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Research Article

HEPATOPROTECTIVE ACTIVITY OF HERBAL PREPARATION (HP-4) AGAINST ALCOHOL INDUCED HEPATOTOXICITY IN MICE

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Abstract

Free radicals include both Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). When free radicals are produced in a regulated manner in a healthy human body it is scavenged efficiently by antioxidant defense system. But excess generation of pro-oxidants by continuous chain reaction in the form of ROS and RNS cause several human diseases. The shift of the balance in the favour of pro-oxidants results in a condition called "oxidative stress". Alcohol is primarily metabolized in the liver to generate ROS and RNS, leading to diseases such as cirrhosis, fatty liver and chronic hepatitis. Alcohol induced damage is associated with oxidative stress. The excess generation of pro-oxidants and reduced antioxidant levels provide an effective model of Hepatotoxicity which is noteworthy. Recent trend is to discover polyherbal formulation of medicinal plants which have hepatoprotective function. In the present study 80% alcoholic extract of leaves of *Aloe vera*, *Bacopa monniera*, *Moringa oleifera* and rhizome of *Zingiber officinale* were utilized to prepare Herbal Preparation or HP-4. Further the hepatoprotective effects of HP-4 was tested in alcohol induced Hepatotoxicity in mice. Silymarin is a well known hepatoprotective drug was used as a standard for comparison. Biochemical and histopathological studies provided ample evidence that HP-4 provided a hepatoprotective role in alcohol induced hepatotoxicity which was comparable to drug Silymarin. The presence of phytochemicals in HP-4 provided a synergistic, supra-additive and co-operative effects in the hepatoprotective function in alcohol induced hepatotoxicity mice model.

Keywords: alcohol; antioxidants; free radicals; hepatoprotection; phytochemicals.

Introduction

Free radicals are atoms with unpaired electrons in excited states which lead to further free radical generation by chain reaction or that result from free radical reactions. Generally, free radicals are very short-lived, with half lives in milli, micro or nanoseconds. Free radicals include both Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) and implicated as the cause in several human diseases. However, ROS and RNS are both produced in regulated manner in normal healthy tissues and maintain cellular homeostasis as well as act as signaling molecules. In a normal healthy human body, the generation of pro-oxidants in form of ROS and RNS are efficiently scavenged by the antioxidant defense mechanisms. When the delicate balance is shifted in the favour of pro-oxidants, the resulting condition is known as "oxidative stress" (Devasagayam *et al.*, 2004).

There is recent interest in the use of naturally occurring antioxidants for the preservation of foods and in the management of many diseases which involve free radicals. It has been established that there is inverse relationship between the dietary intake of antioxidant rich foods and the incidence of human diseases. Protective action of well -

known antioxidants have a significant role in diseases eg vitamin C, vitamin E, β - carotene and plant phenolics. Excessive use of synthetic antioxidants like butylated hydroxyl toluene (BHT) and butylated hydroxyanisole (BHA), may cause hepatic damage and hence their use is restricted. (Walia *et al.*, 2007).

Liver is the primary organ for the metabolism of ingested alcohol (Shanmugam *et al.*, 2010). The liver is the largest, important organ and the site for essential biochemical reactions in the human body. It has the function to detoxify toxic substances and synthesize useful biomolecules. Therefore, damage to the liver leads to grave consequences. Alcohol induces oxidative stress which is known to cause liver injury that is many biochemical metabolic reactions occur as a result of it. Some of these include redox state changes, production of reactive acetaldehyde, damage to the mitochondria of cells, cell membrane damages, hypoxia, effects on immune system, altered cytokine production, induction of CYP2E1 and mobilization of iron (Baskaran *et al.*, 2010).

Alcoholic liver disease is a worldwide health problem which has three manifestations in form of fatty liver/steatosis, alcoholic hepatitis and liver cirrhosis.

At least 80% of chronic alcoholic consumers may develop steatosis, 10-35% alcoholic hepatitis and approximately 10% liver cirrhosis. Intake of alcohol causes accumulation of reactive oxygen species (ROS) like superoxide, hydroxyl radical and hydrogen peroxide in the hepatic cells that oxidize the glutathione which leads to lipid peroxidation of cellular membranes, oxidation of protein and DNA resulting in hepatic damage (Muhammad *et al.*, 2009).

Initially, in the liver alcohol is metabolized into the highly toxic acetaldehyde by the enzyme alcohol dehydrogenase. Acetaldehyde is then oxidized to acetate by acetaldehyde oxidase or xanthine oxidase giving rise to ROS via cytochrome P₄₅₀ 2E1. Prolonged consumption of alcohol increases nitric oxide (NO) level which leads to formation of toxic oxidant peroxynitrite. Low capacity of antioxidants in this situation leads to damage of the cells of the hepatic cells and the cell organelles with the release of reactive aldehydes and ROS (Saalu *et al.*, 2012).

Treatment options available for common liver diseases such as cirrhosis, fatty liver and chronic hepatitis are inadequate in modern medicine. Conventional drugs used in the treatment of liver diseases such as corticosteroids, antiviral, immunosuppressant may lead to serious adverse effects; they may even cause hepatic damage on prolonged use. Therefore, alternative drugs in the form of herbal medicines which are now used for the treatment of liver diseases are sought instead of currently used drugs of doubtful efficacy and safety (Vetrivelan *et al.*, 2010).

Now the prime focus is to follow systematic research methodology and explore scientific basis for the therapeutic properties of traditional herbal medicines that assert to possess hepatoprotective activity. A single drug in the form of herbal medicine cannot be effective for all types of liver diseases. It is therefore considered necessary to develop an effective formulation or combination using indigenous medicinal plants with support of pharmacological experiments and clinical trials based on the concept of polypharmacy (Arun *et al.*, 2011).

Aloe vera L. (syn: *Aloe barbadensis* Miller) is a perennial, succulent plant belonging to the Liliaceae family. The *Aloe vera* leaf possesses many pharmacological activities such as purgative, antibacterial, anticancer, antifungal and antioxidant to name a few (Patil, 2010). In the study reported by Saka *et al.*, 2011, the hepatotherapeutic effect of *Aloe vera* may be contributed by its antioxidant properties of its bioactive principles via membrane-stabilizing mechanisms. This study is also the pharmacological evidence and the support of the traditional folklore medicinal usage of the plant in the treatment of alcohol-induced hepatic damage.

Bacopa monniera belongs to the family *Scrophulariaceae*, the local name being "brahmi". The name brahmi is derived from the word "brahma" meaning "creator" in Hindu

mythology. *Bacopa monniera* is a small herb which grows in the wet, marshy areas in tropical regions. It is a creeping herb with many branches, small, fleshy oblong leaves. Flowers and fruits appear in summer season. The stem and leaves of the plant are widely used as extracts proven to show anti-ulcerogenic, anti-cancer and hepatoprotective properties. (Srivastava *et al.*, 2009).

Bacopa monniera has been classified as a medhyarasayana, a drug to improve memory and intellect (medhya). There is reference of the herb in the "Charaka Samhita" recommending it for mental conditions including anxiety, poor cognition, lack of concentration, as a diuretic, vitalizer of nervous system and heart, as nootropic, digestive aid (Gohil *et al.*, 2010).

Moringa oleifera Lam is the most widely distributed species geographically of the Moringaceae family. It is commonly known as horseradish tree or drumstick tree. Found in mainly Asian countries; has medicinal as well as nutritional values. It has been used both in the Ayurvedic and Unani systems of medicines in India. The leaves are highly nutritious when compared to other equally beneficial parts, being source of protein, β -carotene, vitamin A, B, C and E, riboflavin, nicotinic acid, folic acid, pyridoxine, amino acids, minerals and various phenolic compounds (Fakurazi *et al.*, 2012). In a study reported by Nadro *et al.* in 2006 involving *Moringa oleifera* leaves extract against alcohol induced hepatotoxicity lead to conclusion that pretreatment of the extract protected the liver from acute alcohol induced damage, while post-treatment with the extract exhibited therapeutic effect. That means both preventive and curative properties are demonstrated which has been attributed to the rich antioxidant content of the leaves extract which was exhibited in a dose-dependent manner.

Zingiber officinale Roscoe or commonly known as "ginger" belongs to the family *Zingiberaceae*. The part widely used is the rhizome in traditional medicines. It has been used to treat headaches, rheumatism, burns, peptic ulcer, dyspepsia, depression, impotence and in liver diseases (Abdullah *et al.*, 2004).

In the fresh ginger rhizome the polyphenolic compounds gingerols in the form of 6-gingerol, 8-gingerol and zingerone are the active components. 6-gingerol is responsible for the pungent taste and the most abundant in the rhizome ginger (Nemat *et al.*, 2010). It was concluded from the study of Shanmugam *et al.* that congestion of renal tubules and degenerative changes in the renal tissues in alcohol treated male albino rats were reversed by the treatment with ginger extracts and confirmed its efficacy.

In the present study the leaves of *Aloe vera*, *Bacopa monniera*, *Moringa oleifera* and rhizome of *Zingiber officinale* are used in combination to prepare an Herbal Preparation (HP-4) as reported earlier. Further the hepatoprotective effects of the Herbal Preparation were

tested in alcohol induced hepatotoxicity in mice. Silymarin a commonly used well-known hepatoprotective drug was used as a standard drug for comparison. *Silybum marianum* belongs to the Family Asteraceae is a world-wide known plant with black, shiny seeds. Extracts of *Silybum marianum* seeds has been used in folk medicine for the treatment of liver diseases (Soufy., 2012).

Materials and Methods

The leaves of *Aloe vera*, *Bacopa monnieri*, *Moringa oleifera* and also rhizome of *Zingiber officinale* were collected from Loni, Maharashtra. The herbs were identified by a Professor of Botany, Loni. The leaves & rhizome were shade-dried for 4-6 weeks then finely powdered and sieved twice to obtain a fine powder.

100 gm dried powder each of leaves of *Aloe vera*, *Bacopa monnieri*, *Moringa oleifera* and rhizome of *Zingiber officinale* were separately extracted with Soxhlet extractor using 80% alcohol till solvent was colourless. The extract was dried till constant weight was obtained .25 mg of each extract was mixed together and dissolved in 10 ml methanol, boiled in water bath for 5 minutes ,cooled and centrifuged at 4000rpm for 10 minutes. The clear supernatant obtained was labeled Herbal Preparation (HP-4) as reported earlier (Padmanabhan and Jangle, 2012 a).

Animals: Swiss albino male mice weighing 25-30 g bred in Animal Resource Centre for Medical Research, PIMS Loni were used. The animals were allowed standard food pellets and water *ad libitum*. They were maintained in standard laboratory conditions ($25 \pm 2^\circ\text{C}$)

The study protocol was approved by "The Institutional Animal Ethics Committee" PIMS/AH/215/2011 PIMS Loni and CPCSEA Reg No. 366/01/a CPCSEA.

Study Design

20 mice of body weight 25-30 g were divided into 5 groups of 4 animals each (Nadro *et al.*, 2006).

Group I: Normal Control: The animals received distilled water 5 ml/kg b.w. p.o. for 6 days.

Group II: Toxicant Alcohol Group: Received alcohol 5g/kg b.w. of 25% w/v alcohol p.o. for 6 days.

Group III: Toxicant + Silymarin Group: The animals received 50mg/kg b.w. p.o. was given for 6 days. The animals received alcohol 5g/kg b.w. of 25% w/v alcohol p.o. for 6 days half an hour after vehicle.

Group IV Toxicant + HP- 4 250 mg/kg Group: HP-4 250 mg/kg was p.o. for 6days. The animals received alcohol 5g/kg b.w. of 25% w/v alcohol p.o. for 6 days.

Group V Toxicant + HP-4 500 mg/kg Group: HP-4 500mg/kg was p.o. for 6 days. The animals received alcohol 5g/kg b.w. of 25% w/v alcohol p.o. for 6 days.

Biochemical Parameters: On the 7th day after overnight fast the blood was collected from retro-orbital plexus. The blood was allowed to clot and centrifuged (Remi-R 8C Centrifuge) at 2500 rpm for 10 minutes. The serum was separated and used for the assay of alanine transaminase [ALT] EC 2.6.1.2 (Bradley *et al.*, 1972), aspartate transaminase [AST] EC 2.6.1.1. (Wolf *et al.*, 1972) , alkaline phosphatase [ALP] EC 3.1.3.1 (Young *et al.*, 1972) , γ -glutamyl transferase [γ GT] EC 2.3.2.2 (Persijn *et al.*, 1976) and lactate dehydrogenase [LDH] EC 1.1.1.27(Lum *et al.*, 1974) by using standard methods using enzyme assay kits. Transasia Bio-medicals Ltd Kit for ALT, AST, LDH and Accurex Biomedicals Ltd Kit for γ GT & ALP. The enzyme assays were performed on a semiautoanalyser ERBA Chem7.

The animals were sacrificed by cervical dislocation and liver was washed with saline and dried with tissue paper .After keeping aside some portion of liver for histopathological studies, the remaining part of the liver was homogenized in phosphate buffer 0.2 M, pH 7.4 (Lal *et al.*, 2007).

The 10% homogenized liver tissue was made using a tissue homogenizer (MC Dalal & Co).The supernatant obtained after centrifuging at 4000rpm for 10 mins was used for estimation of SOD, GPx , GR, TBARS and GSH. Total proteins in the supernatant were estimated by Biuret Method (Flack *et al.*, 1984)

Estimation of Superoxide dismutase {SOD} EC 1.15.1.1: SOD was determined by the method described by Marklund and Marklund in 1974 .The SOD activities of the supernatant of tissue homogenate were estimated by measuring the % inhibition of the pyrogallol autoxidation by SOD .2.5 ml of Tris Buffer pH 8.2, 0.05M, 0.5 ml of 1mM EDTA, 0.5ml Pyrogallol 0.2mM freshly prepared were added to 50 μ l of the supernatant of tissue homogenate. OD at 420 nm after 5 minutes was recorded. One unit of SOD was defined as the enzyme activity that inhibited the autoxidation of pyrogallol by 50 percent.

Estimation of Glutathione peroxidase {GPx} EC 1.11.1.9: GPx activity was measured by the method described by Rotruck *et al* 1973 .Briefly, reaction mixture contained 0.2ml of 0.4 M Tris HCL buffer pH 7.0, 0.1ml of 10mM sodium azide, 0.2 ml of the supernatant of liver tissue homogenate, 0.2 ml glutathione 60mg%, 0.1ml of 0.2mM H₂ O₂ .The contents were incubated at 37 $^\circ\text{C}$ for 10 minutes. The reaction was arrested by 0.5 ml of 10% TCA and centrifuged. Supernatant was assayed for glutathione content by using Ellmans reagent {19.8mg of 5, 5'-dithiobis-(2 nitro benzoic acid) DTNB in 0.5g Sodium Citrate in 50ml double distilled water}

Estimation of Glutathione reductase {GR} EC 1.6.4.2: activity was measured spectrophotometrically according to the method of Calberg and Mannervick 1985.The reaction

mixture contained 1mM oxidized glutathione GSSG as substrate, 2mM NADPH and tissue homogenate in phosphate buffer (pH=7.4). The decrease in absorbance at 340nm in terms of NADPH oxidation was measured. One unit of enzyme activity is defined as 1nmol of NADPH oxidized in one minute per mg protein.

Estimation of Thiobarbituric acid reactive substances {TBARS}: TBARS in tissues was estimated by the method of Fraga et al 1981. To 0.5 ml of supernatant of tissue homogenate, 0.5 ml saline and 1.0 ml 10% TCA were added, mixed well and centrifuged at 3000 rpm for 20 minutes. To 1.0 ml of protein free supernatant, 0.25 ml Thiobarbituric acid (TBA) reagent was added, the contents were mixed well and boiled for one hour at 95 °C. The tubes were then cooled to room temperature under running tap water and absorbance was measured at 532 nm.

Estimation of Reduced Glutathione {GSH}: Spectrophotometric quantification of reduced glutathione {GSH} has been carried out using 5, 5'-dithiobis-(2-nitrobenzoic acid) DTNB reagent according to method proposed by Moron et al 1979. Briefly, 200 µl of supernatant of tissue homogenate (10%) was added to 800 µl distilled water and then 2 ml of sodium phosphate-EDTA buffer (0.1 M of sodium phosphate, 0.005 M EDTA buffer pH 8.0) containing 0.6M DTNB were added. The optical density of the yellow coloured complex developed by the reaction of GSH and DTNB was measured at 412nm using a UV-Vis spectrophotometer.

Histopathology: The part of the lobe of liver kept aside for histopathological studies were transferred to formal saline solution. The liver tissues were processed for paraffin embedding and sections 5 µm thick were taken in a microtome. After staining with hematoxylin and eosin,

slides were examined under the microscope for histopathological changes (Bancroft and Cook., 1984).

Result

The activities of serum AST, ALT, ALP, γGT and LDH (hepatic marker enzymes for liver damage) were increased markedly in alcohol fed animals as compared to normal control mice; this indicating liver damage. However, administration of herbal preparation HP-4 at doses of 250 and 500 mg/kg lowered the alcohol induced elevation of serum AST, ALT, ALP, γGT and LDH (Table 1). Silymarin a well known hepatoprotective drug was also shown to cause a decrease in elevated enzyme action compared to alcohol fed group.

Since alcohol is a hepatotoxicant which induces oxidative damage, the levels of liver antioxidant enzymes SOD, GPx, GR were measured. The toxicant group showed reduced levels of antioxidant enzymes as compared to Normal Control Group. Administration of both dosages of HP-4 significantly raised the antioxidant enzymes when compared with the Toxicant group. Silymarin treated group showed similar hepatoprotective effect. The results are depicted in Table 2.

TBARS level was increased and reduced total glutathione was found to be decreased in Toxicant group when compared to Normal Control. The increased TBARS level is indicative of oxidative damage and reduced total glutathione due to protection offered against the stress. These results are shown in Table 3. Treatment with HP-4 at doses 250 and 500mg/kg reversed the increase of TBARS and reduced glutathione when compared to alcohol fed group. The result was comparable to Silymarin treated group.

Table 1: Effect of Herbal Preparation HP-4 on the Liver Function Tests as a marker of liver damage in alcohol induced hepatotoxicity in mice

Sr.No	Units in IU/L				
	AST	ALT	ALP	LDH	γGT
Group I- Control	36.5 ± 3.80	26.08 ± 2.78	89.75 ± 9.90	342.25 ± 35.08	3.00 ± 0.40
Group II Toxicant- Alcohol	60.08 ± 7.40^a	152 ± 16.07^a	350.25 ± 36.89^a	1946 ± 196.78^a	13.26 ± 1.47^a
Group III Alcohol +Silymarin 50mg/kg	34.23 ± 3.60^b	88.45 ± 9.40^b	256 ± 27.60^b	1474 ± 148.25^b	3.9 ± 0.40^b
Group IV Alcohol + HP-4 250 mg/kg	38.60 ± 3.40^c	79 ± 8.13^c	255.66 ± 26.48^c	1372 ± 138.96^c	3.66 ± 0.38^c
Group V Alcohol + HP-4 500 mg/kg	40.9 ± 4.30^d	81.28 ± 9.20^d	224 ± 22.21^d	1333.5 ± 134.95^d	3.5 ± 0.36^d

a p<0.05 Toxicant as compared to control, significantly increased

b p<0.05 Group III as compared to GroupII, significantly decreased

c p<0.05 Group IV as compared to GroupII, significantly decreased

d p<0.05 Group V as compared to GroupII, significantly decreased

Table 2: Effect of Herbal Preparation HP-4 on liver antioxidant enzymes activities levels on Alcohol induced hepatotoxicity in mice

S.N.	SOD Units /100 mg protein	GPx-Nanomoles GSH utilized/min/mg protein	GR-Nanomoles NADPH /100mg protein
Group I- Control	1.99 ± 0.25	44.31 ± 4.97	4.15 ± 0.46
Group II Toxicant-Alcohol	0.88 ± 0.18^a	27.74 ± 2.82^a	3.98 ± 0.40^a
Group III Alcohol +Silymarin 50mg/kg	2.43 ± 0.25^b	58.87 ± 5.51^b	8.76 ± 0.96^b
Group IV Alcohol + HP-4 250 mg/kg	1.71 ± 0.18^c	44.21 ± 4.68^c	7.58 ± 0.81^c
Group V Alcohol + HP-4 500 mg/kg	1.37 ± 0.15^d	42.51 ± 4.97^d	6.40 ± 0.65^d

a p<0.05 Toxicant as compared to control, significantly increased

b p<0.05 Group III as compared to GroupII, significantly decreased

c p<0.05 Group IV as compared to GroupII, significantly decreased

d p<0.05 Group V as compared to GroupII, significantly decreased

Table 3: Effect of Herbal Preparation HP-4 on liver weight, total proteins, TBARS and Reduced GSH on Alcohol hepatotoxicity in mice

Sr.No	Wt of the liver in grams	TBARS nmoles/100mg protein	Reduced GSH mg GSH /100mgprotein
Group I- Control	1.85 ± 0.19	24.19 ± 2.89	2.12 ± 0.20
Group II Toxicant- Alcohol	2.89 ± 0.30^a	57.28 ± 5.96^a	1.35 ± 0.17^a
Group III Alcohol +Silymarin 50mg/kg	2.75 ± 0.28^b	38.70 ± 4.12^b	2.79 ± 0.30^b
Group IV Alcohol + HP-4 250 mg/kg	2.43 ± 0.25^c	26.54 ± 3.10^c	2.55 ± 0.27^c
Group V Alcohol + HP-4 500 mg/kg	1.88 ± 0.19^d	25.50 ± 2.60^d	1.90 ± 0.20^d

a p<0.05 Toxicant as compared to control, significantly increased ,

b p<0.05 Group III as compared to GroupII, significantly decreased

c p<0.05 Group IV as compared to GroupII, significantly decreased

d p<0.05 Group V as compared to GroupII, significantly decreased

Discussion

Alcohol feeding causes elevation in serum activities of AST, ALT, ALP, γ GT and LDH enzymes which are markers of liver damage. In the study reported by Vidhya et al (2009) ethanol treated group resulted in significant increase in AST and ALT activities, which is an indication of hepatocellular damage in rats, whereas treatment with quercetin reduced ethanol –induced toxicity as indicated by the lowering of marker enzymes. In alcohol intoxication as a result of structural changes, an increase in the cell membrane permeability to ions results. The increase in membrane permeability causes leakage of ALT and AST into blood circulation as shown by abnormally high levels of serum hepatic markers. The observations by Vermaulen *et al.*, (1992) which indicated that ALT, AST, γ GT and ALP are normally located in the cytoplasm and released into circulation after hepatic cellular damage. The results of present study is in agreement with studies by Ighodaro et al (2012) in which aqueous leaf extract of *Ocimum gratissimum* causes concomitant reduction in the serum enzyme activities of ALT, AST, γ GT and ALP as compared to the toxicant ethanol treated group, whereas in present study such a reduction in serum enzyme levels is due to the

Herbal Preparation (HP-4) is also observed. As postulated by Saalu et al (2012) and Pari et al (2007) the protective effects of *Moringa oleifera* extracts and caffeic acid in their respective studies of alcohol mediated hepatotoxicity may be attributed to the cell membrane stabilizing ability of the extracts preventing the hepatic enzymes leakage and translocation into the serum.



Fig-1: Control Group –I
Clearly visible nuclei of hepatocytes, central vein with portal triads and normal hepatocytes visible

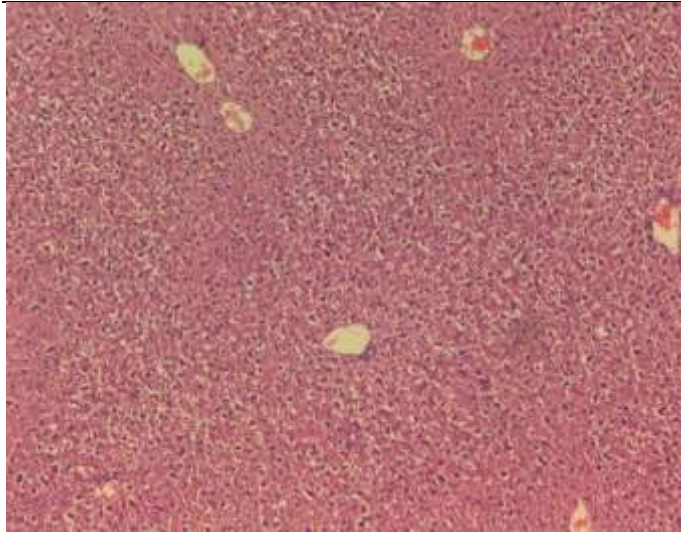


Fig-2: Toxicant Alcohol treated-Group-II
Congestion of sinusoids, central vein, centrilobular fatty change and necrosis of hepatic cells.

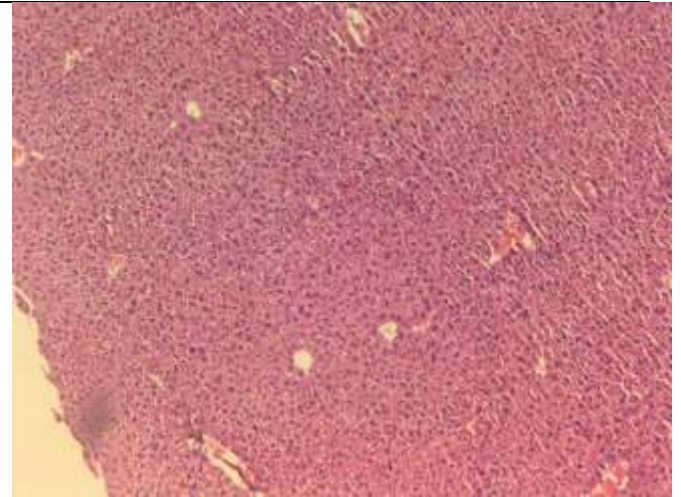


Fig-5: HP-4 500 mg/kg +Alcohol treated-GroupIV
Greater extent of reduction in cloudy swelling, fatty changes exhibit recovery

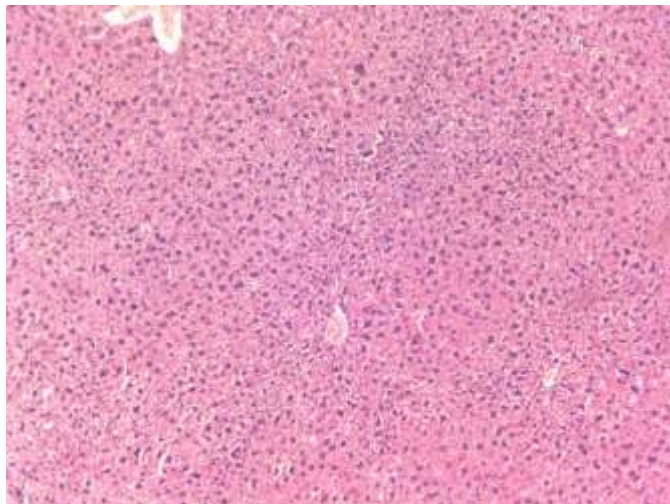


Fig-3: Standard Silymarin +Alcohol treated - Group III
Lesser sinusoidal congestion and fatty change

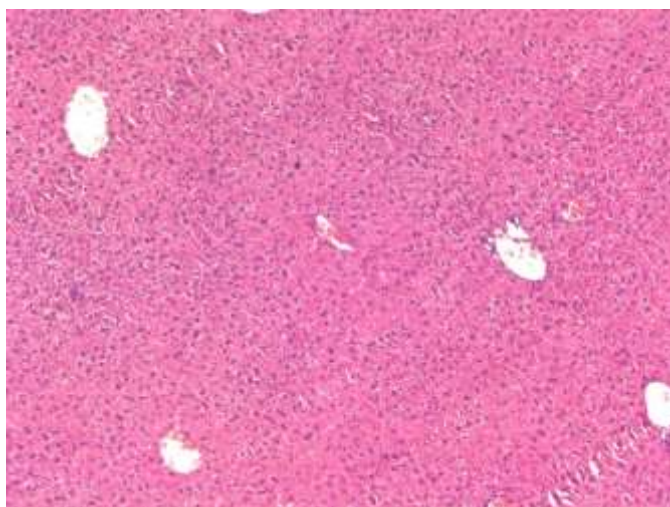


Fig-4: HP-4 250mg/kg +Alcohol treated-GroupIV
Marked reduction in the fatty changes and sinusoidal congestion

In the present study, the hepatic endogenous antioxidant enzymes SOD, GPx and GR reduced in the alcohol treated toxicant group and then shows a dose –dependent increase on the treatment with the Herbal Preparation (HP-4). Superoxide dismutase (SOD) is the most sensitive enzymatic index in liver cells damage. It scavenges the superoxide anion to form hydrogen peroxide and diminishes the toxic effects caused by the radical (Kharpate *et al.*, 2007). SOD is the major attention seeking metalloprotein in the antioxidant family. The increased synthesis of SOD against superoxide anions radical (O_2^-) production is a response of cell to synthesize increases mitochondrial SOD through gene transcription enhancement. In the present study also low levels of SOD in toxicant alcohol group was due to low level of zinc (metal co-factor of SOD); this was also observed in a study by Maruthappan et al (2009) on extract of *Azadiracta indica* which promoted hepatoprotection in alcohol –induced hepatotoxicity by elevating free radical scavenging activity. In the present study Silymarin treated group showed significant increase in SOD, GPx and GR activities of enzymes, similar mechanism may be effective for HP-4.

SOD activity was decreased with ethanol toxicity and intake harmed the liver, heart, brain, kidney, muscle and serum of rats. The reduced activity of SOD in alcohol toxicity may cause the accumulation of O_2^- ; H_2O_2 or the products formed by their degradation. The elevation of SOD on treatment with ginger extracts in alcohol hepatotoxicity in a study reported by Shanmugam et al (2010) was due to antioxidant compounds like gingerols, shogaols, ketone compounds and phenolic compounds which scavenge the superoxide anions.

GPx and GR values showed reduction in the liver homogenate on chronic exposure to alcohol treatment in study reported by Nazeema et al (2009); due to increase in free radical damage and induced lipid peroxidation. Co-

administration with *Mimosa pudica* showed curative nature of the system against free radicals and reduction in GPx and GR values. This is in unison with our present study using Herbal Preparation (HP-4). GPx is a seleno-enzyme; 2/3 rd of which is present in liver cytosol and 1/3rd in mitochondria. The reaction of hydroperoxides with reduced glutathione is catalyzed by GPx. The products formed are glutathione disulphide (GSSG) as well as hydroperoxides. The GR or glutathione reductase enables the reverse reaction of reformation of reduced GSH with the co-enzyme NADPH required for the reaction to occur (Bishayee *et al.*, 1995). The standard hepatoprotective drug Silymarin causes significant increase in antioxidant enzymes in liver in the present study which is an agreement with study by Soufy (2012) using the extract of *Silybum marianum* plant showing potent antioxidant effect due to the flavonoids present.

In the present study the TBARS level increased and reduced GSH levels showed a reduction in the alcohol –toxicant group but showed reversal in activity on pretreatment with Herbal Preparation (HP-4). Elevated lipid peroxidation is considered as an initial mechanism of cell membrane damage. It also initiates nitric oxide free radical leading to the formation of peroxynitrite at cytotoxic high concentrations. Studies by Das *et al* (2010) showed elevated TBARS and nitrite levels in chronic ethanol administration but protective lowering effects on administration of resveratrol and vitamin E in ethanol induced oxidative damage in mice. Even studies by Arun *et al* (2011) showed elevated levels of TBARS in alcohol administered rats and treatment with *Phyllanthus amarus* and *Eclipta prostrata* significantly reduced formation of TBARS. The increased lipid peroxidation as reflected in TBARS results in changes in cellular metabolism of hepatic and extrahepatic tissues, which ultimately leads to whole cell deformity and death. The administration of hepatoprotective drugs *Azadirachta indica* and drug Silymarin showed reduction of TBARS level in alcohol induced hepatotoxicity (Maruthappan *et al.*, 2009). This is in unison with our present study using Herbal Preparation (HP-4) and standard drug Silymarin.

Glutathione (GSH) is the major non-protein, thiol and plays a central eminent role in co-ordinating the body's antioxidant defense processes. Besides controlling the glutathione related enzymes, it acts as free radical scavenger and assists in the maintenance of protein sulphhydryl groups. Depletion of GSH as in our study as well as studies by Das *et al* (2010) and Nazeema *et al* (2009) demonstrate that the hepatic cells are more susceptible to oxidative stress.

GSH is an important source of reducing equivalents during oxidative stress generated by ROS. The higher levels of alcohol intake cause cirrhosis and liver damage by enhancing lipid peroxidation in the liver. Acetaldehyde the toxic metabolite of alcohol depresses liver GSH by

conjugating with sulphhydryl groups of GSH (Maruthappan *et al.*, 2009).

In both acute and chronic administration of alcohol causes formation of cytokines in large amounts, particularly TNF- α by hepatic Kupffer cells, which plays a major role in causing hepatic damage. Moreover, chronic administration of alcohol results in accumulation of hepatic lipids, lipid peroxides leading to the autooxidation of hepatic cells acting as pro-oxidants and decreasing antioxidant levels thereby resulting in a noteworthy hepatotoxicity. (Eswar Kumar *et al.*, 2013).

The alcoholic liver injury is due to the toxic effects of acetaldehyde which maybe mediated by acetaldehyde altered proteins. Chronic alcohol intake results in hypercholesterolemia, hyperlipidemia and hypertriglyceridemia. In chronic lipid accumulation the liver cells become fibrotic and lead to impaired liver function. Enhanced lipid peroxidation has been reported hyperlipidemia which is alcohol intake induced. Recently free radical induced lipid peroxidation has gained prime importance because of its involvement in many diseases. Therefore protection of the cell membrane from lipid peroxidation becomes a necessity to prevent, cure or delay the aforesaid pathologies (Maruthappan *et al.*, 2009).

Alcohol-induced damage is associated with oxidative stress and immunological changes. It alters cytokines levels in a variety of tissues including plasma, lung, liver and brain. These cytokines play a distinct role in cellular communication, activation, inflammation, cell death, cell proliferation, migration and healing mechanisms. The acute and chronic alcoholism can increase the permeability to endotoxins and impair the reticuloendothelial function of the liver and thereby further stimulates production of cytokines. The low level of GSH and increased level of IFN- γ might augment the pro-inflammatory cytokine TNF- α level which plays a key factor in various aspects of liver diseases (Das *et al.*, 2010).

The histopathological examination of liver sections of Normal Control group showed normal arrangement of hepatocytes, with clearly visible nuclei, central vein and portal triad (Fig 1). In Toxicant alcohol treated group areas of congestion of sinusoids, central vein congestion, centrilobular fatty change and necrosis of hepatic cells were observed (Fig 2). There was marked reduction in sinusoidal congestion, cloudy swelling and fatty change and areas of regeneration in Herbal Preparation treated groups (Fig 3 & 4).

The in-vitro antioxidant potential of Herbal Preparation (HP-4) used in the present study were reported by Padmanabhan and Jangle in (2012a). The phytochemicals were found as phenolic compounds, flavonoids and flavonols in Herbal Preparation (HP-4). Moreover DPPH radical scavenging activities and reducing power of HP-4

denoted synergistic effects. The synergistic and supra-additive effect of Herbal Preparation (HP-4) maybe due to the increased possibility of interaction that allows more cooperative effects (Padmanabhan and Jangle, 2012b). Moreover, the anti-inflammatory properties of HP-4 was also detected which may contribute to the mechanism of hepatoprotection (Padmanabhan and Jangle, 2012c).

Polyherbal formulations are considered better than just a single herb. Since the plants in an herbal mixture will have different modes of action for therapeutic properties it shows. In combination it shows synergistic or enhanced activity than that of individual herb. Components of the plant which are less active themselves can act to improve the stability, bio-availability and half life of active components. Hence a particular component in pure form will have only a fraction of the pharmacological activity than it has in its plant matrix, which again highlights the importance of using the plant as a whole or a mixture of plants for therapeutic purposes (Lal *et al.*, 2007).

Therefore both biochemical parameters and histopathological studies reveal that Herbal Preparation (HP-4) a polyherbal formulation of extract of leaves of *Aloe vera*, *Bacopa monniera*, *Moringa oleifera* and rhizome of *Zingiber officinale* offered a synergistic hepatoprotective effects due to the various phytochemicals present in it in alcohol induced hepatotoxicity.

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