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Original Research

GESDAV

Preliminary evaluation of hepatoprotective potential of the polyherbal formulation

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ABSTRACT

Aim: The aim of this study was to investigate the antioxidant and hepatoprotective effects of the polyherbal formulation (PHF)containing *Cajanus cajan* (L.)Millsp., *Lawsonia inermis* L. Linn, *Mimosa pudica* L., *Uraria picta* (Jacq.)DC. and *Operculina turpethum* (L.)Silva Manso on carbon tetrachloride (CCl_4)induced acute liver damage in albino rats. **Materials and Methods:** The groups of animals were administered with PHF at the doses 100, 200 and 400 mg/kg b.w. (per oral [p.o.])once in a day for 7 days and at day 6th and 7th the animals were administrated with Carbon tetrachloride (1.0 mL/kg b.w. 50% v/v with olive oil,; p.o.). The effect of PHF on serum glutamine pyruvate transaminase (SGPT), serum glutamine oxaloacetate transaminase, alkaline phosphatase (ALP)and total bilirubin were determined in CCl_4 - induced hepatotoxicity in rats. Further, the effects of PHF on glutathione (GSH), superoxide dismutase (SOD)level and lipid peroxidation (LPO)activity were also investigated. **Results:** The results demonstrated that PHF (400 mg/kg b.w.)significantly reduces the CCl_4 induced increase in level of serum SGPT, serum ALP and total bilirubin. PHF (400 mg/kg b.w.)prevents the depletion level of GSH and decrease in the activity of SOD in CCl_4 -induced liver injury in rats. In addition, PHF also showed a significant decrease in the LPO levels signifying the potent antioxidant activity. **Conclusion:** All our findings suggest that PHF could protect the liver cells from CCl_4 - induced liver damages and the mechanism may be through the anti-oxidative effect of PHF.

KEY WORDS: Antioxidant, carbon tetrachloride, hepatoprotective, a polyherbal formulation

INTRODUCTION

Liver regulates the various physiological processes of human body and plays a magnificent role in the metabolism of endogenous and exogenous agents. It has great capacity to detoxify toxic substances and synthesize useful metabolites. Indiscriminate use of certain therapeutic drugs such as paracetamol, anti-malarial drugs, anti-tubercular drugs, oral contraceptives, analgesics, antidepressants, anti-arrhythmic drugs and toxic substance (carbon tetrachloride [CCl_4], thioacetamide, aflatoxin)etc. are threatening the integrity of the liver. Uncontrolled consumption of alcohol, various infections and some autoimmune disorder are also facilitating hepatic damage [1]. These hepatotoxic agents are one of the leading causes for hepatitis, cirrhosis, liver cancer and at last death [2]. Overproduction of the reactive oxygen species (ROS)due to unnecessary exposure of toxic chemicals and depletion of antioxidant defense mechanisms attribute toward oxidative stress and culminating into severe hepatic injury [3].

A variety of medicinal plants are used for the treatment of the liver diseases in various systems of medicine [4-8]. Plants contain many phyto-constituents, but sometimes the individual phyto-constituent may not be enough to achieve the desired therapeutic effect. Therefore, the polyherbal formulation (PHF) was prepared in order to enhance the therapeutic effectiveness and improve the bioavailability. Besides synergism attenuates, reduction in undesirable side-effects is a key benefit of the formulation.

Operculina turpethum (L.)Silva Manso is a stout perennial climber which is reported to posses hepatoprotective, anticancer, antioxidant, antisecretory, ulcer protective, and antimicrobial activity [9].

Mimosa pudica L. is reported to possess wound healing, antimicrobial, analgesic, anti-inflammatory, anticonvulsant, antidiarrhoeal, antifertility, anti oxidant, an anti-hepatotoxic and anthelminthic activity [10].

Lawsonia inermis L. is much branched, deciduous, glabrous, sometime spinescent shrub. It possess hypoglycemic, nootropic, antimicrobial, antioxidant, cytotoxic and immunomodulatory activity [11].

Uraria picta (Jacq.)DC. is reported to have acaricidal [12]and anti-inflammatory [13]activity.

Cajanus cajan (L.)Millsp posses antimicrobial, hepatoprotective, hypocholesterolemic and analgesic activity [14].

In the present study, a PHF consisting *C. cajan* (L.)Millsp., *L. inermis* L., *M. pudica* L., *U. picta* (Jacq.)DC. *and O. turpethum* (L.)Silva Manso has been formulated and evaluated for hepatoprotective activity against CCl_4 induced liver toxicity in rats. All the plants of the formulation are taken from the Ayurvedic classical text "Chakradatta," where these are mentioned as ingredient of Ayurvedic formulation "Brihad Panchgavya Ghrit" indicated for jaundice [15].

MATERIALS AND METHODS

Materials

The plants materials *C. cajan* (Whole plant, 1 part), *O. turpenthum* (Root, 2 part), *M. pudica* (Root, 1 part), *U. picta* (Root, 1 part)and *L. inermis* (Leaves, ½ part)were collected from the Rajiv Gandhi South Campus, Banaras Hindu University, Barkachha, Mirzapur, Uttar Pradesh. The botanical authentication of the specimens was done by Dr. Anil Kumar Singh, Professor, Department of Dravyaguna, Faculty of Ayurveda, Institute of Medical Science, Banaras Hindu University; Varanasi, India. For further reference the voucher specimens (APRL/HERB/12-13/112-116)of plant materials were deposited in Rajiv Gandhi South Campus, Banaras Hindu University, Barkachha, Mirzapur, Uttar Pradesh, India.

Preparation of Formulation

The shade dried plant material was coarsely powdered separately by using a mechanical grinder then mixed in a definite proportion. Extraction was performed with 98% methanol by cold maceration technique. After that extract was concentrated under vacuum evaporator and the dried extract was obtained. The dry polyherbal extract was suspended in 5% carboxymethyl cellulose solution before oral administration to animals.

Drug and Chemical

CCl_4 (Central drug house, New Delhi), Pentobarbitone (Ranbaxy India Pvt. Ltd., Mumbai, Maharastra, India), silymarin (Ranbaxy India Pvt. Ltd., Mumbai, Maharastra, India), NADH (Sisco Research Laboratories Pvt. Ltd., Mumbai, Maharastra, India)and NBT (Sisco Research Laboratories Pvt. Ltd., Mumbai, Maharastra, India)were used for the experimental purpose. All the reagents used were of analytical grade. Biochemical estimation kits (Span Diagnostic Surat, Gujarat, India)were used for serum glutamine pyruvate transaminase (SGPT), serum glutamine oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), total bilirubin estimation.

Preliminary Phytochemical Analysis (Qualitative Analysis)

PHF was subjected to preliminary phytochemical screening for the detection of the presence of various phytoconstituents like alkaloid, tannin, saponin, phenolics etc [16].

Hepatoprotective Activity of PHF

Animals

Adult Charles foster albino rats (140 ± 20 g)of either sex were procured from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University (Registration No-542/AB/CPCSEA); Varanasi. The animals were kept in a temperature-controlled room (22 ± 2°C)with humidity (55 ± 10%)and 12 h light and 12 h dark cycle. The animals were provided with standard pelleted feed (Amrit Pvt., Ltd., Pune, Maharastra, India)and fresh water *ad libitum*. Rats were kept at standard laboratory environment for at least 1 week before the experiment. The study has been approved by the Institutional Animal Ethical Committee (Dean/13-14/CAEC/318).

Acute toxicity study

The acute oral toxicity study was performed as per the OECD guideline 425 (2008)[17]. The 18 h fasted rats were divided into 3 groups, each group consisting of 3 animals. The PHF was given in various doses (1000, 2000 and 4000 mg/kg of b.w.)per oral (p.o.). The signs and symptoms of toxicity or any abnormalities coupled with administration of PHF were observed at every 30 min for the first 2 h, then at every one hour for the next two hours. Observation was continued for next 14 days, once a day. Any behavioral changes such as convulsion, salivation, dizziness, diarrhea, sleep and coma were noticed. Any death within 14 days was also noted.

CCl_4 induced liver toxicity

Overnight fasted thirty-six adult rats were divided into six groups of six animals in each group. Group I and II received normal saline for seven days. Group III was treated with standard drug silymarin (100 mg/kg b.w.), Group IV, V and VI were treated with PHF 100, 200 and 400 mg/kg b.w. respectively for seven days. All the animals except the group I were treated with CCl_4 (1.0 mL/kg b.w. 50% v/v with olive oil,; p.o.)on 6th and 7^{th} day [18].

Estimation of biochemical markers

After 24 h of the last administered dose, the rats were anesthetized using ether and sacrificed by cervical dislocation. Blood sample was collected by cardiac puncture method in a heparinized 1.0 mL tuberculin syringe and serum was separated for the estimation of biochemical parameters. The biochemical parameters were properly estimated with standard procedure by the diagnostics kits [19].

Histopathological examination

From sacrificed animals, liver was taken out and cut into small pieces and separated and weighed accurately. The liver was washed with normal saline and was preserved in formalin solution (10%). After fixation, processing of livers was done with routine histopathologic procedure and embedded in paraffin wax. Several 4-5 μm sections of each liver blocks were stained with hematoxylin and eosin. The sections were examined under high-resolution microscope [18].

Assay of antioxidant activity

The level of lipid peroxidation (LPO)were estimated and expressed in terms of malondialdehyde (MDA)as per the method of Ohkawa *et al*. 1979 [20]The activity of superoxide dismutase (SOD)were estimated as per procedure of Kakkar *et al.* (1984)[21]based on reduction of NBT to blue colored formazan in presence of phenazine methasulfate. The levels of glutathione (GSH)were expressed as μmol/g of wet tissue after estimation as per method of Sedlak and Lindsay, 1968 [22].

Pentobarbitone induces seep test

The animals were divided into five groups with 6 animals in each group. All groups were given pentobarbitone (45 mg/kg b.w., i.p.) after 2 h of administration of CCl_4 (1.0 mL/kg b.w. 50% v/v with olive oil; p.o.). Group II was treated with standard drug silymarin (100 mg/kg body weight p.o.). Group III, IV and V receive PHF 100, 200 and 400 mg/kg b.w.; p.o. respectively. The latency and duration of the sleeping time was noted in the each group [23].

Stastical Analysis

All results are expressed as mean ± standard error of the mean (n = 6 in each group). Statistical comparison was done by one-way ANOVA, followed by the Tukey's multiple comparison tests using Graph Pad Prism Software Version 5.01 (Fay Avenue, La Jolla, CA, USA).

RESULTS

Preliminary Phytochemical Analysis

Preliminary phytochemical screening shows the presence of flavonoids, tannins, saponins, steroids in the methanol extract [Table 1].

Table 1: Preliminary phytochemical investigation

Chemical test	Methanol extract
Test for alkaloids	−
Test for carbohydrates	−
Test for flavonoids	+
Test for tannins (phenolic compound)	+
Test for saponins	+
Test for steroids	+
Test for protein	−

+: Present, −: Absent

Acute Toxicity Study

No mortality or any behavioral changes were observed at all the doses 1000, 2000 mg/kg b.w. and 4000 mg/kg b.w. in the acute toxicity study. Hence, the PHF was considered to be safe up to 4000 mg/kg b.w. Therefore, doses of 100, 200 and 400 mg/kg b.w. were selected for the pharmacological studies.

Effect of PHF on Serum Biochemical Levels

The animals treated with CCl_4 (1.0 mL/kg)showed significant ($P < 0.05$)increase in the level of serum enzymes SGPT, SGOT and ALP when compared to the normal control group. The animals treated with silymarin as well as PHF 400 mg/kg showed significant ($P < 0.05$)reduction in the level of SGPT, SGOT and ALP when compared to the CCl_4 treated group. PHF 400mg/kg showed significant ($P < 0.05$)reduction level of serum enzymes SGPT, SGOT and ALP when compared to the PHF 100 and PHF 200mg/kg [Figure 1a-c].

The animals treated with CCl_4 showed significant ($P < 0.05$) increase in the level of total bilirubin and direct bilirubin as compared to the normal control group. The animals treated with silymarin, as well as PHF 400 mg/kg, showed significant ($P < 0.05$)reduction in the level of total bilirubin and direct bilirubin as compared to the CCl_4 treated group. PHF in the dosages of 100 and 200 mg/kg p.o. showed insignificant reduction in the level of total bilirubin and direct bilirubin as compared to animal treated with silymarin. PHF in the dosage level of 400 mg/kg showed significant ($P < 0.05$)reduction level of total bilirubin and direct bilirubin as compared to the PHF 100 and PHF 200 mg/kg [Figure 1d and e].

In vivo Assay of Antioxidant Activity

Effect of PHF on LPO level

The animal treated with CCl_4 showed significant ($P < 0.05$) increase level of LPO as compared to the normal control group. Silymarin treated group, PHF 200 and PHF 400 mg/kg showed significant ($P < 0.05$)reduction in the level of LPO as compared to the CCl_4 treated group. At the dosage level of PHF 100 mg/kg, it showed insignificant ($P < 0.05$)reduction in the level of LPO as compared to the silymarin treated group [Figure 2a].

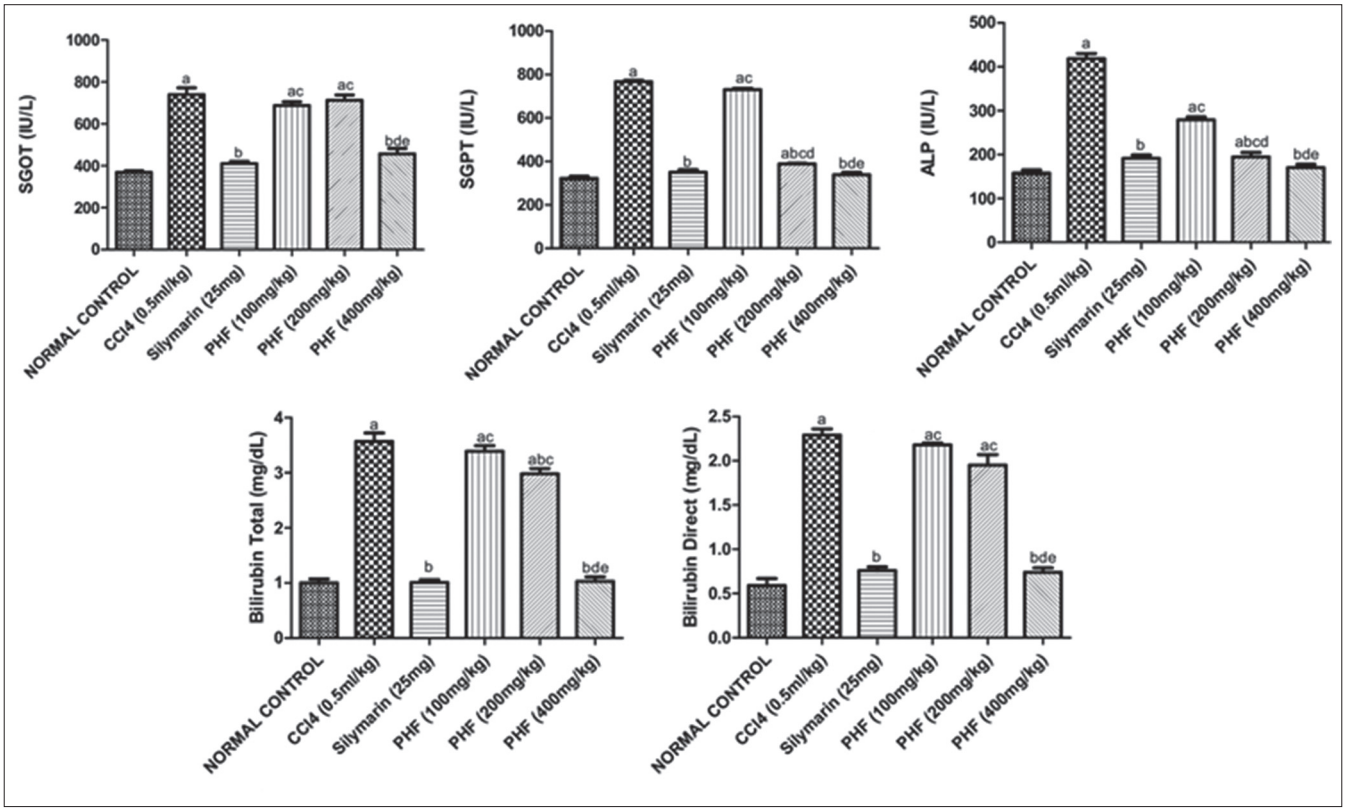

Figure 1: Effect of polyherbal formulation on biochemical parameters in carbon tetrachloride induced hepatic injury in rats

Effect of PHF on SOD level

The animal treated with the CCl_4 showed significant ($P < 0.05$) reduction in activity of SOD as compared to the normal control group. The standard drug silymarin and animals treated with test group PHF 100, PHF 200, PHF 400 mg/kg, p.o. showed significant ($P < 0.05$)increase in activity of SOD as compared to the animal treated with CCl_4 [Figure 2b].

Effect of PHF on GSH level

The animal treated with the CCl_4 showed significant ($P < 0.05$)decrease in the level of GSH as compared to the normal control group. The standard drug silymarin, as well as PHF, showed significantly ($P < 0.05$)increase in the level of GSH as compared to the CCl_4 treated group [Figure 2c].

Histopathological Result

Figures 3a and b shows the liver cells of rat in the normal control group. Liver cells were normal in shape and size with prominent nucleus. Cordlike arrangement of liver cells which was separated by sinusoids and central vein (CV)is clearly visible.

CCl_4 - treated animal's liver histopathology [Figure 3c and d] shows that the structural design of liver was totally damaged as compared to normal rats. Prominent cell vacuolation, pyknotic and degenerated nuclei along with damaged bile capillaries were observed. Cell lysis and aggregation of nuclei was also visible at some places. Wide spaces were formed at some sinusoids. These cellular changes were greatly reduced in CCl_4 with silymarin treated groups. A healthy population of hepatocytes interspersed with patches of mild necrosis was observed [Figure 3e and f].

In the liver cells of rats treated with PHF 400 and intoxicated with CCl_4 [Figure 3k and l], the nucleus were not very clear as in normal hepatocytes, but as compared to the CCl_4 treated group the number of hepatocytes with normal nucleus were much more. Cell vacuolation and pyknotic nuclei were observed to be low. Endothelium was disrupted at places and hepatic cells adjoining to intralobular vein shows atrophy. The overall architecture of the liver appears to be normal. The liver of PHF 100 [Figure 3g and h]and PHF 200 [Figure 3i and j]treated rats showed insignificant reversal of pathological alterations done by CCl_4.

Effect of PHF on Pentobarbitone Induced Sleeping Time

The animals treated with CCl_4 and pentobarbitone (45 mg/kg i.p.)showed a significant increase in sleeping time as compared to animals treated with only pentobarbitone. The animals treated with standard drug silymarin and test drugs PHF 200, 400 mg/kg showed significant ($P < 0.05$)reduction in the duration of sleep as compared to the CCl_4 treated group [Figure 4].

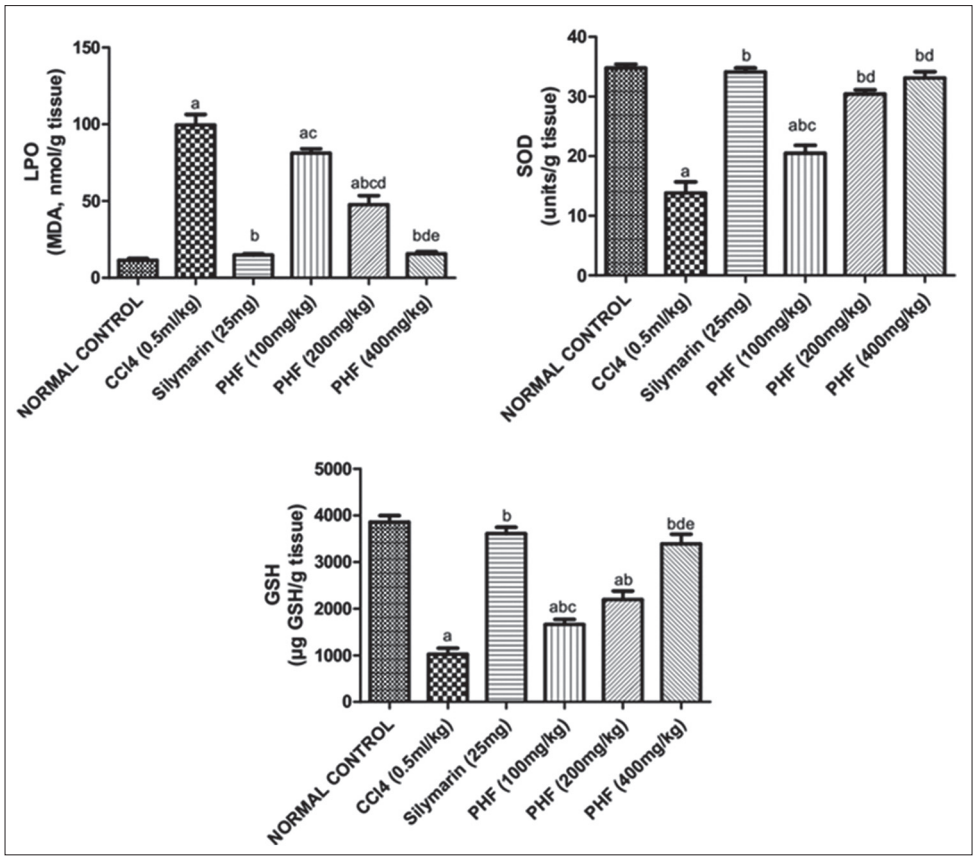

Figure 2: Effect of polyherbal formulation graded dose on the levels of lipid peroxidation, superoxide dismutase and glutathione in carbon tetrachloride induced hepatic injury in rats

DISCUSSION

In today's world liver diseases become a global health problem, lacking helpful curative approach. There are so many plants that are used as a hepatoprotective agent in traditional medicine systems [2,18,19,23]. It is, therefore, necessary to assess the scientific basis for the reported hepatoprotective activity of herbal drugs in the form of PHF. In this connection, a polyherbal formulation was prepared and evaluated against CCl_4 induced liver toxicity in rats.

It has been established that CCl_4 is metabolically activated by cytochrome P_{450} - dependent mono-oxygenases to form highly reactive free radical metabolites, tri-chloro-methyl free radical ($CCl_3^{\bullet}$)which later convert into more toxic tri-chloro-methyl-peroxyl radical (CCl_3OO^-)in presence of oxygen. The same is capable to produce disturbance in the transport function of the hepatocytes which leads to leakage of enzymes (SGOT and SGPT)from cells, hyper bilirubinaemia as well as rise in the level of serum ALP [24]. The result of the present study reveals that the pre-treatment with PHF antagonizes elevated enzyme parameters. The tendency of these enzymes to return towards the normal range in the PHF administered group was clearly indicating that the PHF 400 mg/kg challenge to protect liver tissue from CCl_4 injury. It was reported and accepted that serum levels of SGOT and SGPT return to normal with the healing of liver parenchyma and the regeneration of hepatocytes [25].

In the agreement with other reports, CCl_4 treated rat's recorded significant decrease in hepatic non enzymatic (GSH) and enzymatic (SOD)antioxidant markers [18]. Therefore, reduction in free radicals generation is a possible mechanism in the defense of the liver against different injuries. In this study dramatic increase in LPO after CCl_4 treatment suggested that natural antioxidant defense mechanism to scavenge excessive free radicals has been compromised [26]. Prophylactic treatment with PHF significantly inhibited the formation of MDA in the liver. These findings are in accordance with previous reports for other hepatoprotective agents [19].

In the present study, the PHF (400 mg/kg)showed a potential *in vivo* antioxidant activity as it elevates the reduced levels of liver cytosolic SOD and GSH. These antioxidant enzymes are involved in the reduction of ROS and peroxidase produced in the living organism thus play a vital role in the maintenance of a balance redox status. The restoration of the SOD towards a normal value indicates that the PHF can help in cellular defense mechanisms by preventing cell membrane oxidation. The PHF also restores some vital molecules such as NAD, Cytochrome, and GSH as indicated by increased peroxidase activity [27].

Although barbiturate sleeping time is not a direct measure of hepatic injury but it shows the status of the liver. It has been established that the phenobarbitone induced sleeping time is

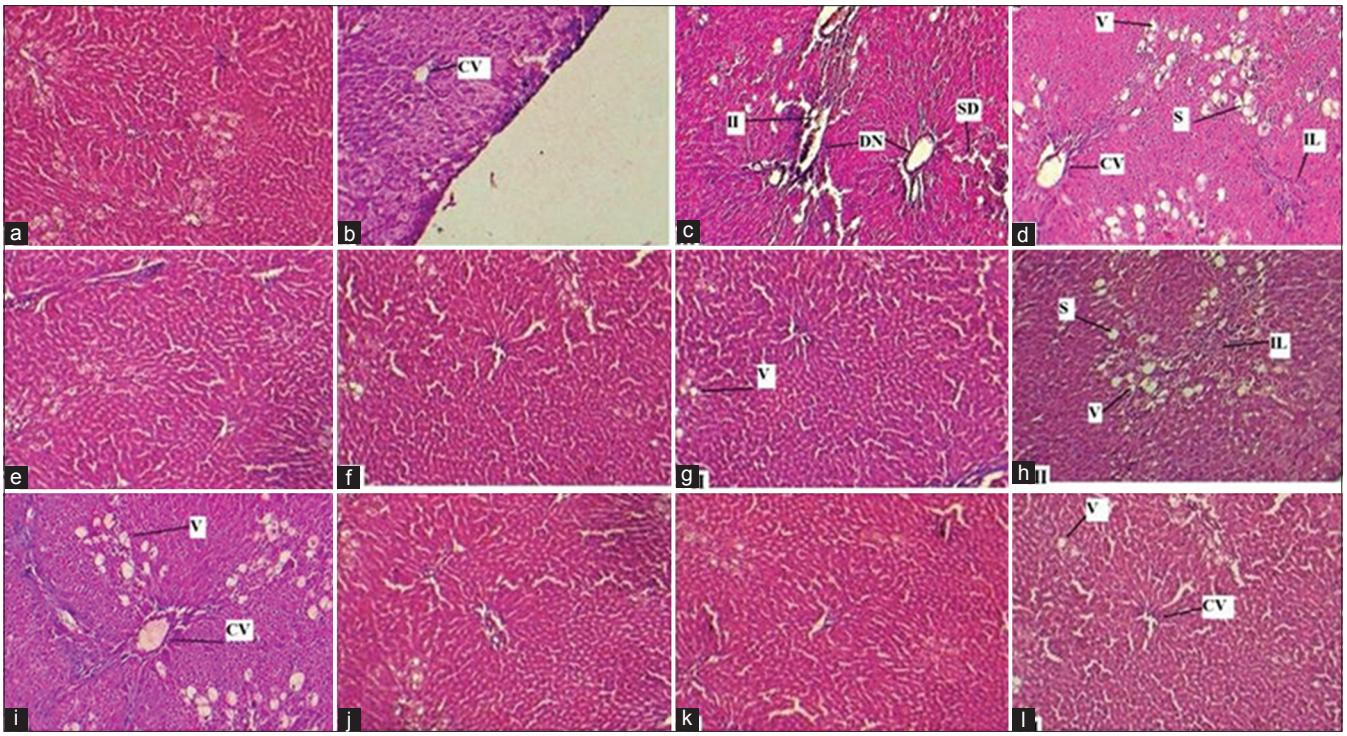

Figure 3: Photomicrographs of liver sections from: (a and b) Normal control group; (c and d) Carbon tetrachloride (CCl_4) (1:1 in olive oil) 1 mL/kg i.p., (e and f) silymarin (100 mg/kg) + CCl_4, (g and h) polyherbal formulation (PHF) 100 mg/kg + CCl_4, (i and j) PHF 200 mg/kg + CCl_4, (k and l) PHF 400 mg/kg + CCl_4, CV: Central vein, SD: Sinusoidal dilatation, V: Vacuolation, S: Steatosis, IL: Infiltration of lymphocytes, DN: Degenerated nuclei, II: Inflammatory infiltration

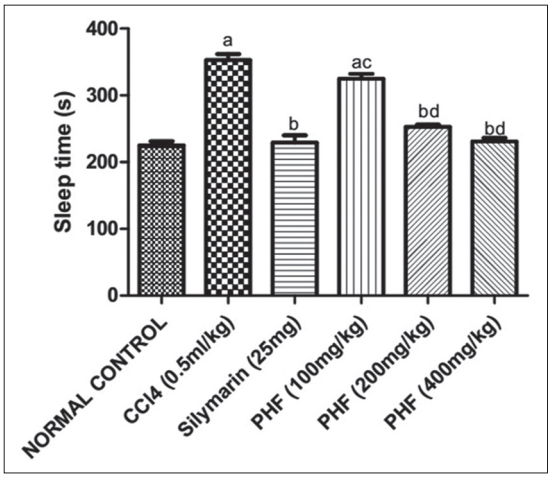

Figure 4: Effect of polyherbal formulation graded dose on the duration of barbiturate induced sleep in carbon tetrachloride induced hepatic injury in rats

a measure of hepatic metabolism since the barbiturates are metabolized primarily in the liver [28]. If there is any existing liver damage by CCl_4 intoxication, the amount of the hypnotic metabolized per unit time will be less, which results in prolonged sleeping time after the given dose of barbiturates [23]. In this study, there is a considerable reduction and restoration of Pentobarbitone sodium-induced sleeping time after PHF administration i.e. PHF was effective in regulating the level of microsomal drug metabolizing enzymes in the liver. This outcome supported the use of this formulation in the chronic hepatic diseases.

The experiential hepatoprotective activity of the PHF may be on account of the presence of polyphenolic compounds found in the preliminary phytochemical screening [Table 1] as these compounds are reported to have the free radical scavenging ability, which stabilizes lipid oxidation. Flavonoids correspond to a group of polyphenolic compounds, which exhibits a wide range of biological activities primarily due to their antioxidant property, probably due to their free radical scavenging activity and ability to reduce free radical formation [29]. Some studies suggest a correlation between phenolic content and hepatoprotective activities [30]. Saponins present in the PHF are also effective against hepatotoxins [25]. Report also indicates that some steroids may be responsible for hepatoprotective effect [31].

The biochemical findings were also confirmed by histopathological observation. Histopathological studies under light microscope confirm the curative efficacy of PHF against CCl_4 induced liver damage. Vacuolated hepatocytes along with fatty deposition, necrosis and degenerative changes were observed in CCl_4 treated rats. In this experimental group infiltration of inflammatory cells in the CV was also evident. This could be due to the formation of highly reactive free radicals because of oxidative stress caused by CCl_4. These set of changes have also been reported by other researchers following CCl_4 treatment [32].

This severity of toxic effects was much less when seen in sections treated with PHF and was comparable to silymarin. Simultaneous treatment of PHF with CCl_4 exhibits less damages to the hepatic cells as compared to the rats treated with CCl_4 alone. Intralobular veins were found to damaged but to a lesser extent. Endothelium is disrupted at places. Hepatic cells are adjoining to Intralobular vein show atrophy. Pyknotic nucleus and cell vacuolation are observed to be low. The correlation between liver biomarkers and histopathological changes suggested that they could be used for early detection of acute liver damage. Reduction of biochemical and histological damages exerted by PHF confirms its hepatoprotective potential.

Results indicated improvement in metabolic activity and cellular stability. On the whole hepatoprotective effect of PHF is most likely due to antioxidant action. This data provide a primary base for the use of these five plants in the form of an herbal preparation for the treatments of hepatic disorders.

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