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Hepatoprotective activity of herbal preparation (HP-4) against carbontetrachloride induced hepatotoxicity in mice

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ABSTRACT

Oxidative stress is a condition resulting due to increased generation of free radicals and decrease in antioxidant levels. Several diseases such as liver diseases are caused due to free radicals formation in the body. Carbontetrachloride or CCl_4 is a potent hepatotoxicant. It is metabolized in the liver to generate free radicals, which react with cellular lipids and proteins, eventually leading to cell death. 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. The hepatoprotective role of HP-4 is compared to standard drug Silymarin in CCl_4 model of hepatotoxicity in mice. Control group or Group I fed on normal saline p.o. for 7 days. Toxicant or Group II injected with CCl_4 diluted with paraffin (1:1) on first day of dosage 1.5 ml/kg b.w. i.p. Group III received 50mg/kg Silymarin p.o. for 7 days and single similar dose of CCl_4 on day 1 as in Group II. Group IV and V fed with HP-4 of dosages 250mg/kg and 500mg/kg p.o.for 7 days and single similar dose of CCl_4 on day 1 as in Group II. Serum and liver homogenate derived from mice was utilized for biochemical studies. A part of liver was preserved for histopathological studies which provide a direct means to ascertain HP-4 efficacy. It is concluded that HP-4 a polyherbal formulation exhibits synergistic action of phytochemicals alone and in combination to exhibit hepatoprotective action, which is evidenced by biochemical and histopathological studies.

Keywords: free radicals, antioxidants, carbontetrachloride, hepatoprotection, phytochemicals.

INTRODUCTION

Normally free radicals of different forms are generated at a low level in cells to help in the modulation of several physiological functions. Free radicals are quenched by an integrated antioxidant system in the body [1]. Oxidative stress in cells and tissues result from the increased generation of free radicals or reactive oxygen species and decrease in antioxidant defense potential [2].

The role of free radicals in disease pathology is well established. Liver disease remains a serious health problem [3].

Liver plays an important role in the metabolism, synthesis, storage and also detoxification of many endogenous and exogenous compounds and converting to less toxic substances for excretion [4]. Carbon tetrachloride (CCl_4) is toxic to the liver and its toxicity is dose dependent and is based on the time of exposure [5]. According to the findings by the International Programme on Chemical Safety (IPCS 1999), CCl_4 was shown to be an outstanding and potent hepatotoxicant [6].

In the liver, CCl_4 is metabolized into highly reactive trichloromethyl radical. This free radical causes autooxidation of the fatty acids present in the cytoplasmic membrane phospholipids, resulting in functional and morphological changes in the cell membrane. Trichloromethyl free radical combines with lipids and proteins in the presence of

oxygen to form trichlomethylperoxyl radical .This radical elicits lipid peroxidation, destruction of Ca^{2+} homeostasis and finally results in cell death [5].

The fact that reliable liver protective drugs are explicitly inadequate in allopathic medicine, exhorted the scientists to explore herbal remedies [7]. In India around 20,000 medicinal plant species have been recorded recently, but more than 500 traditional communities use about 800 plant species for curing different diseases. Standardization of herbal formulations is an essential factor in order to assess the quality, purity, safety and efficacy of drugs based on the concentration of their active principles [9].There are a number of medicinal preparations in the Ayurvedic system of Indian medicine recommended for the treatment of liver disorders [8]. To promote the proper use and to determine their potential as sources for new drugs, it is essential to study the medicinal plants [10].

Silybum marianum has high safety, causes no adverse effect on body weight and produces hepatoprotective ands antioxidant effects on CCl_4 poisoned rats [11].

Leptadenia reticulata showed significant hepatoprotective activity. The ethanolic extract is more potent in hepatoprotection in CCl_4 –induced liver injury model as compared with aqueous extract [12].

Caesalpinia sappan Linn. heartwood was hepatoprotective and was able to restore the biochemical levels to normal which was altered due to CCl_4 intoxication in freshly isolated rat hepatocytes and also in animals [13].

Aloe barbadensis, Miller (commonly known as *Aloe vera*) is a perennial plant belonging to the family Liliaceae. *Aloe vera* not only possesses hypoglycemic activity, it is a blood purifier and also hepatoprotective [14].

Aqueous extract of *Aloe barbadensis* is significantly capable of restoring the integrity of hepatocytes indicated by improvement in physiological parameters, excretory capacity and also stimulation of bile flow. That represents the hepatoprotective potential of *Aloe barbadensis* Mill against CCl_4 induced hepatotoxicity [15].

Bacopa monnieri L. (Family Scrophulariaceae) commonly known as *brahmi* .It is reported that the plant contains tetracyclic triterpenoids, saponins, bacoside A and B , hersaponin , alkaloids viz. herpestine ,brahmine and flavonoids [3].

The hepatoprotective function of ethanolic extract containing bacoside A of *in –vitro* grown *Bacopa monnieri* was evaluated and ascertained in CCl_4 intoxicated albino mice [16].

Moringa oleifera popularly known as the *drumstick tree*, belongs to the family Moringaceae as the only member of the family. Some authors have called this plant, the miracle tree with countless nutritional and therapeutic benefits. Among these benefits reported are antihelminthic, antibiotic, antimicrobial activities, antioxidant and hepatoprotective properties [17].

The potent hepatoprotective activity of leaves of *Moringa oleifera* Lam. was found to reduce total bilirubin, direct bilirubin, SGPT and SGOT in CCl_4 treated albino rats [18]. Leaves of *Moringa oleifera* have been used as antiulcer, diuretic, anti-inflammatory and for wound healing. Ethanolic extract of leaves have shown antifungal activity against a number of dermatophytes, whereas methanol extract has a potent CNS depressant action. The aqueous extract of the leaves has been found to possess antifertility activity [19].

Zingiber officinale Roscoe .or commonly known as *ginger* belongs to the family Zingiberaceae. The rhizome is commonly used in cooking and traditional medicine. The oleoresin from the rhizome contained 6-gingerol and its homolog which have shown to possess anti-inflammatory, anti-pyretic, analgesic, cardiotonic and anti-hepatotoxic properties [20]. It is a common condiment for various foods and beverages. It has a long history of medicinal use dating back 2,500 years in China and India for conditions such as headaches, nausea, rheumatism, and colds [21].

The results of the study on normal and CCl_4 induced hepatotoxic rats suggested that the consumption of ginger –based diet maintained the integrity of the liver and protects it against damage [22].

On the basis of results obtained the combined ethanolic extract of *M.azedarach* and *P.longum* (BHE) exerts more hepatoprotective activity than when they are administered separately or when compared to standard drug Silymarin and may serve as a useful adjuvant in several liver diseases. This may be attributed to the synergistic activity of both herbal drugs when given in combination [5].

O.basilicum and *T. foenum –graecum* are rich sources of flavonoids which have been shown to possess various biological properties related to antioxidant mechanisms .Perhaps the flavonoids and saponins present in *O.basilicum* and *T. foenum –graecum* were responsible for their hepatoprotective effects as compared to Silymarin in CCl_4 induced hepatotoxicity in goat liver [23].

The polyherbal formulation (PHF08) composed of extracts of *Tinosporia cordifolia*, *Emblica officinalis*, *Withania somnifera*, *Curcuma longa*, *Glycyrrhiza glabra*, *Bacopa monnieri*, *Terminalia chebula*, *Asparagus racemosus*, *Terminalia arjuna* and *Aloe barbadensis* showed promising hepatoprotective properties as compared to standard drug Silymarin in CCl_4 induced oxidative damage in rats being related to its antioxidant and free radical scavenging activity [24].

In the present study, the hepatoprotective activity of herbal preparation (HP-4) containing 80% alcoholic extract containing *Aloe vera*, *Bacopa monnieri*, *Moringa oleifera* leaves and rhizome of *Zingiber officinale* in equal proportion was assessed against silymarin, a standard drug in CCl_4 induced hepatotoxicity in mice. Silymarin is a flavanolignan, extracted from the fruits and seeds of the plant milk thistle *Silybum marianum* .L.Gaertn) [25].

EXPERIMENTAL SECTION

The leaves of *Aloe vera*, *Bacopa monnieri* and *Moringa oleifera* and the 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 and powdered finely in a mixture and sieved twice to obtain a fine powder.

100 gm dried powder of leaves of each *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. 25mg of each extract was mixed together and dissolved in 10 ml water, boiled in water bath for 5minutes, cooled and centrifuged at 4000 rpm for 10 minutes. The clear supernatant obtained was labeled as Herbal Preparation (HP-4) as reported earlier [26].

Animals: Swiss albino male mice weighing 25-30 g bred in Animal Resources 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 ± 2 ˚C). The study protocol was approved by "The Institute Animal Ethical Committee" PIMS /AH/215 /2011 PIMS Loni and CPCSEA Reg No 366/01/a/CPCSEA.

Study Design: 20 male mice of weight 25-30 g were divided into 5 Groups of 4 animals each.

Group I Normal Control: The animals received normal saline passed orally for 7 days.

Group II Toxicant CCl_4 Group: The animals received a single dose of CCl_4 diluted with liquid paraffin in ratio 1:1 and 1.5 ml/kg b.w. i.p. on 1st day.

Group III: Standard Silymarin Group: The animals received Silymarin 50mg /kg p.o. was given for7 days. The animals received a single dose of CCl_4 diluted with liquid paraffin in ratio 1:1 and 1.5 ml/kg b.w. i.p. on 1st day.

Group IV Toxicant + HP-4 250mg/kg Group: HP-4 250mg/kg was p.o for 7 days. The animals received a single dose of CCl_4 diluted with liquid paraffin in ratio 1:1 and 1.5 ml/kg b.w. i.p. on 1st day.

Group V Toxicant + HP-4 500mg/kg Group: HP-4 500mg/kg was p.o for 7 days. The animals received a single dose of CCl_4 diluted with liquid paraffin in ratio 1:1 and 1.5 ml/kg b.w. i.p. on 1st day.

Biochemical Parameters: On the 8th 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 assay of alanine transaminase (ALT) EC 2.6.1.2 (Bradley et al 1972)[27], aspartate transaminase (AST) EC 2.6.1.1 (Wolf et al1972)[28] ,alkaline phosphatase (ALP) EC 3.1.3.1(Young et al)[29] , γ glutamyl transferase (γ GT) EC 2.3.2.2 (Persijn et al 1976) [30] and lactate dehydrogenase (LDH)EC 1.1.1.27 (Lum G and Gambino SR)[31] 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 Chem 7.

Histopathology: The animals were sacrificed by cervical dislocation & liver was excised, washed with saline and dried with tissue paper. A part of the lobe of liver was transferred to Formasaline solution for histopathological

studies. 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 [32].

The remaining part of the liver was weighed and was homogenized in phosphate buffer 0.2M, pH =7.4 [7] .The 10% homogenized liver tissue was made using a tissue homogenizer (MC Dalal & Co).The supernatant obtained after centrifuging at 4000rpm for 10 minutes was used for estimation of SOD, GPx, GR,TBARS & GSH. Total proteins in the supernatant were estimated by Biuret Method [33].

Determination of superoxide dismutase {SOD} EC 1.15.1.1: SOD was determined by the method described by Marklund and Marklund in 1974 [34].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 50µl of the supernatant of tissue homogenate. OD at 420nm after 5 minutes was recorded. One unit of SOD was defined as the enzyme activity that inhibited the autoxidation of pyrogallol by 50 percent.

Determination of glutathione peroxidase {GPx} EC 1.11.1.9: GPx activity was measured by the method described by Rotruck et al 1973 [35] .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 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 (19.8mg of 5, 5' dithiobis-(2 nitro benzoic acid) DTNB in 0.5g Sodium Citrate in 50ml double distilled water).

Determination of glutathione reductase {GR} EC 1.6.4.2: activity was measured spectrophotometrically according to the method of Calberg and Mannervick 1985[36].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.

Determination of thiobarbituric acid reactive substances {TBARS}: TBARS in tissues was estimated by the method of Fraga et al 1981[37].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.

Determination 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[38].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.

RESULTS AND DISCUSSION

Table 1: Effect of Herbal Preparation HP-4 on the Liver Function Tests as a marker of liver damage in carbontetrachloride (CCl_4) induced hepatotoxicity in mice

Sr.No	Units in IU/L				
	AST	**ALT**	**ALP**	**LDH**	**γGT**
Group I Control	36.26 ± 4.15	28.70 ± 3.22	88.31 ± 8.49	300.15± 32.80	3.65 ± 0.32
Group II Toxicant (CCl_4)	80.05 ± 8.50 [a]	54.10 ± 5.20 [a]	376.3 ± 36.96 [a]	555.20 ± 56.3 [a]	7.40 ± 0.88 [a]
Group III CCl_4+ Silymarin 200mg/kg	53.61 ± 6.60 [b]	38.14± 3.30 [b]	196.28 ±20.92 [b]	282.46± 28.80 [b]	4.80 ±0.50 [b]
Group IV CCl_4+ HP-4 250 mg/kg	40.71 ± 4.40 [c]	40.55 ± 4.20 [c]	100.61± 10.10 [c]	329.76± 33.10 [c]	5.16 ± 0.62 [c]
Group V CCl_4+ HP-4 500 mg/kg	48.44 ± 4.60 [d]	43.41 ± 4.05 [d]	120.32± 13.10 [d]	269.24± 28.35 [d]	6.14± 0.66 [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' <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 CCl4 induced hepatotoxicity in mice

Sr.No	SOD Units /100 mg protein	GPx-Nanomoles GSH utilized/min/mg protein	GR-Nanomoles NADPH /100mg protein
Group I Control	1.42 ± 0.14	49.38 ± 4.50	4.72 ± 0.45
Group II Toxicant (CCl_4)	0.75 ± 0.08 [a]	26.19 ± 2.13 [a]	2.35 ± 0.26 [a]
Group III CCl_4+Silymarin	1.20 ± 0.18 [b]	57.41 ± 5.82 [b]	8.02 ± 0.78 [b]
Group IV CCl_4+ HP-4 250 mg/kg	1.21 ± 0.13 [c]	60.38 ± 6.43 [c]	8.41 ± 0.87 [c]
Group V CCl_4+ HP-4 500 mg/kg	1.53 ± 0.17 [d]	72.30 ± 7.40 [d]	8.11 ± 0.85 [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: Effect of Herbal Preparation HP-4 on liver weight, total proteins, TBARS and Reduced GSH on CCl_4 hepatotoxicity in mice

Sr.No	Wt of the liver in grams	TBARS nmoles/100mg protein	Reduced GSH mgGSH /100mgprotein
Group I Control	1.855 ± 0.19	25.28 ± 2.92	2.16 ± 0.23
Group II Toxicant (CCl_4)	2.046 ± 0.25 [a]	94.12 ± 10.24 [a]	1.32 ± 0.17 [a]
Group III CCl_4+Silymarin	1.713 ± 0.19 [b]	32.32 ± 3.84 [b]	2.35 ± 0.24 [b]
Group IV CCl_4+ HP-4 250 mg/kg	1.922 ± 0.23 [c]	29.77 ± 2.83 [c]	2.09 ± 0.21 [c]
Group V CCl_4+ HP-4 500 mg/kg	2.132 ± 0.24 [d]	33.30 ± 3.58 [d]	2.39 ± 0.29 [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.

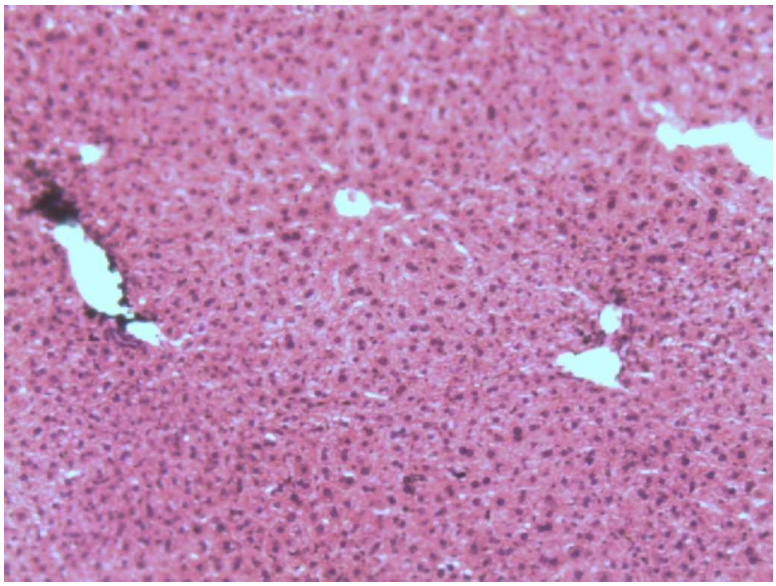

Figure 1: Histopathology of Control or Group I

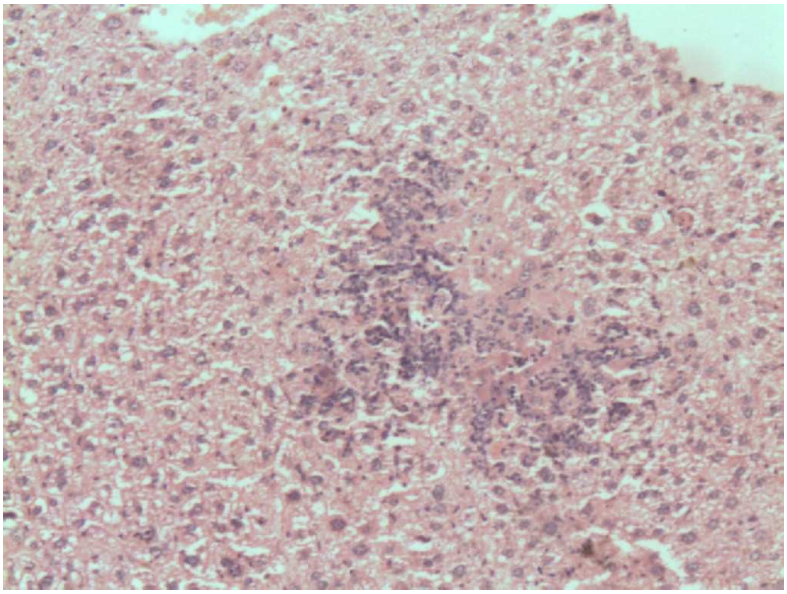

Figure 2: Histopathology of Toxicant or Group II

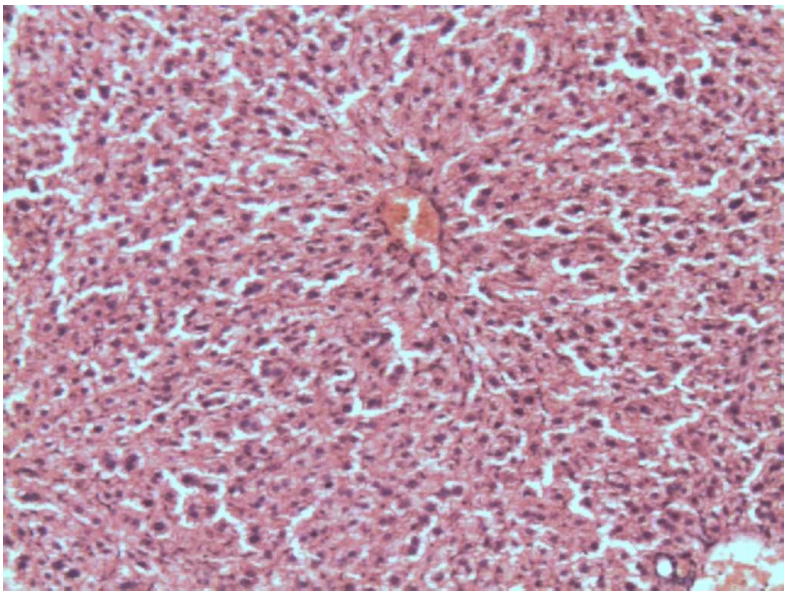

Figure 3: Histopathology of Group III

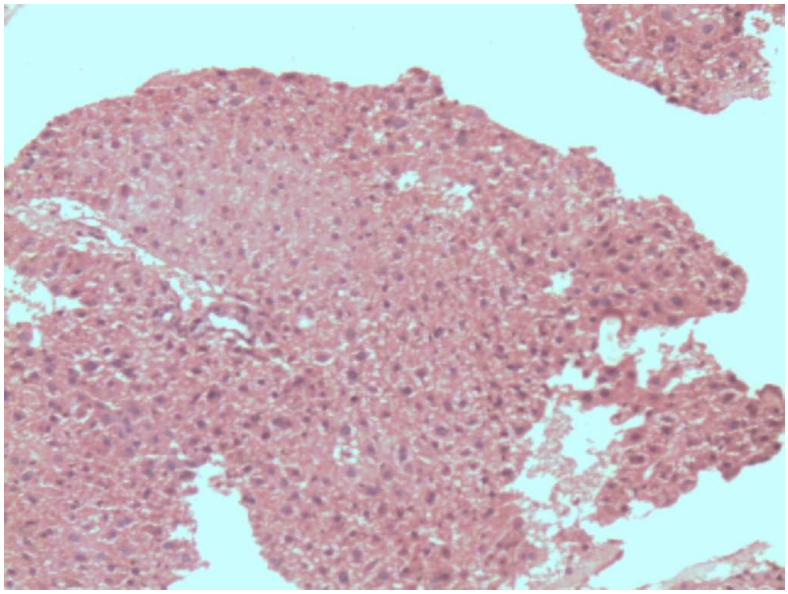

Figure 4: Histopathology of Group IV

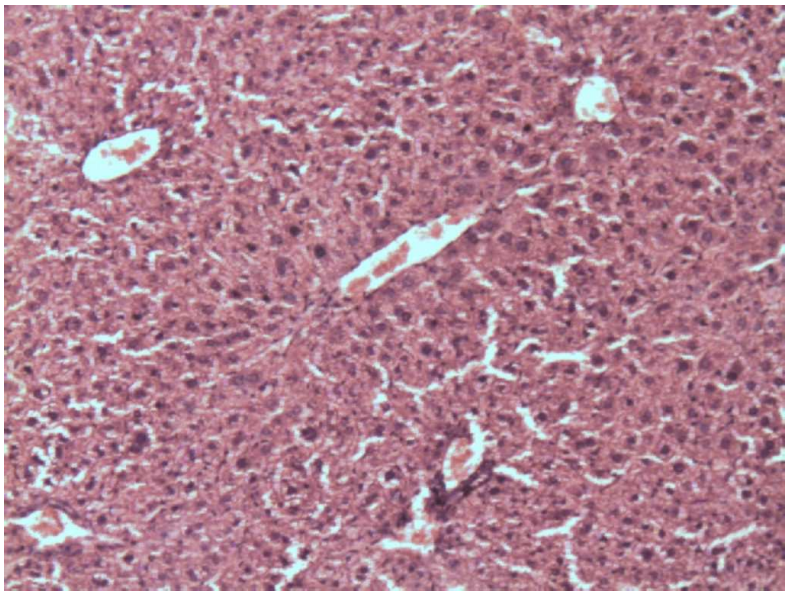

Figure 5: Histopathology of Group V

The activities of serum AST, ALT, ALP, γ GT and LDH (hepatic marker enzymes for liver damage) were markedly elevated in treated CCl_4 animals compared to normal control mice, indicating liver damage. Administration of herbal preparation HP-4 at doses of 250 and 500 mg/kg remarkably prevented CCl_4 induced elevation of serum AST, ALT, ALP, γ GT and LDH in Table 1.Silymarin a well known hepatoprotective drug administration in CCl_4 treated mice resulted in decrease in enzyme activities.

Since oxidative stress contributes to the development of CCl_4 induced hepatotoxicity the levels of liver antioxidant enzymes SOD, GPx, GR were measured. Values for the antioxidant enzymes were reduced in the Toxicant Group as compared to Normal Control .Treatment with both dosages of HP-4 significantly raised the antioxidant enzyme levels as compared to the mice treated with CCl_4. Results are depicted in Table 2.Administration of Silymarin 50mg/kg p.o. and HP-4 in 250 mg/kg & 500mg/kg p.o. reversed the trend of TBARS and GSH when compared with CCl_4 alone. Silymarin administration in CCl_4 treated mice resulted in increase in activity compared with CCl_4 treated mice.

TBARS level was increased whereas total glutathione was found to be decreased in Toxicant Group as compared to Normal Control. These results are shown in Table 3.

CCl_4 is activated by cytochrome P 450 2E1 (CYP2E1), CYP2B1 OR CYP2B2and possibly CYP3A to form trichloromethyl radical (CCl_3·).This toxic free radical reacts with biologically important substances such as proteins, lipids, nucleic acids present in the cell. The radical can react with oxygen to form CCl_3OO· a highly reactive species. CCl_3OO· Initiates a chain of lipid peroxidation which attacks and destroys polyunsaturated fatty acids associated with phospholipids affecting the permeability of mitochondrial endoplasmic reticulum and plasma membrane resulting in loss of calcium ions sequestration and homeostasis resulting in cellular damage [39] [40].

CCl_4 causes oxidative damage and disrupted the hepatocellular plasma membrane causing the release of enzymes into blood from the cytoplasm. Hepatic injury due to toxins could result in defective excretion of bile by the hepatocytes which were reflected in the increased activities of ALP ,γ GT in serum [41].

Treatment with Silymarin 50mg/kg p.o. and HP-4 at 250 mg/kg and 500mg/kg p.o resulted in causing the decrease in elevation of the serum enzymes showing the hepatoprotective activity. The protective effects maybe due to the stabilization of plasma membrane thereby preserving the structural integrity of cells as well as the repair of the hepatic tissue damage caused by CCl_4 [42].

CCl_4 induced hepatic injury decrease the activity of cytochrome P450 and thereby the metabolic functional activity of the hepatocytes [43].It may be possible that the antioxidant activity of HP-4 maybe protecting against CCl_4 induced hepatic cytochrome P450 enzyme inactivation as observed in the case of curcumin [44] and Silymarin [45].

In the present study, the activities of SOD, GPx and GR are found to be decreased in CCl_4 treated rats. Such decrease in the activation of antioxidant enzymes in CCl_4 treated rats were reported by Venukumar and Latha [46].

Escobar et al [47] reported that enhanced free radical concentration resulting from oxidative stress conditions can cause loss of enzyme activities. Ahmad et al [48] are of opinion that the reactive intermediate formed in the course of bioactivation of CCl_4 may bind to those enzyme activities.

Venukumar and Latha [46] reported that the decreased activities of antioxidant enzymes were partially elevated by administration of methanolic extract of *C.orchioides* rhizomes to CCl_4 treated rats and are of the opinion that the natural antioxidants present in the extract are responsible for this.

Similar observations were reported by Lin et al [49] with *Boehmeria nivea var nivea* and *B. nivea var tenacissma.*Lee et al [50] concluded that the hepatoprotective activity of *Salvia miltiorrhiza* may be due to its ability to decrease the metabolic activation of CCl_4 by an increase in cyt P450 2E1 protein content and its antioxidant activity associated with less increase in hepatic iNOS protein content. Hepatoprotective effects of Silymarin in CCl_4 treated rats were reported by Sharma et al [51].Silymarin is reported to possess antioxidant ,cytoprotective, membrane stabilizing, anti-inflammatory action [52] .

Components of HP-4 & HP-4 has shown to possess antioxidant [26] and anti-inflammatory action [53] due to its flavonoids, alkaloids, phenolic contents which may be responsible for partial protection to CCl_4 injury in mice.

In the present study, the levels of serum enzymes ALT, AST, ALP γ GGT, LDH were increased in hepatotoxicity due to CCl_4 .The herbal preparation (HP-4) could however lower the ALT, AST, and ALP, γ GGT and LDH in the mice treated with the toxin CCl_4. Assessment of liver damage can be made by estimating the activities of serum enzymes ALT, ALP, γ GGT and LDH which are originally present in higher concentration in cytoplasm. When there is hepatopathy, these enzymes leak into the blood stream in conformity with the extent and type of liver damage [5].This finding correlates with marked increase in serum ALT, AST, ALP, γGT and LDH. Silymarin is used as standard hepatoprotective compound in the present study since it is reported to have a protective effect on the plasma membrane of hepatocytes [13].The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms that have been disturbed by a hepatotoxin is the index of its protective effects [54].

In the present study SOD, GPx and GR levels are reported to have decreased in livers of mice toxicated by CCl_4.Thereafter the standard drug Silymarin and the herbal preparation (HP-4) increases the levels of the antioxidant enzymes. It is reported that flavonoids of *Silybum marianum* contributes to antioxidant activity and interacts directly with the cell membrane components to prevent any abnormalities in the content of lipid fraction and maintains normal fluidity [11]. A previous study on pretreatment of *Emblica officinalis* on rats showed effect on the metabolizing enzymes GPx and GR. Liver possess growth ability after cellular loss in hepatotoxicant induced liver injury. Regenerating liver provides a model to study the factors that modulates the process of regeneration. Acute

hepatic necrosis on CCl_4 administration is followed by proliferative response that reaches peak at 48 hour, with scarce mitotic cells [55].

In the present study elevated TBARS and decreased GSH levels were similar to study of Meera et al in 2009 (23) on goat liver on CCl_4 administration by the plants *O. basilicum* and *T. foenum –graecum*. There is increase activity of lipid peroxidation system and excessive free radical formation on elevated TBARS formation. The elevated TBARS formation is elevated significantly and comparable to that of standard drug Silymarin. GSH is a tripeptide (γ – glutamyl cysteinyl glycine) non –protein thiol abundant in liver that plays pivotal role in the detoxification processes including conjugation of reactive intermediates and maintenance of GSH redox cycle as antioxidant armoury.

The increase of GSH in the herbal preparation (HP-4) treated group provides idea regarding the mode of protection. Similar studies by Arun Sam Lal et al in 2007 [7] mentioned that enrichment of glutathione in the liver leads to reduced free radical formation (trichloromethyl and trichloromethylperoxy radicals) which leads to reduced lipid peroxidation and it could be assumed that it is the antioxidant and free radical scavenging properties of the herbal mixture which protects the liver from CCl_4 induced liver injury. Hence it is possible that the mechanism of hepatoprotection of HP-4 is due to antioxidant effect.

In –vitro antioxidant effect of HP-4 in various experimental models were reported by authors Padmanabhan & Jangle in 2012[26] .HP-4 is previously reported to have phenolic compounds, flavonoids, and flavonols. Moreover the HP-4 was reported to have synergistic antioxidant effect on DPPH scavenging activity and reducing power [56].The hepatoprotection by HP-4 maybe due to synergistic action of various phytochemicals having antioxidant action.

Padmanabhan and Jangle in 2012 have reported the in-vitro anti-inflammatory activity of HP-4, which may be one of the mechanisms of hepatoprotection of HP-4. [53]

HP-4 is a mixture of *Aloe vera, Bacopa monnieri, Moringa oleifera and Zingiber officinale.*

According to Sharma in 2012 [15] the possible mechanism of hepatoprotective action of aqueous extracts of *Aloe barbadensis* maybe due to its antioxidant activity as indicated by protection against lipid peroxidation and maintained glutathione contents.

A study by Gudipati et al in 2012 [16] that bacoside A is the efficient component in the *in-vitro Bacopa monnieri* cultures that prevented CCl_4 induced damage in albino mice model as indicated by the levels of the serum markers of hepatic damage.

A separate study by Senthil Kumar et al in 2012[18] lead to the conclusion that potent hepatoprotective activity of leaves of *Moringa oleifera* Lam. was found to reduce the level of total bilirubin, direct bilirubin, SGPT and SGOT in CCl_4 treated animals.

Also reported is study by Kazeem et al in 2012 [22] that the ginger –supplemented diet afforded protection against CCl_4 induced liver damage by ginger maybe due to its free radical scavenging activity thereby intercepting those radicals involved in CCl_4 metabolism by microsomal enzyme .This protective role of ginger maybe due to minerals and antioxidant chemicals.

A study by Shukla et al in 2006 [57] suggested that the possible potential of polyherbal formulation(PHF) maybe due to the cumulative effect of these plants and their antioxidant properties which hinder the formation of free radicals produced by CCl_4

On the basis of study by Rajeshwary et al in 2011 [5] it was concluded that combined ethanolic extract of *M.azedarach* and *P.longum* (biherbal extract) exerts more hepatoprotective activity that when they are administered separately and may serve as a useful adjuvant in several clinical conditions associated with liver damage. This maybe attributed to the synergistic activity of both herbal drugs when given in combination. Antioxidant principles from herbal sources are multifaceted in their effects and provide enormous scope in correcting the imbalance through regular intake of a proper diet.

Polyherbal formulation are thus preferred than just a single herb. Since the different plants in the herbal mixture will have different modes of action for curing the disease and in combined form may sometimes exhibit synergistic activity(enhanced activity than that of individual herb) .Components of the plants which are less active themselves ,can act to improve the stability, solubility ,bio-availability or half –life of the active components. Hence a particular

principle 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 in using the plant as a whole or a mixture of plants for treating a disease [7].

The histopathological studies of the liver were direct means of studying the efficacy of the drug HP-4.Histological profile of control animals showed normal hepatocytes. Section of liver of the toxicant CCl_4 treated animals exhibited severe intense centrilobular necrosis, vacuolization and macrovesicular fatty changes. The Silymarin and HP-4 treated groups of animals showed significant liver protection against CCl_4 induced liver damage as evident by the presence of normal hepatic cords, absence of necrosis and fatty infiltration.

CONCLUSION

Hence HP-4 is a polyherbal formulation of *Aloe vera*, *Bacopa monnieri*, *Moringa oleifera* and *Zingiber officinale* the phytochemicals present in the mixture alone and their combination in a synergistic way are exhibiting their hepatoprotective action as evidenced by biochemical and histopathological studies in carbon tetrachloride induced hepatotoxicity model in mice.

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