protein, acrolein readily carbonylates lysine, cysteine and histidine side chains. There is increasing evidence for an involvement of toxic carbonyls in human diseases (Calingasan et al., 1999; Tanaka et al., 2001). Many different types of protein oxidative modification can be induced directly by ROS or indirectly by reactions of secondary byproducts of oxidative stress (Berlett and Stadtman, 1997). Cysteine and methionine are particularly prone to oxidative attack by almost all ROS. Protein modifications elicited by direct oxidative attack on Lys, Arg, Pro, Thr, or by secondary reaction of Cys, His or Lys residues with reactive carbonyl compounds, can lead to the formation of protein carbonyl derivatives (aldehydes and ketones). Thus, protein carbonyl content is the most general indicator and is the most commonly used marker of protein oxidation (Berlett and Stadtman, 1997; Shacter, 2000; Beal, 2002). Prior administration of an aqueous extract of *Phyllanthus fraternus* offered complete protection (99%) and prevented the rise in the carbonyl content (Fig. 4).

Oxidants, inhibitors of enzymes containing an iron–sulfur center, free radicals and other reactive species cause the oxidation of biomolecules (e.g., protein, amino acids, lipid and DNA), which leads to cell injury and death (McCord, 2000; Freidovich, 1999). For example, radiation-induced ROS markedly alters the physical, chemical and immunologic properties of SOD, which further increase oxidative damage in cells. The cytotoxic effect of free radicals is deleterious to mammalian cells and is responsible for the pathogenesis of many chronic diseases. At the same time it is also responsible for the killing of pathogens by activated macrophages and other phagocytes in the immune system. Thus, there are "two faces" of free radicals in biology in that they serve as signaling and regulatory molecules at physiological levels but as highly deleterious and cytotoxic oxidants at pathological levels (Freidovich, 1999). Significant decrease of the total sulphydryl groups due to the administration of allyl alcohol was protected to an extent of 59% by prior administration of an aqueous extract of *Phyllanthus fraternus* (Fig. 4).

Superoxide is generated from O_2 by multiple pathways (Wu and Morris, 1998; Freidovich, 1999; Gilbert, 2000; Evans and Halliwell, 2001). Under physiological conditions, there is a well-managed balance between the formation and neutralization of ROS by these systems. Oxidative stress can occur when ROS production is accelerated or when the mechanisms involved in scavenging ROS is impaired. Increased production of ROS is thought to occur more frequently than dimnished antioxidant defence, and is postulated to play a role in the pathogenesis of several diseases (Halliwell and Gutteridge, 1999). Prior administration of the aqueous extract of *Phyllanthus fraternus* offered 53% protection on superoxide level and prevented the significant increase due to the administration of allyl alcohol (Fig. 4).

Petrol extract of whole plant (Bhatnagar et al., 1961) and the leaf extract (Bhowmick and Chaudhary, 1982) of *Phyllanthus fraternus* are reported to have antifungal activity. Ethanolic extract of whole plant showed anticancer activity in the mouse and antispasmodic activity on isolated guinea pig ileum (Dhar et al., 1968). The aqueous extract from its leaves is reported to have hypoglycaemic effect in normal as well as alloxan-diabetic rabbits (Ramakrishnan et al., 1982). The hydroalcoholic extract (HE) given intraperitoneally, produced significant inhibition of acetic acid-induced abdominal constrictions (Santos et al., 2000). Ahmed et al. (2002) have reported that the methanolic fraction was the most active among all fractions studied in protecting liver against carbon tetrachloride induced toxicity.

It was reported earlier from our laboratory that mitochondrial dysfunction caused by the administration of an alcohol (Sebastian and Setty, 1999) or thioacetamide (Padma and Setty, 1997) or carbon tetrachloride (Padma and Setty, 1999) could be prevented by prior administration of aqueous extract of *Phyllanthus fraternus*. This study mainly involved on mitochondrial function and lipid peroxidation. The present study is extended to protein oxidations and superoxide levels also. Hepatotoxins like alcohol, carbon tetrachloride, thioacetamide and allyl alcohol induce toxicity and the mechanism by which they induce is different for each agent. For example alcohol is known to induce ROS by decreasing the oxidation rates by electron transport chain and also it depletes SH groups. Carbon tetrachloride is converted to tri chloro methyl radical which is responsible for hepatotoxicity. Allyl alcohol used in this study is known to deplete GSH and induces toxicity.

Reduced glutathione (GSH), the major intracellular non-protein thiol, is mainly known as a nucleophilic scavenger and an enzyme-catalyzed antioxidant in electrophilic/oxidative tissue injury (Lieberman et al., 1996). GSH plays an important role in the maintenance of the intracellular redox state. The intracellular level of GSH, which differ from one cell type to another, may be crucial for ROS-induced NF-κB response (Nanxin and Michael, 1999). The extracellular GSH catabolism may be involved in the modulation of cell signalling and activation of transcription factors (Marie-Jose et al., 2000).

NF-κB is a ubiquitous transcription factor that regulates inflammatory mediators, and several structural proteins that are involved in infection, inflammation, stress responses and apoptosis (Baeuerle and Henkel, 1994). The exact and complex molecular mechanisms involved in the regulation of NF-κB remain to be elucidated (Nanxin and Michael, 1999).

It is widely recognized that the ROS are involved in the activation of NF-κB (Nanxin and Michael, 1999). They can play a role as second messengers, when they are produced in physiological concentrations. ROS, particularly H_2O_2 possess several

properties that make it ideal for the second messenger's role. They are, of small size and therefore diffuse rapidly through biological membranes, and their synthesis and degradation are fast (Baeuerle et al., 1996).

Chemical studies of this plant show that the leaves of *Phyllanthus fraternus* contain a number of lignans and alkaloids. Phyllanthin (a bitter constituent) and hypophyllanthin (a non-bitter compound) were isolated from the leaves of *Phyllanthus fraternus* and identified as lignans. The hexane extract of the leaves gave three additional lignans, niranthin, nirtetralin and phyltetralin. Among phyllanthin, hypophyllanthin, triacontanal and tricontanol isolated from a hexane extract of *Phyllanthus fraternus*, phyllanthin and hypophyllanthin showed a protective effect against carbon tetrachloride and galactosamine induced cytotoxicity in primary cultured rat hepatocytes, while triacontanal was protective only against galactosamine induced toxicity (Syamasunder et al., 1985). Two alkamides (E, E-2,4-octadienamide and E,Z-2,4-decadienamide) have been isolated from *Phyllanthus fraternus*, a plant that is used in Ghanaian traditional medicine to treat malaria. The compounds possess an alpha, beta, gamma, delta-unsaturated conjugated amide, a feature believed to enhance antiplasmodial activity. In vitro assay of the two alkamides was found to possess moderate antiplasmodial activity (Sittia et al., 1998).

Animal studies have shown that dietary phytochemical antioxidants are capable of removing free radicals. Among them, phenolic and polyphenolic compounds, such as flavonoids and catechin in edible plants, exhibit potent antioxidant activities (Decker, 1995). A large body of the literature has documented the beneficial effects of tea polyphenolic compounds on scavenging superoxide and hydroxyl radicals (Fang et al., 1998), and on their role in the prevention and therapy of disease. These polyphenols also enhance Cu, Zn-SOD activity and decrease malondialdehyde concentrations (Cui et al., 2000).

A diet rich in Brussels sprouts (300 g/day) markedly decreases the urinary excretion of 8-hydroxydeoxyguanosine in humans, indicating a reduction of DNA oxidation (Verhagen et al., 1995). Similarly, dietary supplementation of cabbage and broccoli extracts to rats decreases free radical-induced tissue damage brought about by irradiation (Fang et al., 1987). Moreover, phytic acid has a high chelation potential and can be supplemented to diets for suppressing iron-catalyzed oxidative reactions and potentially for reducing the incidence of colonic cancer and inflammatory bowel disease (Graf and Eaton, 1990). Collectively, these studies suggest that phytochemicals may be used as effective antioxidants for improving human health.

In conclusion the administration of an aqueous extract of *Phyllanthus fraternus* prior to allyl alcohol administration showed significant protection on the allyl alcohol-induced oxidative stress in rats. Different parameters on which the beneficial effects were seen include, rate of respiration, oxidative phosphorylation, lipid peroxidation, protein carbonyls, sulphydryl groups and the generation of superoxide radicals. Most of these parameters are the markers of oxidative stress. The extract of *Phyllanthus fraternus* showed good antioxidant potential and prevented oxidation of proteins and lipids. By virtue of its ability to scavenge ROS, it probably can modulate transcription factors and regulate the levels of antioxidant enzymes. The work in this line is in progress.

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