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Hepatoprotective Activity of Herbal Preparation (HP-4) Against D-Galactosamine Induced Hepatotoxicity in Mice

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ABSTRACT
Oxidative stress in mammals results from imbalance between generation of free radicals and the rate of their suppression by antioxidant. Hepatotoxicity may result as an effect of excessive free radical formation due to exogenous chemicals or metabolic reactions. D-Galactosamine (D-GalN) is a well known hepatotoxicant. Herbal medicines have been utilized to manage hepatotoxicity according to recent trends. In the present study Herbal Preparation or HP-4 is a combination of 80% alcoholic extract of leaves of *Aloe vera*, *Bacopa monniera*, *Moringa oleifera* and rhizome of *Zingiber officinale* has been utilized to study its efficacy on mice model of D-GalN hepatotoxicity. D-GalN hepatotoxicity induces liver injury closely resembling human viral hepatitis with necrosis, inflammation and regeneration. Evidenced by biochemical and histopathological studies it is concluded that polyherbal formulation HP-4 offered a synergistic protection due to the phytochemicals present which provide hepatoprotective activity induced by D-GalN hepatotoxicity.

Keywords: free radicals, antioxidants, hepatotoxicity, viral hepatitis, phytochemicals, hepatoprotection.

INTRODUCTION

In living organisms, reactive oxygen species (ROS) and reactive nitrogen species (RNS) cause deleterious cytotoxic effects to mammalian cells. These free radicals include various forms of activated oxygen and nitrogen such as superoxide anion (O_2^-),hydroxyl (OH^-), nitric oxide radicals (NO^-) and non –free radical species such as hydrogen peroxide, nitrous acid (HNO_2) which lead to generation of free radicals. [1]

These free radicals are continuously formed inside the human body as a result of exposure to exogenous chemicals in the environment or due to various endogenous metabolic reactions involving bioenergetic electron transfer and redox enzymes. [2]

Oxidative stress in mammalian life can be defined as the imbalance between generation of reactive oxygen species (ROS) and the rate of their suppression by antioxidants. [3]

Antioxidants have been reported to scavenge free radicals by interfering with the oxidation process and chelating metal ions. Thus oxidative stress is prevented by the action of antioxidants. [4]

Various forms of synthetic antioxidants such as tertiary butylated hydroxyquinone, butylated hydroxytoluene, butylated hydroxyanisole, gallic acid esters have proved to be protective against oxidative stress but they are too costly. Apart from that there are negative effects on health such as increased radio-sensitization, mutagenicity, tumour-incidence etc. Therefore, in the recent times there is increased interest in the therapeutic potentials of medicinal plants which act as antioxidants and reduce the harmful effects of free radicals. [5]

Hepatotoxicity in most of the cases is due to the effects of free radicals. Most of the hepatotoxic chemicals cause damage to the liver cells by lipid peroxidation and inducing oxidative damage. Hepatotoxicity is one of the common diseased conditions leading to serious consequences ranging from metabolic disorders to even death. [4]

Hepatic injury may be caused by different agents such as viruses, chemicals, alcohol, autoimmune diseases and D-galactosamine. D-galactosamine (D-GalN) is a well known hepatotoxicant. It induces liver injury closely resembling human viral-hepatitis with necrosis, inflammation and regeneration. The toxicity of D-GalN is associated with the depletion of uridine pools, limited ribonucleic acid (RNA) and protein synthesis thus overall affecting hepatocellular function. [6]

Management of liver diseases is still a great challenge to the modern medicine. At present only a few hepatoprotective drugs from natural sources are available and there is lack of

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effective allopathic medication available for treatment of liver disorders. [7]

Recently, it has been observed that there is a shift in the universal trend from synthetic to herbal medicine or a "Return to Nature". Medicinal plants have been valued in developing countries of the world for primary health care due to better cultural acceptability, better compatibility with human body and lesser number of side effects. Since India has been bestowed with huge wealth of medicinal plants, it is rightly referred to as "Medicinal Garden of the World". [8]

Aloe vera is a perennial herb with short, stout & thick stem. Leaves are pale green, sessile, lanceolate fleshy with spiny margins and contain fresh gel or mucilage. [9] *Aloe barbadensis* Miller. also known as *Aloe vera* has been reported to show anti-inflammatory, wound healing properties, against burns, ulcerative colitis, psoriasis, as well as hepatoprotective agent. It belongs to the family Liliaceae. [10-11] The name *Aloe vera* is derived from Arabic word "Alloeh" meaning shining bitter substance while "vera" in Latin meaning "true". It is called as "plant of immortality" by Egyptians. [12]

Bacopa monniera Wettst belongs to family Scrophulariaceae is commonly known as *water hyssop* or *brahmi*. It is a perennial, creeping herb inhabiting wetlands and muddy shores. Leaves of the plant are succulent, thick and oblanceolate and arranged oppositely on the stem. The flowers are small; white with four to five petals. [13] It is reported that the brahmi plant contains tetracyclic triterpenoids, saponins, bacoside A and B, hersaponin, alkaloids viz. herpestine, brahmine, flavonoids. [14]

Moringa oleifera Lam. syn *M. pterygosperma*, Gaertn belongs to family Moringaceae. It is the *horse-radhish* tree or *drumstick* tree. The foliage is light-green and tri-pinnately compound and flowers are white, sweetly scented flowers. Every part of the tree i.e. bark, root, fruit, flowers, leaves, seed and gum is widely used being rich in proteins, vitamins and minerals. The plant has antipyretic, anti-inflammatory, hypocholestrolemic, wound healing, anti-thyroid, anti-microbial, antihyperglycemic, antioxidant, anti-tumour properties to name a few. [15]

Zingiber officinale Roscoe commonly known as *ginger* belongs to Zingiberaceae family. The part of the plant widely used is the rhizome. The plant produces orchid-like flowers which are greenish yellow streaked with purple colour. Ginger is cultivated in regions of abundant rainfall. [16] The name of the genus *Zingiber* derived from the Sanskrit word meaning "horn-shaped" referring to the protrusions on the rhizome. [17] Ginger has been used as medicine since the Vedic period and is called "Maha- aushadhi" means the great medicine. [18]

Since, the hepatoprotective activity of the four medicinal plants mentioned has not been assessed in D-galactosamine hepatotoxic model in mice; the present study has been undertaken. In the present study hepatoprotective activity of herbal preparation (HP-4) containing 80% alcoholic extract containing *Aloe vera, Bacopa monniera, Moringa oleifera* leaves and rhizome of *Zingiber officinale* in equal proportion was assessed against Silymarin, a standard drug in N-Galactosamine induced hepatotoxicity in mice.

Silymarin is standardized hepatoprotective extract of *Silybum marianum* (Compositae). It reverses hepatotoxin-induced alterations of biochemical parameters. It has been for long the most thoroughly investigated of all hepatoprotective medicinal plants in preventing liver damage induced by carbontetrachloride, D-GalN and paracetamol in rat models. [19] Silymarin has been used as a standard for comparison in our study.

MATERIALS AND METHODS

The leaves of *Aloe vera, Bacopa monniera, 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.

Dried powder of weight 100 g each, of leaves of *Aloe vera, Bacopa monniera, 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 4000 rpm for 10 minutes. The clear supernatant obtained was labeled Herbal Preparation (HP-4) as reported earlier. [20]

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 (12:12 hr L: D cycle and 25± 2ºC). 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

Mice of body weight 25-30 g were selected. Total number of 20 mice was divided into 5 groups of 4 animals each.

Group I: Normal Control: The animals received distilled water 5 ml/kg b.w. p.o. for 8 days.

Group II: Toxicant N-Galactosamine Group: Also received distilled water 5ml/kg b.w. p.o. for 8 days. A single dose of D-Gal N in D.W. 200 mg/kg b.w was given i.p. after one hour of vehicle on the 8th day.

Group III: Standard Silymarin Group: The animals received 100 mg/kg b.w. p.o. was given for 8 days. The animals received a single dose of D-Gal N in D.W 200 mg/kg b.w i.p after 1 hour of vehicle on the 8th day.

Group IV Toxicant + HP- 4 250 mg/kg Group: HP-4 250 mg/kg was p.o. for 8 days. A single dose of D-Gal N in D.W 200 mg/kg b.w. was given i.p. after 1 hour of vehicle on the 8th day.

Group V Toxicant + HP-4 500 mg/kg Group: HP-4 500 mg/kg was p.o. for 8 days. The animals received single dose of D-GalN in D.W.200 mg /kg b.w i.p. after 1 hour of vehicle on the 8th day. [21]

Biochemical Parameters: On the 9th 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 [22], aspartate transaminase [AST] EC 2.6.1.1 [23], alkaline phosphatase [ALP] EC 3.1.3.1 [24], γ –glutamyl transferase [γ GT] EC 2.3.2.2 [25] and lactate dehydrogenase [LDH] EC 1.1.1.27 [26] by using standard methods using enzyme assay kits. Transasia Bio-medicals Ltd Kit for ALT, AST, LDH and Accurex Biomedicals Ltd Kit for γ GGT & ALP. The enzyme assays were performed on a semiautoanalyser ERBA Chem7.

Table 1: Effect of herbal preparation HP-4 on the liver function tests as a marker of liver damage in D-Galactosamine (D-GalN) induced hepatotoxicity in mice (Units in IU/L)

S. No	AST	ALT	ALP	LDH	γGT
Group I Control	34.75±3.55	26.75 ± 3.67	87.66 ± 9.49	318.67 ± 33.54	3.33 ± 0.39
Group II Toxicant (D-GalN)	178±18.35[a]	182.5± 19.06[a]	274.5±28.53[a]	1536.75±154.1[a]	12.13±1.30[a]
Group III D-GalN +Silymarin (100mg/kg)	112 ±12.69[b]	109 ± 10.77[b]	230.33±24.36[b]	1100.7 ± 120.7[b]	5.33 ± 0.56[b]
Group IV D-GalN+HP-4 (250 mg/kg)	146 ±15.75[c]	88.3 ± 9.10[c]	268.33±27.98[c]	1165.33 ±117.7[c]	7.66 ± 0.77[c]
Group V D-GalN+HP-(500mg/kg))	150 ±16.13[d]	75.5 ± 8.20[d]	228.25±23.57[d]	1297.9 ± 129.8[d]	8.75 ± 0.88[d]

Values Mean ±SD of triplicate determinations.
a $p<0.05$ Toxicant as compared to control, significantly increased, b $p<0.05$ Group III as compared to Group II, significantly decreased, c $p<0.05$ Group IV as compared to Group II, significantly decreased, d $p<0.05$ Group V as compared to Group II, significantly decreased

Table 2: Effect of herbal preparation HP-4 on liver antioxidant enzymes activities levels on D-GalN induced hepatotoxicity in mice

S. No	SOD Units/100mg protein	GPx-Nanomoles GSH Utilized/min/mg protein	GR-Nanomoles NADPH/100mg protein
Group I Control	1.96 ± 0.22	47.94 ± 4.49	4.83 ± 0.49
Group II Toxicant (D-GalN)	0.68 ± 0.08[a]	27.32 ± 2.80[a]	2.05 ± 0.23[a]
Group III D-GalN +Silymarin (100mg/kg)	1.80 ± 0.29[b]	52.60 ± 6.19[b]	6.05 ± 0.81[b]
Group IV D-GalN+HP-4(250mg/kg)	1.63 ± 0.18[c]	56.33 ± 5.52[c]	4.61 ± 0.70[c]
GroupV D-GalN+HP4(500mg/kg)	1.84 ± 0.20[d]	55.32 ± 5.61[d]	5.95 ± 0.57[d]

Values Mean ±SD of triplicate determinations
a $p<0.05$ Toxicant as compared to control, significantly increased, b $p<0.05$ Group III as compared to Group II, significantly decreased, c p<0.05 Group IV as compared to Group II, significantly decreased, d $p<0.05$ Group V as compared to Group II, significantly decreased

Table 3: Effects of herbal preparation HP-4 on liver weight, total proteins, TBARS and reduced GSH on D-GalN hepatotoxicity in mice

S. No	Wt of the liver in grams	TBARS nanomoles/ 100mg protein	Reduced GSH mgGSH /100mg protein
Group I Control	1.884 ± 0.18	26.07 ± 2.77	2.91 ± 0.31
Group II Toxicant (D-GalN)	1.945 ± 0.20[a]	71.24 ± 8.72[a]	0.95 ± 0.10[a]
Group III D-GalN +Silymarin (100mg/kg)	1..989 ± 0.22[b]	41.03 ± 4.48[b]	1.04 ± 0.11[b]
Group IV D-GalN+HP-4(250mg/kg)	1.990± 0.21[c]	51.30 ± 5.53[c]	1.90 ± 0.20[c]
Group V D-GalN+HP-500mg/kg)	1.893 ± 0.22[d]	41.70 ± 4.47[d]	1.12 ± 0.15[d]

Values Mean ± SD of triplicate determinations.
a p<0.05 Toxicant as compared to control, significantly increased, b p<0.05 Group III as compared to Group II, significantly decreased, c p<0.05 Group IV as compared to Group II, significantly decreased, d p<0.05 Group V as compared to Group II, significantly decreased.

The animals were sacrificed by cervical dislocation & liver was excised, 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 weighed. It was homogenized in phosphate buffer 0.2 M, pH 7.4. [27]

The 10% homogenized liver tissue was made using a tissue homogenizer (MC Dalal & Co).The supernatant obtained after centrifuging at 4000 rpm for 10 minutes was used for estimation of SOD, GPx, GR, TBARS and GSH. Total proteins in the supernatant were estimated by Biuret Method. [28]

Estimation of Superoxide dismutase {SOD} EC 1.15.1.1: SOD was determined by the method described by Marklund and Marklund in 1974. [29] 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,
0.05 M, 0.5 ml of 1mM EDTA, 0.5ml Pyrogallol 0.2mM freshly prepared were added to 50µl 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.

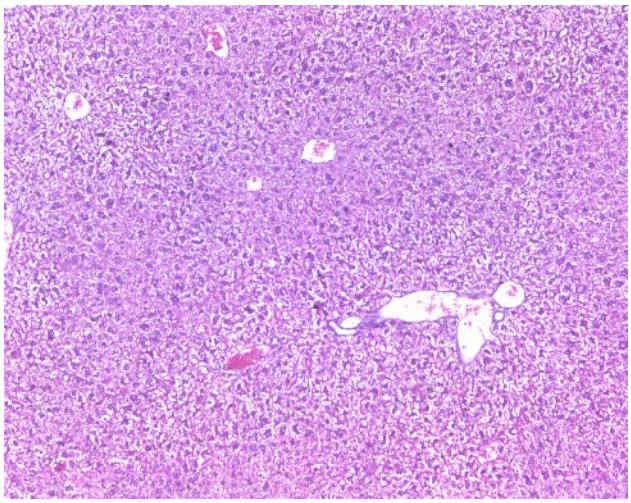

Fig. 1: Histopathology slide of Toxicant that is D-Galactosamine (200 mg/kg in distilled water) treated mice or Group II

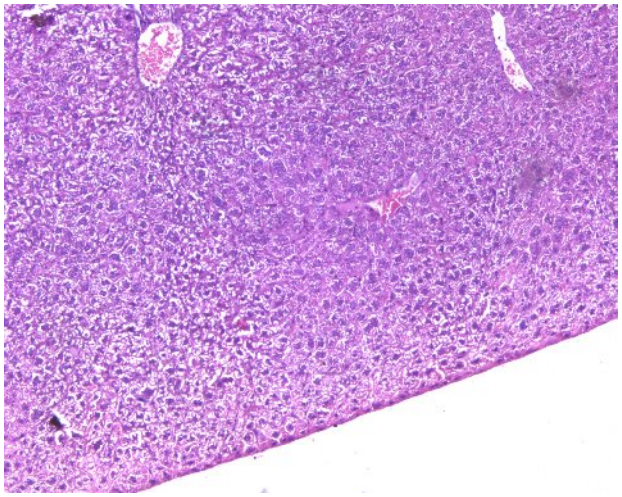

Fig. 2: Histopathology slide of D-Galactosamine (200 mg/kg in distilled water) +Silymarin (100mg/kg) treated mice or Group III

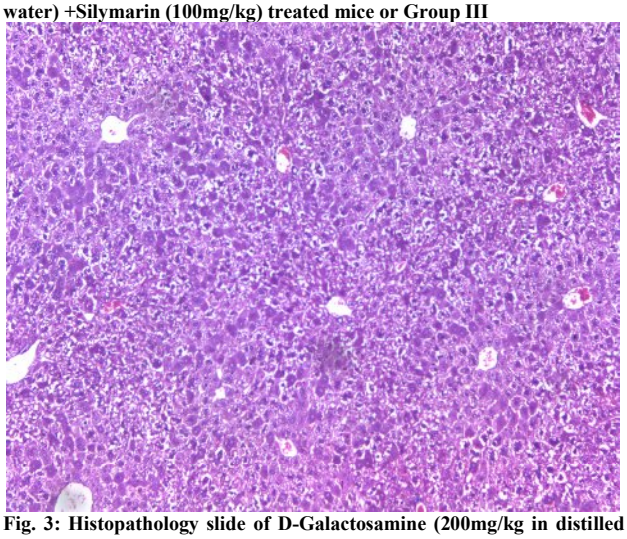

Fig. 3: Histopathology slide of D-Galactosamine (200mg/kg in distilled water) + HP- 4 (250 mg/kg) treated mice or Group IV

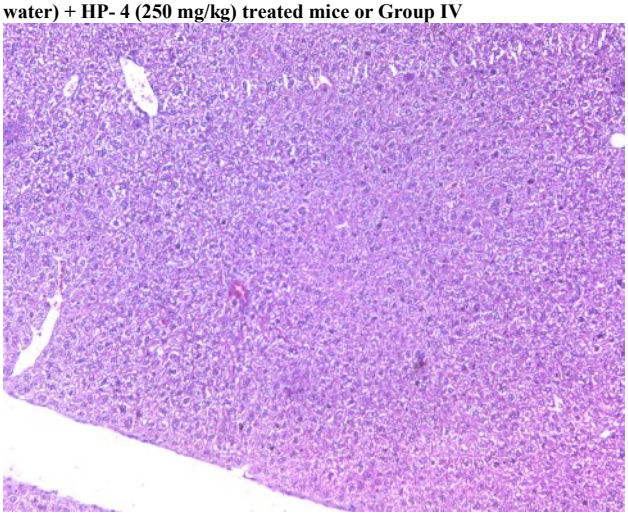

Fig. 4: Histopathology slide of D-Galactosamine (200mg/kg in distilled water) + HP- 4 (500 mg/kg) treated mice or Group V

Estimation of Glutathione peroxidase {GPx} EC 1.11.1.9: GPx activity was measured by the method described by Rotruck *et al* 1973. [30] Briefly, reaction mixture contained 0.2 ml of 0.4 M Tris HCL buffer pH 7.0, 0.1 ml of 10 mM sodium azide, 0.2 ml of the supernatant of liver tissue homogenate, 0.2 ml glutathione 60 mg%, 0.1 ml of 0.2 mM H_2O_2 .The contents were incubated at 37°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 [19.8 mg of 5, 5′ dithiobis-(2 nitro benzoic acid) or DTNB in 0.5 g Sodium Citrate in 50 ml 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. [31] The reaction mixture contained 1mM oxidized glutathione GSSG as substrate, 2 mM NADPH and tissue homogenate in phosphate buffer (pH=7.4).The decrease in absorbance at 340 nm 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. [32] 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 nitro benzoic acid) DTNB reagent according to method proposed by Moron et al 1979. [33] Briefly, 200µl of supernatant of tissue homogenate (10%) was added to 800µl 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 0.6 M 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 Formasaline solution. The liver tissues were processed for paraffin embedding and sections 5µm thick were taken in a microtome. After staining with hematoxylin and eosin, slides were examined under the microscope for histopathological changes. [34]

Statistical Method: Result Table values are Mean ± SD of triplicate determinations. Student's unpaired t-test applied with $p<0.05$ as level of significance. Values obtained as $p<0.05$ considered as statistically significant.

RESULTS

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

Since D-Galactosamine 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 as compared to the Toxicant group. Silymarin group shows 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.

DISCUSSION

D-GalN is a well established hepatotoxicant, which is widely used model which closely resembles human viral hepatitis in its morphologic and functional characteristics therefore considered very useful for evaluation of hepatoprotection. [35-36] D-GalN hepatotoxicity is considered as an experimental model of acute hepatitis although it does not affect other organs. [37]

Liver damage induced by D-GalN usually shows disturbances of liver cell metabolism which leads to characteristic changes in the liver serum enzymes. [38] The increased levels of AST, ALT, ALP, γ-GT and LDH which are hepatic serum marker enzymes in the toxicant D-GalN group, maybe interpreted as a result of the liver cell destruction or changes in the cell membrane permeability indicating severity of hepatocellular damage induced by D-GalN hepatotoxicant .which according to previous reports. [39] Pre-treatment with Herbal Preparation (HP-4) in dosages of 250 mg/kg and 500 mg/kg attenuated the increased serum levels of hepatic enzymes. On comparison with the standard hepatoprotective drug Silymarin used in dosage 100 mg/kg also showed significant reduction in elevated serum levels of hepatic enzymes. Hepatocellular necrosis leads to an increase in serum levels of both AST and ALT which are released from the liver into the blood stream. Among the two enzymes, ALT is a better index of liver injury ,as liver ALT activity represents 90% of total enzyme activity present in the body. [40]

ALP activity when considered is related to the hepatocyte function. That means an increase in its activity is due to elevated synthesis in presence of increased biliary pressure. [41]

The reduction of AST and ALT towards normal values by the administration of herbal preparation (HP-4) is an indication of stabilization of plasma membranes as well as repair of damage tissues caused by D-GalN. This effect is in perfect agreement with the fact that serum levels of transaminases return to normal with healing of the liver parenchymal cells and healing of hepatocytes as also reported by studies by Gupta *et al*. [21] An anti-cholestatic effect of silymarin being reported by Saraswat *et al* [42] showed the effect of Silymarin in decreasing elevated levels of serum transaminases and alkaline phosphatase in isolated rat hepatocytes with galactosamine induced damage.

In the present study hepatic antioxidant enzyme levels of SOD, GPx and GR levels are reported to be decreased in liver of mice treated with hepatotoxicant D-GalN. Thereafter the standard hepatoprotective drug Silymarin and the herbal preparation (HP-4) increases the levels of the antioxidant enzymes. The hepatoprotective effects of silymarin from *Silybum marianum* extracts on liver cells is due to the presence of flavonoids and its antioxidant effects. [43]

The decrease in SOD, GR by treatment with hepatotoxicant D-GalN and restoration to normal value with protein isolated from leaves of herb *Cajanus indicus* were reported by Sinha *et al*. [44]

The levels of TBARS were increased and total reduced GSH levels were decreased in the animals treated with toxicant D-GalN in our study. This indicated elevated lipid peroxidation since TBARS is an indicator of lipid peroxidation and was found significantly increased in liver homogenates of D-GalN treated rats as reported by Seçkin *et al,* 2008. [45] In the study in which protein isolated from leaves of *Cajanus indicus*, the protein extract ameliorated the increased levels of TBARS and enhanced reactive oxygen species scavengers like total reduced GSH both prior and post to the D-GalN intoxication.

D-GalN induced acute liver viral hepatitis is a useful model to study hepatic injury which is easy and convenient to replicate experimentally. Its damage resembles that due to human viral hepatitis. D-GalN induces a decrease in liver uracil nucleotides which rapidly inhibits both RNA and protein synthesis that is transcription and translation processes. Rapid depletion of uridine diphosphate glucose appears to be the first biochemical lesion, followed by defects in glycoprotein synthesis which is secondary biochemical lesion. This leads to eventual damage of cellular membranes and ultimately to spotty liver cell necrosis. Then the cellular damage provokes inflammatory reaction or tertiary reactions, resulting in a picture closely resembling viral hepatitis. [19]

These changes induce cellular damage of the hepatocytes and subsequent development of acute hepatitis with disseminated hepatocellular necrosis and infiltration of polymorphonuclear leukocytes (PMNL). The other important mechanism to explain mode of development of GalN hepatitis have also been well documented. Evidences support the fact that the release of reactive oxygen species (ROS) and cytokines such as tumour necrosis factor (TNF-α) and Interleukin-1 (IL-1) by Kupffer cells (KC) in the liver also contribute towards the hepatocyte damage in D-GalN hepatotoxicity. It has been also been proved that depletion of KC by gadolinium chloride, substantially decreases D-GalN hepatitis in rats. In addition, it is also well known that when animals are treated with ROS inhibitors or scavengers such as N-acetylcysteine, cystiamine, silymarin and others the D-GalN induced hepatotoxicity is attenuated. [46]

The *in-vitro* antioxidant potential of the Herbal preparation (HP-4) used in the present study were reported by the authors Padmanabhan *et al*, 2012. [20] The phytochemicals phenolic compounds, flavonoids and flavonols were also detected. Moreover the DPPH radical scavenging and reducing power of HP-4 that is its antioxidant activity denoted synergistic effects as compared to the individual plant extracts when used alone. The synergistic and supraadditive effect of herbal preparation HP-4 may be due to increased possibility of interaction amongst phytochemicals that allows more co-operative effects. [47] Apart from this the anti-inflammatory properties of Herbal preparation (HP-4) was also detected which may contribute to the mechanism of hepatoprotection and therapeutic value. [48]

In a recent study by Singh *et al* in 2012 [49] the protective effects of Silymarin in lower doses and also its synergism in

combination with plant extracts *Picorrhiza kurroa, Tephrosia purpurea, Phyllanthus amarus* and *Asparagus racemosus* in experimental liver injury using paracetamol and carbon tetrachloride induced hepatotoxicity model in rats. The synergistic action was demonstrated in potentiation of cellular protective mechanisms like anti-oxidative, anti-inflammatory, membrane stabilization effects of the polyherbal combination used in the study by Singh et al as compared to Silymarin used alone at lower doses.

Moreover, herbal formulations have often been found to function better in a synergistic manner than when used alone. One such polyherbal formulation is LIV 52, which is a mixture of extracts from *Capparis spinosa, Cinchorium intybus, Solanum nigrum, Cassia occidentalis, Terminalia arjuna, Achillea millefolium, Tamarix gallica, Eclipta alba, Phyllanthus niruri, Berberis aristata, Taphanus sativus, Phyllanthus emblica, Plumbaga zeylanica, Boerhavia diffusa, Tinospora cordifolia, Embelia ribes, Terminalia chebula* and *Fumaria officinalis.* [50]

Polyherbal formulation are therefore preferred than just a single herb. Since the different modes of action of therapeutic properties it shows. In combined form may sometimes exhibit synergistic activity (enhanced activity than that of individual herb). Components of the plant which are less active they, can act to improve stability, solubility, bio-availability or half-life of active components. Hence a particular component in the pure form may 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 mixture of plants for therapeutic purpose. [27]

The histopathological studies in the transverse liver sections showed normal parenchymal architecture with cords of hepatocytes, portal tracts and central veins in the control group with no cell injury or cirrhosis found. In the D-GalN treated groups, mild lobular disarray with inflammatory cells in the sinusoids of the lobules were present. Ballooning degeneration of the hepatocytes as well as focal necrosis within lobules is noted. The Herbal Preparation pre-treated groups in dosages of 250 mg/kg and 500 mg/kg and Silymarin group showed absence of necrotic lesions in liver sections.

The present study involves the D-GalN hepatotoxicity in mice however the uncovered area of research is chronic dosages in mice. Thus both biochemical parameters and histopathological studies provide evidence that Herbal Preparation HP-4 a polyherbal formulation of *Aloe vera, Bacopa monniera, Moringa oleifera* and *Zingiber officinale* offered a synergistic protection due to phytochemicals present in its hepatoprotective activity in D-GalN mediated hepatotoxicity in mice.

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