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Potential of Selected Underutilized Leafy Vegetables Against CCl4 Induced Oxidative Stress on Wistar Rats

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Abstract

Nutritional immunology might become even more important in prevention of disease. Underutilized leafy vegetables are the richest source of many bioactive compounds especially phenolic compounds with excellent antioxidant properties. Increased consumption of diets containing leafy vegetables may give positive results to human health. The underutilized leafy vegetables which includes **Indian pennywort, Sessile joyweed, Red amaranth, Red spinach, Asiatic day flower, Indian sorrel, Roselle leaves** are quantified for its phenols, flavonoids and vitamins contents by HPLC-LC MS method. The phenols, flavonoids and vitamins ranged from 0.186 – 12014 µg/g, 0.018 – 785.25 µg/g and 0.361 – 18466.56 ng/g in the underutilized leafy vegetables. Based on the bioactive compounds present, herbal preparations are developed and evaluated for hepatoprotective property against CCL4 induced intoxication in wistar rat model. Biochemical estimation in blood serum (AST/SGOT, ALT/SGPT, ALP, Total bilirubin) and antioxidant enzyme estimation in liver homogenate (SOD, GOD, LPO) showed positive results to reduce hepatotoxicity in CCL4 intoxicated wistar rat model. This exploration can helps in addressing importance of bioactive components present in underutilized leafy vegetables in combating various degenerative diseases and also proved the nutraceutical potential of leafy vegetables. Further which can be used for development of functional foods with the present pre-clinical data.

Keywords: Underutilized leafy vegetables, Nutraceutical potential, Hepatoprotective

Introduction

The field of nutritional immunology is one that is expanding quickly. The role of nutritional immunology in disease prevention may increase. Non-conventional greens are very rich source of photochemical which helps in boosting the immune system. The SARS and MERS outburst, we have gained knowledge about the mechanisms of bioactive plant ingredients against the attachment and replication of COVID-19 as well as overshooting immune responses. Using bioactive chemicals, this might be used to the design of COVID-19 studies. Several functional foods interact with the epigenetic regulation of viral infection and mechanisms of senescence. This study also concentrates on the link between bioactive plant ingredients that improve immune system. The usage of herbal medicines in

improving human health is gaining popularity over time, which surge the need for proper utilization of these products. According to the report by WHO International Expert Meeting reviews, analyzes clinical reports on combination treatments for SARS which combine both Traditional and Western medicine. It was summarized not only mechanisms of infections and case studies, but also various combinations of therapy. Nutraceuticals and functional foods have wide potential for preventing the mechanisms of viral infection and altering immune responses (Haselberger *et al*., 2020).

One of the body's most vital organs is the liver. It performs a fundamental play in the regulation of diverse physiological processes and its activity is related to different vital functions. Additionally, the liver participates in the biochemical processes of development, nutrition delivery, energy production and reproduction. It also facilitates the secretion of bile, the metabolism of fats and carbohydrates and the storage of vitamins. Damage to the cells, tissues, structure or function of the liver is referred to as hepatic disease. This damage can be brought on by biological agents (bacteria, viruses and parasites) autoimmune conditions (immune hepatitis, primary biliary cirrhosis) as well as by the action of various chemicals and excessive alcohol consumption. Studies are available that have looked into the effects that various phytochemicals have on health that may be found in the literature. Although there have been a variety of studies focused on assessing their hepatoprotective potential, the majority of research has focused on examining their sedative, analgesic, antipyretic, cardioprotective, antibacterial, antiviral, antiprotozoal and anticarcinogenic properties. Along with this, there is a long history of empirical evidence supporting the use of natural treatments for the treatment of hepatic diseases. This area of study has developed into a cutting-edge one with the main aim of analysing the composition of conventional orunderutilised foods as also the various phytochemicals are extracted from these foods. In general, liver- protective fruits and plants, contain a variety of chemical compounds, such as phenols, coumarins, lignans, monoterpenes, glycosides, essential oils, alkaloids, carotenoids, flavonoids, organic acids and xanthenes (Santillan *et al.,* 2014).

The recent evidences lend more weight on the role that vegetables and medicinal plants in preventing diseases such as hypertension, cancer and heart related diseases. Phyto-chemicals present have always been an essential source of medicines for human health problems. The WHO has recommended for evaluation of the plant’s effectiveness in condition where we lack safe modern drugs. This has lead to increasing demand for research on natural products which with minimum or no side effects. Because of many phyto-chemical compounds present in green leafy vegetables, they play an essential role in evolving potent therapeutic agents.

Recent studies based on clinical studies have clearly specified the importance of vegetables, fruits, medicinal plants and active constituents or their extracts in the combating various diseases. There are limited studies available related to the polyherbal preparation for the management of hepatic disease. By considering the efficacy of herbs, the research on developing polyherbal formulation from natural plants of locally available herbs appears to be attractive and pioneering.

The polyherbal preparation emerging from the research work shall provide medicinal health benefit at a reduced price and with no side effect than synthetic or conventional medicine. In this background, the present research was undertaken to focus mainly developing and evaluating polyherbal preparation from selected leafy vegetables against CCL_4 induced hepatotoxicity.

Materials and methods

Development of herbal preparations

Herbal powders of all the treatments were mixed in different proportions based on phenols and flavonoids profiling by HPLC for development of herbal preparations

List of ingredients used in herbal preparations (HP)

Treatment	T1	T2	T3	T4	T5	T6	T7	TOTAL(Grams)
HP1	0	5	10	15	20	25	25	100 g
HP2	25	0	5	10	15	20	25	100 g
HP3	25	25	0	5	10	15	20	100 g
HP4	20	25	25	0	5	10	15	100 g
HP5	15	20	25	25	0	5	10	100 g
HP6	10	15	20	25	25	0	5	100 g
HP7	5	10	15	20	25	25	0	100 g

T1:Centella asiatica L.; T2: Alternanthera sessilis L.; T3: Amaranthus cruentus L.; T4: Celosia argentea L.; T5: Commelina communis L., T6: Hibiscus cannabinus; T7: Oxalis carniculata L.

The details of treatments are as follows

Treatments 10
Replication 06
Statistical Design : Dunnet's test
Sample size : 42 Female wistar ratsAnimal body weight : 170 – 200 g

Acute oral toxicity study in mice

The acute oral toxicity study was carried out in female mice by the 'fix dose' method according to OECD (Organization for Economic Co-operation and Development) guideline number 425. The fixed-dose method, test procedure with a starting dose of 2000 mg/kg body weight, was adopted. The mice fasted overnight and the next day the herbal preparation was administered orally at a dose level of 2000 mg/kg body weight. Then the mice were observed continuously for 3 hours for general behavioral and finally for mortality after 24 hours till 14 day. No mortality was observed during 14 days. The test sample was found to be safe up to a dose of 2000 mg/kg. From the results obtained, 400 mg/kg was chosen for further experimentation as the maximum dose to be administered.

Experimental animals

Albino wistar rats weighing 170-200 g of female rats were used. Animals were obtained from the Central Animal House of Hanagal Shri Kumareshwar College of Pharmacy and Research Centre, Bagalkote. The animals were housed under standard conditions at room temperature with 12 h light/dark cycle and fed with standard laboratory animal feed (pellet diet) and provided with clean drinking water. The experimental protocols were approved by Institutional Ethical Committee.

Induction of experimental hepatotoxicity by carbon tetrachloride (CCl4) in wistar rats and oral

administration of herbal preparations

The animals were divided into ten groups of six animals each. The rats of group I served as normal, received normal saline 10 ml/kg b.wt; group II served as control (CCl4- treated). Rats of group III served as standard, received 200 mg/kg body weight of Silymarine and group IV, V and VI are received 400 mg/kg b.wt of herbal preparation for a period of 19 days by oral administration. On days 15th, 17th and 19th the rats of group II, III, IV, V, VI received 1 ml/kg body weight of carbon tetrachloride in liquid paraffin (1:1) orally with the respective assigned treatments. After 24hrs the last dose of CCl_4 administration, all the groups of rats were sacrificed. Liver tissue homogenates of all experimental animals were collected and estimated for enzyme assay.

Treatment details for evaluation of hepatoprotective property of herbal preparations in CCl4 induced wistar rats

up	Treatment details	Si Single-dose (mg/kg body weight)	No. of rats	Duration
Group 1	Normal group	-	6	21 days
Group 2	Control (CCl_4 - treated)	-	6	21 days
Group 3	CCl_4 + Silymarine	200 mg/kg	6	21 days
Group 4	HP1	400 mg/kg	6	21 days
Group 5	HP2	400 mg/kg	6	21 days
Group 6	HP3	400 mg/kg	6	21 days
Group 7	HP4	400 mg/kg	6	21 days
Group 8	HP5	400 mg/kg	6	21 days
Group 9	HP6	400 mg/kg	6	21 days
Group 10	HP7	400 mg/kg	6	21 days

Biochemical estimation in blood serum

Each animal's blood was drawn by retro-orbital plexus and centrifuged (3000 rpm for 10 min) to separate serum for the measurement of AST, ALT, total bilirubin, total protein, and HDL-cholesterol after the 24 hours of the last dose of CCl_4 with their respective assigned treatment.

Serum glutamic oxaloacetic transaminase (SGOT/AST)

The conversion of the amino group from L-aspartate to alpha-ketoglutarate is catalyzed by Aspartate Aminotransferase (AST), which results in the production of oxalacetate and L-glutamate. The indicator reaction in which malate dehydrogenase (MDH) is catalyzed involves the reduction of oxaloacetate and the conversion of NADH to NAD. As a result, the rate at which absorbance at 340 nm falls is proportional to the activity of the AST. Lactate dehydrogenase (LDH) is introduced to prevent interference from endogenous pyruvate, which is typically present in serum. The reagent's reaction mechanism increases the stability of the working reagent by regenerating NADH.

Serum glutamic pyruvic transaminase (SGPT/ALT)

The amino group is transferred from L-alanine to α-ketoglutarate by the presence of ALT in the sample, which results in the formation of L-glutamate and pyruvate. L-lactate is produced when pyruvate is reduced in the presence of NADH and lactate dehydrogenase (LDH). NADH is oxidized to NAD^+ during this reaction. The rate at which the absorbance at 340 nm decreases as a result of NADH being converted to NAD+ is used to track the reaction. The reagent has a mechanism for regenerating NADH in order to prolong the working reagent's stability.

Total bilirubin (mg/dL)

Method: Diazo method of Pearlman & Lee.

Total protein (g/dL)

Method: Modified Biuret, End Point Assay

HDL-Cholesterol (mg/dL)

Method: Phosphotungstic Acid Method, End point

Antioxidant enzyme estimation in liver homogenate

Following the collection of blood samples, the rats were slaughtered, and their livers removed. The liver was then perfused with ice-cold normal saline to eliminate any remaining blood constituents. Chloroform was used to sacrifice the animals. After the liver was taken out and cleaned in 0.9% ice-cold saline, it was fully perfused with cold normal saline to remove all traces of blood. It was then placed on ice, blotted on filter paper, weighed, and homogenized in cold phosphate buffer (0.1 M, pH 7.4). To determine the amount of total protein and lipid peroxidation, the homogenates were centrifuged with a post-mitochondrial supernatant (PMS; MPW-350R, Korea) at 10,000 rpm for 10 min at 4^o C. The supernatant was once more centrifuged at 17000 rpm for 1 hour at 4^o C. The resulting supernatant was utilised to estimate SOD, GSH, LPO, and total protein in greater detail.

Superoxide dismutase (SOD)

Superoxide dismutase activity was determined by SOD's ability to stop adrenaline from auto-oxidizing to adrenochrome at an alkaline pH. After adding 0.1 mM adrenaline in carbonate buffer (pH 10.2) to 25 µl of the liver homogenate supernatant from centrifugation, adrenochrome production was observed at 295 nm18. To calculate the SOD activity (U/mg of protein), the standard plot was utilized.

Glutathione (GSH)

To summarise, proteins were precipitated using 10% TCA, centrifuged, and 0.5 ml of the supernatant was combined with 0.006 mM DTNB and 0.2 M phosphate buffer (pH 8.0). After 10 minutes of incubation, the absorbance of this mixture was measured at 412 nm using the relevant blanks. The standard plot was used to calculate the glutathione content under the same experimental setup.

Lipid peroxidation (LPO)

As a result of lipid peroxidation, a variety of cytotoxic products are produced, the majority of which are aldehydes. Examples of these products include malondialdehyde and 4-hydroxynonenal. Peroxides and

hydroperoxides are also produced. Using a standard protocol, the amount of thiobarbituric acid reactive substance (TBARS) in the homogenate was determined. To put it briefly, 0.5 ml of 10% homogenate was incubated with 15% TCA, 0.375 percent TBA, and 5N HCl for 15 minutes at 95°C. After cooling and centrifuging the mixture, the absorbance of the supernatant was measured at 512 nm using the appropriate blank. The amount of lipid peroxidation was calculated and expressed as TBARS nmoles/mgof protein using ε = 1.56 x 105 M-1cm-1.

Catalase (U/mg ofprotein)

Catalase activity was assayed by the method of Claiborne. Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05M, pH 7.0), 1.0 ml H2O2 (0.019 M), and 0.05 ml homogenate (10% w/v) with total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of units/mg protein.

Histopathological preparations

To ensure adequate fixation, liver tissues were gathered and placed in 10% formalin. After processing, these tissues were embedded in paraffin wax. Hematoxylin and eosin dye was used to cut and stain sections with a thickness of 5–6 µ. The sections were then examined under a microscope to look for changes in histopathology.

Statistical analysis

A fully randomized block design analysis was performed on the data from experiments I, II, and III. Additionally, the values for experiment IV were reported as mean ± SEM. Every value was given as mean ± SEM. One Way Analysis of Variance (ANOVA) was used to statistically analyze the results, and multiple Dunnet't tests were then performed. A p-value of less than 0.05 was deemed significant. The control group's effects were contrasted with those of the various treated groups.

RESULTS

In-vivo hepatoprotective activity of herbal preparations at a dose of 400 mg/kg against CCl_4 induced hepatic damage in rats was summarized in Table No.1 and Table No. 2

Effect of herbal preparations on serum SGOT

A significant (P<001) increased levels of SGOT (Table 21) was observed in CCl_4 treated group G_2 (542.10 ± 7.51 U/L**)** as compared to normal group G_1 (130.70 ± 6.57 U/L). The groups treated with herbal preparations showed significant (P<001) reduction in SGOT level (G_5 - 151.6 ± 2.842 U/L , G_6 - 153.70 ± 4.0535 U/L, G_7 -155.80 ± 5.860 U/L, G_{10} - 160.1 ± 3.240 U/L, G_4 -163.60 ± 1.529 U/L , G_8 -
170.0 ± 10.11 U/L, G_9 -202.7 ± 7.122 U/L) at a dose of 400 mg/kg. Similarly, Standard Silymarine treated group showed significant (P<001) reduction in SGOT level (G_3 -134.90 ± 3.35 U/L) against control (G_2 - CCl_4 treated) group represented in Table 1. All herbal preparations decreased the serum SGOT level significantly but highest reduction is observed in G_5. The results revealed that induction of hepatotoxicity leads to increased SGOT level in rats. But after oral administration with silymarine and herpal preparations, a decrease in the serum SGOT level was noticed.

Effect of herbal preparations on serum SGPT

A significant (*P*<001) increased levels of SGPT was observed in CCl4 treated group G2 (145.90 ± 9.85 U/L**)** as compared to normal group G1 (67.40 ± 2.65 U/L). The groups treated with herbal preparations showed significant (*P*<001) reduction in SGPT level (G5 - 81.59 ± 3.486U/L , G6 -83.90 ± 3.873 U/L, G7
-89.03 ± 2.018 U/L, G10 - 93.43 ± 1.654 U/L, G4 -96.65 ± 5.229 U/L , G8 -101.00 ± 2.306 U/L, G9 -
106.70 ± 8.344U/L) at a dose of 400 mg/kg (Table 1). Similarly, Standard Silymarine treated group showed significant (*P*<001) reduction in SGPT level (G3 -58.70 ± 6.50 U/L) against control (G2 - CCl4 treated) group represented in Table 20. All herbal preparations decreased the serum SGPT level significantly but highest reduction is observed in G5. The results revealed that induction of hepatotoxicity leads to increased SGPT level in rats. But after oral administration with silymarine and herpal preparations, a decrease in the serum SGPT level was noticed.

Effect of herbal preparations on serum total-bilirubin

A significant (P<001) increased levels of total bilirubin was observed in CCl4 treated group G2 (0.88 ±
0.05 mg/dl) as compared to normal group G1 (0.29 ± 0.48 mg/dl). The groups treated with herbal preparations showed significant (P<001) reduction in total bilirubin level (G5 - 0.39 ± 0.01 mg/dl, G6 - 0.41 ± 0.06 mg/dl, G7 -0.44 ± 0.06 mg/dl, G10 - 0.49 ± 0.04 mg/dl, G4 -0.52 ± 0.04 mg/dl, G8 -0.53 ± 0.03mg/dl, G9 -0.62 ± 0.03 mg/dl) at a dose of 400 mg/kg (Table 1). Similarly, Standard Silymarine treated group showed significant (P<001) reduction in total bilirubin level (G3 -0.35 ± 0.04 mg/dl) against control (G2 - CCl4 treated) group represented in Table 20. All herbal preparations decreased the total bilirubin level significantly but highest reduction is observed in G5. The results revealed that induction of hepatotoxicity leads to increased total bilirubin level in rats. But after oral administration with silymarine and herpal preparations, a decrease in the total bilirubin level was noticed.

Table 1: Effect of oral administration of herbal preparations on serum biochemicalenzymes against CCl4 induced liver damage in Wistar rats

Animal groups	GROUP	SGOT (U/L)	SGPT (U/L)	Total-bilirubin (mg/dl)	Total protein (g/dl)	HDL-Cholesterol (mg/dl)
G1	Normal	130.70 ± 6.57	67.40 ± 2.65	0.29 ± 0.48	10.75 ± 0.69	6.75 ± 0.85
G2	Control (CCl_4-treated)	542.10 ± 7.51ª	145.90 ± 9.85ª	0.88 ± 0.05ª	5.64 ± 0.69ª	3.50 ± 0.64ª
G3	Silymarine (200mg/kg)	134.90 ± 3.35***	58.70 ± 6.50***	0.35 ± 0.04***	11.18 ± 0.38***	9.53 ± 0.27***
G4	CCl_4+HP1(400mg/kg)	163.60 ± 1.529***	96.65 ± 5.229***	0.52 ± 0.04***	16.22 ± 6.66***	10.12 ± 0.58***
G8	CCl_4+HP2(400mg/kg)	151.6 ± 2.842***	81.59 ± 3.486***	0.39 ± 0.01***	21.34 ± 8.46***	11.71 ± 1.09***
G6	CCl_4+HP3(400mg/kg)	153.70 ± 4.0535***	83.90 ± 3.873***	0.41 ± 0.06***	18.86 ± 6.56***	11.33 ± 0.93***
G7	CCl_4+HP4(400mg/kg)	155.80 ± 5.860***	89.03 ± 2.018***	0.44 ± 0.06***	17.43 ± 7.21***	10.71 ± 0.42***
G8	CCl_4+HP5(400mg/kg)	170.0 ± 10.11***	101.00 ± 2.306***	0.53 ± 0.03***	15.52 ± 5.04***	9.71 ± 0.39***
G9	CCl_4+HP6(400mg/kg)	202.7 ± 7.122***	106.70 ± 8.344***	0.62 ± 0.03***	15.12 ± 5.475***	9.40 ± 1.16***
G10	CCl_4+HP7(400mg/kg)	160.1 ± 3.240***	93.43 ± 1.654***	0.49 ± 0.04***	17.13 ± 6.16***	10.90 ± 0.59***

All values were expressed as a Mean ± SEM, n=6, One-way Analysis of Variance (ANOVA) followed by multiple Dunnet's- test. The value significant $^{c}P<0.05$, $^{b}P<0.01$, $^{a}P<0.001$ as compared to normal group and $*P<0.05$, $**P<0.01$,$***P<0.001$ as compared to control group. (HP: Herbal preparations)

Effect of herbal preparations on serum total protein

A significant ($P<001$) decreased levels of Total protein was observed in CCl_4 treated group G_2 (5.64 ± 0.69 g/dl) as compared to normal group G_1 (10.75 ± 0.69 g/dl). The groups treated with herbal preparations showed significant ($P<001$) elevated level of total protein (G_5 - 21.34 ± 8.46 g/dl, G_6 - 18.86 ± 6.56 g//dl, G_7 -17.43 ± 7.21g/dl, G_{10} - 17.13 ± 6.16 g/dl, G_4 -16.22 ± 6.66 g/dl, G_8 -15.52 ± 5.04 g/dl, G_9 -15.12 ± 5.475 g/dl) at dose of 400 mg/kg. Similarly, standard Silymarine treated animals showed significant ($P<001$) increase levels of total protein (G_3 -11.18 ± 0.38 g/dl) against CCl_4 induced hepatotoxicity, as compared to control (G_2 - CCl_4 treated) which is represented in Table 1.

Effect of herbal preparations on serum HDL-Cholesterol

A significant ($P<001$) decreased levels of HDL-Cholesterol was observed in CCl_4 treated group G_2 (3.50 ± 0.64 mg/dl) as compared to normal group G_1 (6.75 ± 0.85 mg/dl). The groups treated with herbal preparations showed significant ($P<001$) elevated level of HDL Cholesterol (G_5 - 11.71 ± 1.09 mg/dl, G_6 -11.33 ± 0.93 mg//dl, G_7 -10.71 ± 0.42 mg/dl, G_{10} - 10.90 ± 0.59 mg/dl, G_4 -10.12 ± 0.58 mg/dl, G_8 -9.71 ± 0.39 mg/dl, G_9 -9.40 ± 1.16 mg/dl) at dose of 400 mg/kg (Table 21). Similarly, standard Silymarine treated animals showed significant ($P<001$) increase levels of HDL-Cholesterol (G_3 -9.53 ± 0.27 mg /dl) against CCl_4 induced hepatotoxicity, as compared to control (G_2 - CCl_4 treated) which is represented in Table 1.

Effect of oral administration of herbal preparations on liver antioxidant enzyme levels against CCl4-induced liver damage in Wistar rats

Effect of herbal preparations on SOD levels

A significant ($P<001$) decreased levels of SOD was observed in CCl_4 treated group G_2 (74.96 ± 1.85 U/mg of protein) as compared to normal group G_1 (181.20 ± 4.08 U/mg of protein). The groups treated with herbal preparations showed significant ($P<001$) elevated level of SOD (G_5 - 158.60 ± 7.72 U/mg ofprotein, G_6 -150.50 ± 10.01 U/mg of protein, G_7 -134.20 ± 6.52 U/mg of protein, G_{10} - 72.30 ± 4.02 U/mg of protein, G_4 -64.55 ± 4.35 U/mg of protein, G_8 -62.90 ± 5.98 U/mg of protein, G_9 -48.71 ± 6.96 U/mg of protein) at dose of 400 mg/kg (Table 22). Similarly, standard Silymarine treated animals showed significant ($P<001$) increase levels of SOD (G_3 -216.70 ± 3.59 U/mg of protein) against CCl_4 induced hepatotoxicity, as compared to control (G_2 - CCl_4 treated) which is represented in Table 2.

Effect of herbal preparations on LPO level

A significant ($P<001$) increase levels of LPO was observed in CCl_4 treated group G_2 (320.20 ± 5.27 nM/mg ofprotein) as compared to normal group G_1 (129.10 ± 3.14 nM/mg ofprotein). The groups

treated with herbal preparations showed significant (P<001) decreased level of LPO (G_5 - 161.70 ± 10.68 nM/mg of protein, G_6 -163.70 ± 14.92 nM/mg of protein, G_7 -171.60 ± 1.25 nM/mg of protein, G_{10} -
175.00 ± 4.96 nM/mg of protein, G_4 -189.80 ± 7.51 nM/mg of protein, G_8 -202.70 ± 16.41 nM/mg of protein, G_9 218.60 ± 14.01 nM/mg of protein) at dose of 400 mg/kg. Similarly, standard Silymarine treated animals showed significant (P<001) decreased levels of LPO (G_3 -136.40 ± 4.96 nM/mg of protein) against CCl_4 induced hepatotoxicity, as compared to control (G_2 - CCl_4 treated) represented in Table No. 2.

Table 2: Effect of oral administration of herbal preparations on liver antioxidantenzyme levels against CCl4 induced liver damage in Wistar rats

Animal gropus	Treatments	SOD (U/mg of protein)	GSH (nM/mgof protein)	LPO (nM/mgof protein)	Total protein (g/dl)	CAT (U/mg of protein)
G1	**Normal**	181.20 ± 4.08	91.68 ± 3.430	129.10 ± 3.14	9.18 ± 0.56	0.6536 ± 0.07529
G2	**Control (CCl4- treated)**	74.96 ± 1.85[a]	8.92 ± 0.63[a]	320.20 ± 5.27[a]	4.01 ± 0.69[b]	0.1382 ± 0.01179[a]
G3	**Silymarine (200mg/kg)**	216.70 ± 3.59***	28.42 ± 1.08***	136.40 ± 4.96***	10.23 ± 0.38***	0.4965 ± 0.04359***
G4	**CCl4+HP1(400mg/kg)**	64.55 ± 4.35	56.73 ± 5.54***	189.80 ± 7.51***	14.22 ± 5.66***	0.4011 ± 0.0216***
G5	**CCl4+HP2(400mg/kg)**	158.60 ± 7.72***	76.92 ± 2.36***	161.70 ± 10.68***	20.34 ± 5.41***	0.6715 ± 0.0644***
G6	**CCl4+HP3(400mg/kg)**	150.50 ± 10.01***	60.12 ± 4.83***	163.70 ± 14.92***	16.56 ± 4.56***	0.5826 ± 0.0632***
G7	**CCl4+HP4(400mg/kg)**	134.20 ± 6.52***	57.68 ±	171.60 ±	18.43 ±	0.5124 ±

			3.70***	1.25***	6.21***	0.0568***
G8	**CCl4+HP5(400mg/kg)**	62.90 ± 5.98	56.35 ± 3.81***	202.70 ± 16.41***	14.52 ± 5.04***	0.3873 ± 0.0417***
G9	**CCl4+HP6(400mg/kg)**	48.71 ± 6.96	49.66 ± 3.16***	218.60 ± 14.01***	14.12 ± 3.475***	0.3048 ± 0.04021***
G10	**CCl4+HP7(400mg/kg)**	72.30 ± 4.02	57.17 ± 3.21***	175.00 ± 4.96***	18.13 ± 7.16***	0.4369 ± 0.0409***

All values were expressed as a Mean ± SEM, n=6, One-way Analysis of Variance (ANOVA) followed by multiple Dunnet's- test. The value significant ^{c}P*<0.05,* ^{b}P*<0.01,* ^{a}P*<0.001 as compared tonormal group and* *P*<0.05,* **P*<0.01,* ***P*<0.001 as compared to control group. (HP: Herbal preparations)***.**

Effect of herbal preparations on total protein

A significant (*P*<001) decreased levels of total protein was observed in CCl_4 treated group G_2 (4.01 ± 0.69 g/dl) as compared to normal group G_1 (9.18 ± 0.56 g/dl). The groups treated with herbal preparations showed significant (*P*<001) elevated level of total protein (G_5 - 20.34 ± 5.41 g/dl, G_7 - 18.43 ± 6.21 g//dl, G_{10} -18.13 ± 7.16 g/dl, G_6 – 16.56 ± 4.56 g/dl, G_4 -16.22 ± 6.66 g/dl, G_8 -14.52 ± 5.04 g/dl, G_4 -14.22 ± 5.66 g/dl), G_4 -14.12 ± 3.47 g/dl), at dose of 400 mg/kg. Similarly, standard Silymarine treated animals showed significant (*P*<001) increase levels of total protein (G_3 -10.23 ± 0.38 g/dl) against CCl_4 induced hepatotoxicity, as compared to control (G_2 - CCl_4 treated) which is represented in Table 2.

Effect of herbal preparations on Serum CAT activity

A significant (*P*<001) decreased levels of CAT was observed in CCl_4 treated group G_2 (0.1382 ± 0.01179 U/mg of protein) as compared to normal group G_1 (0.6536 ± 0.07529 U/mg of protein). The groups treated with herbal preparations showed significant (*P*<001) elevated level of CAT (0.6715 ± 0.0644 U/mg ofprotein, G_6 -0.5826 ± 0.0632 U/mg ofprotein, G_7 -0.5124 ± 0.0568 U/mg ofprotein, G_{10} - 0.4369 ± 0.0409 U/mg of protein, G_4 -0.4011±0.0216 U/mg of protein, G_8 -0.3873 ± 0.0417 U/mg of protein, G_9 - 0.3048 ± 0.04021 U/mg of protein) at dose of 400 mg/kg. Similarly, standard Silymarine treated animals showed significant (*P*<001) increase levels of CAT (G_3 -0.4965 ± 0.04359 U/mg of protein) against CCl_4 induced hepatotoxicity, as compared to control (G_2 - CCl_4 treated) represented in Table 2.

Histopathological study

Histologically, normal group animals showed normal hepatic architecture, absence of centrilobular necrosis and macrovesicular fatty changes and no dilation of portal vein and absence of inflammation

and infiltration of tissue. The control group animals exhibited intense centrilobular necrosis, vacuolization macrovesicular fatty changes and distorted central vein architecture with increased intracellular space. Silymarine treated group animals showed almost similar to normal hepatic architecture. However, the different treatment groups with 400 mg/kg of herbal preparations exhibited significant liver protection against CCl_4 induced liver damage, as evident by the presence of normal hepatic cords, absence of necrosis and fatty infiltration and there is no dilation of portal vein.

DISCUSSION

Serum glutamic oxaloacetic transaminase (U/L)

A serum glutamic oxaloacetic transaminase (SGOT) or aspartate aminotransferase (AST) test measures the levels of the enzyme AST in the blood to assess liver health. It measures one of two liver enzymes called serum glutamic-oxaloacetic transaminase. An AST test is often a more definitive indicator of potential liver damage. The normal level of SGOT usually must lie between 8-45 units/liter of the serum SGOT is found in several areas of body, including liver, kidneys, muscles, heart and brain. If any of these areas are damaged, SGOT levels may be higher than normal. For example, the levels could be raised during a heart attack. In the present study, a significant increased level of SGOT was observed in CCl_4–induced liver damaged control group rats as compared to normal groups. Liver injury contributes to increased serum level of transaminase enzymes due to easy availability of amino acids Bhuyan *et al.* (2018). In hepatic control rats, increase in SGOT level might be due to hepatotoxicity results in hepatocellular damage, thus a variety of enzymes normally located in the cytosol are released into blood stream (Reddy *et al*., 2010). Similarly, in the study of Reddy *et al*. (2018) it was observed that increased SGOT activity was noticed in Isoniazid liver damaged control rats. On treatment with test herbal preparations, it was found that HP_2 (151.6±2.842 U/L) showed significantly higher reduction in the SGOT level followed by HP_3, HP_4, HP_7, HP_1, HP_5 and HP_6 at 400 mg/kg body weight, indicating hepatoprotective effect in the CCl_4–induced liver damaged group rats (Table 1). HP_2 that consisted slightly higher antioxidant levels due to the presence of significant amount of phenols and flavonoids makes this preparations more effective in scavenging and antioxidant in biological systems. Thereby it might have reversed the activity of SGOT level. The protective effect may be the result of stabilization of plasma membrane thereby preserving the structural integrity of cell as well as the repair of hepatic tissue damage caused by CCl_4 (Verma *et al*., 2013).Similarly, standard drug Silymarinetreated animals showed significant reduction in SGOT level against CCl_4 inducedhepatotoxicity, as compared with control (CCl_4 treated) group.

Serum glutamic pyruvic transaminase (U/L)

Serum glutamic pyruvic transaminase (SGPT) testsis performed to identify the level of glutamic pyruvic transaminase (GPT) in the bloodstream. This test is most frequently used to keep a check on the health condition of liver. The cells that are known to produce the most amount of GPT are the heart, kidney, liver and muscle cells. In the current investigation, a significant increased level of SGPT was observed in CCl_4-induced liver damaged control group rats as compared to normal groups. An increase in the levels of this enzyme in serum was due to the leakage of the enzymes from the liver as a result of tissue damage (Mir *et al*., 2007). The treatment with herbal preparations at dose of 400 mg/kg body weight showed marked improvement in diminishing the higher level of SGOP in CCl_4-induced hepatic group rats (Table 21). This might be due to decreased oxidative stress due to its free radical scavenging activity

and antioxidant property which can be evidenced by decrease in lipid peroxidation. However, standarddrug Silymarine treated animals showed significant decreased levels of SGPT against CCl4-induced hepatotoxicity, as compared to control group.

Total bilirubin (mg/dL)

A bilirubin test measures the levels of bilirubin in blood. Bilirubin is a yellowish pigment that is produces during the breakdown of red blood cells. Bilirubin passes through the liver and is eventually excreted out of the body. Higher than usual levels of bilirubin may indicate different types of liver or bile duct problems. Sometimes, higher bilirubin levels may be caused by an increased rate of destruction of red blood cells. In the present experiment, a significant and marked elevation was observed in serum bilirubin levels of CCl4-induced liver damaged control group rats groups as compared to normal group rats. Sivakumar *et al*. (2018) opined that elevated bilirubin might be due to hepatic injury. Our results are in line with findings of Sivakumar *et al*. (2018), it was noticed that Isoniazid-induced liver damaged control rats showed elevated levels of bilirubin. The 400 mg/kg body weight dose of herbal preparations and silymarine treated groups significantly reduced the bilirubin in CCl4-induced hepatic group rats (Table 1). Reduction of elevated bilirubin were observed with the administration of herbal preparations indicating the possibility of preparations to stabilize biliary dysfunction in CCl4 induced chronic injury. This might be due to the inhibitory activity on microsomal acyl coenzyme A: cholesterol acyltransferase. This enzyme is responsible for acylation of cholesterol to cholesterol esters in liver (Matsuda, 1994). The hepatoprotective activity observed with these herbal preparations may be due to the presence of flavonoids, phenols and alkaloids as the preliminary phytochemical investigations revealed the presence of these compounds. Earlier studies with flavonoids, lactones, tannins and alkaloids established the hepatoprotective activity of these compounds (Kanai *et al*., 1998).

Total protein (g/dL)

A total protein test measures the amount of protein in your blood. Proteins are important for the health and growth of the body's cells and tissues. The test can help diagnose a number of health conditions, including liver disease, kidney disease and malnutrition. If your total protein level is low, you may have a liver or kidney problem or it may be that protein isn't being digested or absorbed properly. A high total protein level could indicate dehydration or a certain type of cancer, such as multiple myeloma, that causes protein to accumulate abnormally. In the present investigation, total protein level in the serum was markedly decreased in CCl4-induced liver damaged control group rats as compared to normal groups (Table 1). This might be due to reduction in synthesizing proteins was seen following intoxication of the liver with hepatotoxicants (Sivakumar *et al*., 2018). Similarly, Sivakumar *et al*. (2018) noticed that lower level of total protein in Isoniazid-induced liver damaged control rats. The total protein content had significantly increased in herbal preparations treated CCl4-induced hepatic group rats at dose of 400 mg/kg body weight. This might be due to stabilization of the endoplasmic reticulum leading to protein synthesis (Sureshkumar, S. V, 2007). However, standard drug Silymarine treated animals showed significant increase levels of total protein against CCl4 induced hepatotoxicity, as compared to control.

High-density lipoprotein (mg/dL)

High-density lipoprotein (HDL) cholesterol is known as the "good" cholesterol because it helps remove

other forms of cholesterol from bloodstream. Higher HDL cholesterol levels are better to lower the risk of heart attacks. In the current investigation, a significant decreased level of HDL-Cholesterol was observed in CCl4-induced liver damaged control groups as compared to normal groups (Table 1). This might be due to impaired fat metabolism due to hepatic damage caused by CCL4 metabolites by binding to proteins and lipids. CCL4 changes the composition and secretion of lipoproteins in primary liver cells. Due to the impairment of triacyl glycerides synthesis of VLDL and HDL apoproteins is inhibited (Boll *et al*, 2001). Oral administration of herbal preparations at dose of 400 mg/kg body weight significantly reversed the elevation of HDL level towards normal in CCl4-induced hepatic group rats. This might be due to the antioxidant property of the herbal prepartions in reducing the markers of liver injury induced by CCl4 and restore the oxidative stability, the level of inflammatory cytokines, the lipid profile and the cell survival Akt1 signals (Ebaid *et al*., 2013). However, standard drug silymarine treated animals showed significant increased levels of HDL-Cholesterol against CCl4-induced hepatotoxicity, as compared to control.

Effect of oral administration of herbal preparations on liver antioxidant enzyme levels against CCl4-induced liver damage in Wistar rats

Superoxide dismutase (IU/mg of protein)

Superoxide dismutase (SOD) is an antioxidant enzyme that acts to degrade superoxide, a major causative factor for oxidative stress associated with cancer, cardiovascular disease and various other ailments. SOD inhibits the reaction by converting the superoxide radical to oxygen by its radical scavenging activity. In the present investigation, a significant decreased level of SOD was observed in CCl4-induced liver damaged control group rats as compared to normal groups. This might be due to suppression of the anti-oxidant system. The significantly reduced activities of SOD pointed out the hepatic damage in the rats administered with paracetamol drugs (Sivakumar *et al.*, 2018). Treatment with herbal preparations at dose of 400 mg/kg body weight significantly reversed the SOD activity in the CCl4-induced hepatic rats (Table 2). This might be the hepatoprotective effect of herbal preparation attributed to its herbal ingredients which possess very potent antioxidant and hepatoprotective phytoconstituents and their combined synergistic action of all the ingredients helps to normalize the liver function i.e. antioxidative system activation by suppressing the TNF-α/IL-6 pathway and blocking the TGF-β1 pathway (Lin *et al*., 2019) and thus cure complex liver disorders. However, the phenolics and flavonoids in herbal preparations as bioactive compounds (Table 2) may have an efficient activity to prevent CCl4-induced oxidative stress and liver inflammation, since herbal preparation possess greater antioxidant property (Al-Qabba *et al*., 2020). While standard drug Silymarine treated animals showed significant increase levels of SOD against CCl4-inducedhepatotoxicity, as compared to control.

Glutathione peroxidase (nM/mg of protein)

One of the detectable biomarker in the serum is glutathione. GSH is the most abundant cellular non-protein thiol, attaining concentrations in the high mill molar range in liver. Total antioxidant status was measured by amount of enzymatic GSH. GSH plays the important role in balance the oxidative stress. In the current study, a significant decreased level of GSH was observed in CCl4-induced liver damaged control group rats as compared to normal groups (Table 1). Depletion of reduced glutathione (GSH) is known to result in enhanced lipid peroxidation and excessive lipid peroxidation can cause increased

glutathione consumption. According to Bhuyan *et al*. (2018), in hepatic control groups, the decreased GSH may be due to reduction in GSH synthesis or degradation of GSH by oxidation stress in hepatic animal. But post treatment with silymarin and polyherbal preparation restored the glutathione level showing a dose dependent effect. This might be due to hepatoprotective effect of herbal preparation attributed to its herbal ingredients which possess very potent antioxidant and hepatoprotective phytoconstituents and their combined synergistic action of all the ingredients helps to normalize the liver function i.e. antioxidative system activation by suppressing the TNF-α/IL-6 pathway and blocking the TGF-β1 pathway (Lin *et al*., 2019) and thus cure complex liver disorders. However, the phenolics and flavonoids in herbal prepartions as bioactive compounds (Table 2) may have an efficient activity to prevent CCl4-induced oxidative stress and liver inflammation, since herbal preparation possess greater antioxidant property (Al-Qabba *et al*., 2020). While standard drug silymarine treated animals showed significant increase levels of GSH against CCl4-inducedhepatotoxicity, as compared to control. However, standard silymarine treated animals showed significant increase levels of GSH against CCl4 inducedhepatotoxicity, as compared to control.

Lipid peroxidase (nM/mg of protein)

Lipid peroxidation (LPO) is a free radical-related process that in biologic systems may occur under enzymatic control, e.g., for the generation of lipid-derived inflammatory mediators, or non-enzymatically. Lipid peroxidation occurs under conditions where reactive oxygen species (ROS) readily react with vulnerable lipids on cell membranes i.e. degradation of biomembranes (Palanivel *et al*., 2008). In present study, a significant increase level of LPO was observed in CCl4-induced liver damaged control group rats as compared to normal groups (Table 2). Bhuyan *et al*. (2018) opined that, the oxidative stress induced by CCl4 may lead to imbalance of *in vivo* antioxidant system. Increase in the level of lipid peroxides in liver reflected the hepatocellular damage. The depletion of antioxidant defenses and/or raise in free radical production deteriorates the prooxidant-antioxidant balance, leading to oxidative stress-induced cell death (Sodhi *et al*., 1997: Sivakumar *et al*., 2018). The CCl4-induced liver damaged rats treated with herbal preparations showed significant decreased level of LPO at dose of 400 mg/kg in treatment group. This might be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity (Palanivel *et al*., 2008) i.e. healing of hepatic parenchyma and the regeneration of hepatocytes. Moreover, standard silymarine treated animals showed significant decreed levels of LPO against CCl4 induced hepatotoxicity, as compared to control.

Total protein (g/dL)

Protein levels in the blood can be determined with a total protein test. The body's tissues and cells depend on proteins for growth and health. Liver disease, kidney disease, and malnutrition are just a few of the illnesses that the test can assist in diagnosing. If your total protein level is low, you might be experiencing problems with your kidneys or liver, or you might not be properly digesting or absorbing the protein. Dehydration or a specific kind of cancer, like multiple myeloma, which causes protein to accumulate abnormally, may be indicated by a high total protein level. In the current study, rats in the CCl4-induced liver damage control group had a significantly lower total protein level in the liver homogenate than rats in the normal group (Table 2). This could be because hepatotoxicant-induced liver intoxication resulted in a decrease in the synthesis of proteins (Sivakumar *et al*., 2018). In a similar vein, Sivakumar *et al*. (2018) observed that control rats with liver damage induced by isoniazid had a lower

level of total protein. At a dose of 400 mg/kg body weight, the total protein content of the herbal preparations treated CCl4-induced hepatic group rats had increased significantly. Protein synthesis may result from the endoplasmic reticulum stabilizing, as suggested by previous studies. However, standard drug Silymarine treated animals showed significant increase levels of total protein against CCl4 induced hepatotoxicity, as compared to control.

Catalase (U/mg ofprotein)

Catalase is an enzymatic antioxidant widely distributed in all animal tissues and the highest activity is found in the red cells and liver. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals (Chance *et al*., 1952). Therefore, reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. GSH plays the important role in balance the oxidative stress. In the current study, a significant decreased level of CAT was observed in CCl4-induced liver damaged control group rats as compared to normal groups. Depletion of reduced glutathione (GSH) is known to result in enhanced lipid peroxidation and excessive lipid peroxidation. According to Bhuyan *et al*. (2018), in hepatic control groups, the decreased CAT may be due to reduction in CAT synthesis or degradation of CAT by oxidation stress in hepatic animal. But post treatment with silymarin and polyherbal preparation restored the catalase level showing a dose dependent effect. This might be due to hepatoprotective effect of herbal preparation attributed to its herbal ingredients which possess very potent antioxidant and hepatoprotective phytoconstituents and their combined synergistic action of all the ingredients helps to normalize the liver function i.e. antioxidative system activation by suppressing the TNF-α/IL-6 pathway, and blocking the TGF-β1 pathway (Lin *et al*., 2019) and thus cure complex liver disorders. However, the phenolics and flavonoids in herbal prepartions as bioactive compounds (Table 22) may have an efficient activity to prevent CCl4-induced oxidative stress and liver inflammation, since herbal preparation possess greater antioxidant property (Al-Qabba *et al*., 2020). While standard drug silymarine treated animals showed significant increase levels of CAT against CCl4-induced hepatotoxicity, as compared to control. However, standard silymarine treated animals showed significant increase levels of CAT against CCl4 induced hepatotoxicity, as compared to control.

Assessment of histopathological examination of hepatic tissues in experimental rats

Photomicrographs of hepatic cross-sections of normal, hepatic, hepatic rats treated with silymarine and herbal preparations stained with hematoxylin and eosin. Microscopic observation of hepatic tissue of normal group animals showed normal hepatic architecture, absence of centrilobular necrosis and macrovesicular fatty changes and no dilation of portal vein and absence of inflammation and infiltration of tissue. The control group animals exhibited intense centrilobular necrosis, vacuolization macrovesicular fatty changes and distorted central vein architecture with increased intracellular space. Silymarine treated group animals showed almost similar to normal hepatic architecture. However, the different treatment groups with 400 mg/kg of herbal preparations exhibited significant liver protection against CCl4 induced liver damage (HP2> HP3> HP4> HP7> HP1> HP5> HP6), as evident by the presence of normal hepatic cords, absence of necrosis and fatty infiltration and there is no dilation of portal vein. In the investigations of Antony *et al*. (2006), alloxan injection elicited significant morphological alterations in hepatic rats with swollen cells, necrosis and round cell infiltration. The necrotic changes were also found to be minimal in the hepatic tissues indicating reduced hepatic

injury and slightlyrecovered necrotic change in sylimyrin (200 mg/kg) treated hepatic rats. The rats treated with herbalformulations (400 mg/kg body weight) showed minimal to moderate cell destruction and necrotic changes and representing slightly restored necrotic changes, β-cells with shrunken islets andregeneration of endoplasmic reticulum. This could be due to multiple and beneficial effects of phytoconstituents of different plants in the polyherbal formulation. The higher quantity of phenoliccompounds, flavonoid and tannin contents of the polyherbal formulation may contribute to its highantioxidant property (Abbas *et al*., 2017). Alagammal *et al*. (2012) opined that flavonoids are wellknown to regenerate the endoplasmic reticulum in the alloxan-induced rats.

In another investigation of Ahsan *et al*. (2009), the histopathological study of normal rats showed exhibited normal hepatic cells each with well defined cytoplasm, prominent nucleus and nucleolus and well brought out central vein. However, CCl4 intoxicated group animal showed total loss of hepatic architecture with centrilobular hepatic necrosis, fatty changes, vacuolization and congestion of sinusoids,kupffer cell hyperplasia, crowding of central vein and apoptosis. Whereas, hepatic rats treated with herbal preparations at a dose of 400mg/kg showed moderated to less hepatic cells destruction. The scientists suggested the liver protection is related to glutathione-mediated detoxification as well as free radical suppressing activity of herbal preparations due to the protective and antioxidant effect.

CONCLUSIONS

- Regular oral administration of herbal preparations (HP-2) exhibited significant hepatoprotective potential by governing the serum biochemical and antioxidant activity. Each plant act by different mechanisms to treat hepatic disease. The anti-hepatic activity of herbal preparation is due to combination and presence of phenols, flavonoid, antioxidants, tannins and crude fibre content
- The formulation (HP2) also restored the serum biochemical enzymes (SGOT, SGPT, Total bilirubin, Total protein and HDL-Cholesterol) and liver antioxidant enzymes (SOD, GSH, LPO and CAT) which indicated that they reduce the complications of hepatic disease
- Thus, the plant powders that were combined to create herbal preparations without the need for an extraction process have the potential to be used as a dietary supplement to manage hepatotoxicity.

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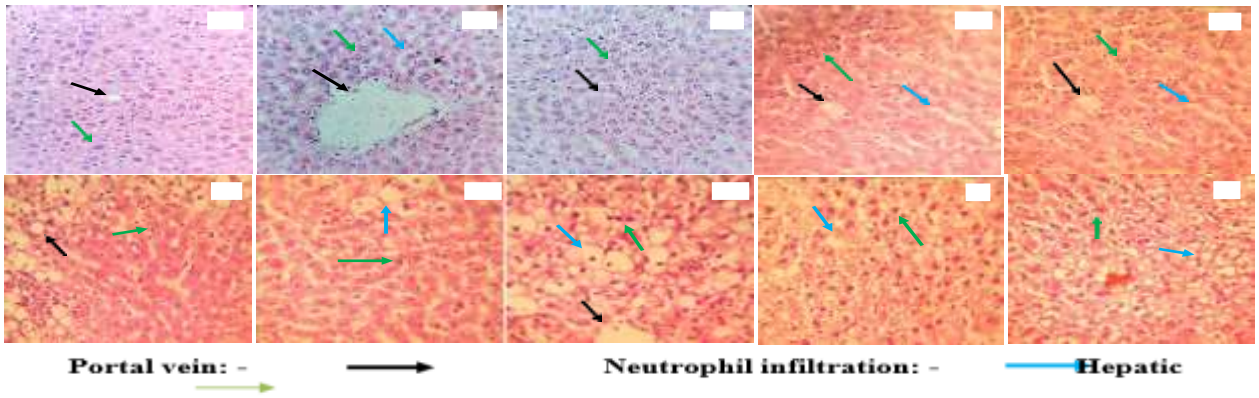

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Cell

Plate 7: Effect of oral administration of herbal preparations on histopathology study against CCl4 induced liver damage in WA rats. In 40 X magnified Photographs of liver from different treatment groups stained with Hematoxylin and Eosin (H&E). Plates; **A:** Normal group, **B:** CCl4 induced Control group, **C:** Silymarine treated Standard group, **D:** CCl4+HP1 (400mg/kg), **E:** CCl4+HP2 (400mg/kg), **F:** CCl4+HP3 (400mg/kg), **G:** CCl4+HP4 (400mg/kg), **H:** CCl4+HP5 (400mg/kg), **I:** CCl4+HP6 (400mg/kg), **J:** CCl4+HP7 (400mg/kg). In plate **A** shows portal vein constriction and plate **B** shows portal vein dilation and also there was completely alerted hepatic cells architecture, centrilobular necrosis, hepatic steatosi, and macrovascular fatty changes noticed. But in Plate **C, D, E, F, G, H, I** and **J** showed almost normal architecture and absence of centrilobular necrosis, hepatic steatosis.

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