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AN OPTIMIZED HERBAL FORMULA DIMINISHES CCL_4-INDUCED HEPATOTOXICITY IN RATS BY INHIBITION OF LIPID PEROXIDATION AND P53-MEDIATED APOPTOSIS

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ABSTRACT: Introduction: In this present study, the heaptoprotective effect of an optimized herbal formula (HF) was evaluated against carbon tetrachloride (CCl_4)-induced liver injuries in rats. **Methods:** *In-vitro* antioxidant activity of the HF was evaluated by reducing power, lipid peroxidation, and DPPH scavenging assays. Hepatoprotective activity of HF (100, 200 and 400 mg/kg b.w) was evaluated against CCl4-induced hepatic damage in rats. Serum enzymatic levels of serum glutamate oxaloacetate transaminase (AST), serum glutamate pyruvate transaminase (ALT), serum alkaline phosphatase (ALP), and Gamma-glutamyltransferase (γ-GT) were estimated along with an estimation of catalase (CAT), superoxide dismutase (SOD), Glutathione (GSH) and TBARS levels in liver tissues. The lipid profile was also examined. Furthermore, histopathological examination of the liver sections and p53 expression assay were performed to confirm and advocate the induction of hepatotoxicity as well as hepatoprotective efficacy. **RESULTS:** The HF exhibited strong *in-vitro* antioxidant activities in terms of reducing power, inhibition of lipid peroxidation, and DPPH radical scavenging. HF restored the significantly elevated serum enzymatic levels of AST, ALT, ALP and γ-GT in a dose-dependent manner with maximum efficacy at 400 mg/kg dose level. The lipid profile was also found to be normalized. His to pathological observations as well as p53 expression assay did further confirmed the biochemical evidence of hepatoprotection. An elevated level of catalase (CAT), superoxide dismutase (SOD), Glutathione (GSH), and reduced TBARS levels in liver tissues further reinforce the heaptoprotective revelations. **Conclusions:** The RESULTS clearly disclose the antioxidant activity and heaptoprotective activity of HF against CCl_4-induced hepatic damage in experimental animals

INTRODUCTION: The liver demonstrates a major role in the metabolism of xenobiotics by regulating the synthesis, secretion and metabolism of xenobiotics. Liver injury and hepatic parenchymal damage are always evidenced to be linked with disarrangement of different liver metabolic pathways and functions [1]. There are numerous etiological modalities, including various infectious agents, viruses, numerous chemical agents, and environmental pollutants responsible for causing hepatotoxicity and damage.

A plethora of growing original research in free radical biology also proposed the pathophysiological role of various free radicals and oxidative stress in liver damage leading to hepatotoxicity. Research also indicated oxidative

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stress and free radicals damage as the mechanism of actions of different potent hepatotoxins such as CCl_4, paracetamol, etc. in the pathophysiology of hepatic injury [2]. CCl_4 is one of the most common hepatotoxins and an industrial solvent. It is the most used experimental animal model of xenobiotic-induced, oxidative stress-mediated hepatotoxicity [3, 5]. Numerous reactive oxygen species (ROS) generated by CCl_4, get attached to the polyunsaturated fatty acids, forming peroxyl and alkoxy radicals to produce lipid peroxide, which in turn causes damage to the cell membrane and alters enzymatic activity [6, 7]. The cascade of lipid peroxidation following the attack by oxygen-derived free radicals becomes pathophysiological basis of various liver diseases including hepatic fibrosis, hepatic injury, necrosis and apoptosis [8].

Moreover, a physiological antioxidant defense system comprising different enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH) *etc*. neutralises the free radicals. Vitamin deficit alongside overrun of free radicals and a reduced level of above-mentioned enzymes is flagged as the foremost perpetrator for fabricating oxidative stress [9, 10]. In this context, neutralizing free radicals and preventing lipid peroxidation have been targeted as the possible treatment and prevention goal of hepatotoxicity and damage [11, 12]. DNA fragmentation brings p53 gene expression, causing arrest of the G-phase of the cell cycle, whereas severe DNA damage induces apoptosis [13]. Therefore, inhibition of p53-dependent hepatocyte apoptosis can be an effective therapeutic approach for treating and preventing hepatic damage.

In addition, the lack of a reliable liver protective drug in the modern system of medicine encouraged the investigation of traditional systems of medicine, including Ayurveda, Siddha, Unani *etc*. for a probable answer to hepatotoxicity [14]. There is a growing focus on the activity of natural antioxidants against chemically induced toxicities. Numerous medicinal plants, as well as herbal modalities, are being explored for an effective hepa to protective remedy. Considering the need and growing concerns, the present study was designed to investigate the pharmacological effects of an optimized herbal formula (HF) on activities of hepatic lipid peroxidation, hepatocellular enzymes, antioxidant enzymes, lipid profiles, and hepatic cell apoptosis as markers for hepatotoxicity and damage in CCl_4-induced rat model of hepatotoxicity.

MATERIALS AND METHODS:

Drugs and chemicals: Silymarin was arranged from Ranbaxy Laboratories, Delhi, India. Malondialdehyde (MDA) and carbon tetrachloride (CCl_4) were purchased from Sigma Chemicals Company, St Louis, MO, USA and E Merck, Mumbai, India, respectively. All other reagents and chemicals used in the experiments were of analytical grade and available commercially.

The Herbal Formula: The herbal formula (HF) was previously developed, characterized, and optimized in the pharmacology laboratory of the department of pharmaceutical sciences, Dibrugarh university. Dibrugarh, Assam, India. The HF comprises four plant extracts, including methanol extract of *Rumex hastetus*, *Alternanthera sessilis*, *Angelica sinensis*, and *Urtica dioica*.

Acute Toxicity Studies: An acute oral toxicity study was performed according to the OECD guidelines for the testing of chemicals, Test No. 423 (OECD, 2001; Acute oral toxicity-Acute toxic class method). Wistar rats (n = 3) of either sex were chosen by a random sampling method for the acute toxicity study. The animals were fasted overnight prior to the experimentation and maintained under standard laboratory environments. The HF was administrated orally in increasing doses up to 2000 mg/kg.

Determination of Total Phenolic Compounds: Total soluble phenolics in the HF was determined by means of the Folin-Ciocalteau method using Quercetin as a standard phenolic compound [15]. The concentration of total phenolic compounds in the HF was presented as gram of Quercetin equivalent (QE) using an equation obtained from the equation of regression line of standard quercetin graph:

$$Y= 0.0031 \times + 0.0552, R2 = 0.9797$$

Where, y is the absorbance and × is the concentration.

***In-vitro* Antioxidant Activity:**

Reducing Power: The reducing power of the HF, BHT and α-tocopherol was determined according to the method of Oyaizu (1986) [16].

Total Antioxidant Activity: The total antioxidant activity of the HF was determined according to the thiocyanate method [17].

DPPH Radical Scavenging Activity: The free radical scavenging activity of the HF was measured by DPPH• using the method described previously [18].

***In-vivo* Hepatoprotective Activity Appraisal:**

Test Animals: Wistar rats (180-240 g) of either sex were procured from the S/N Chakraborty Enterprise, Kolkata, West Bengal, India (Reg. No. 1576/GO/a/11/CPCSEA) for the study. The animals were housed in large, clean polypropylene cages in a temperature-controlled room (22 ± 2 °C with relative humidity (45-55%) under 12 h light and dark cycles. All the animals were acclimatized to the laboratory environment for two weeks prior to experiments.

Animals were provided with a standard rodent pellet diet and clean drinking water *ad libitum*. The care and use of laboratory animals were strictly in accordance with the guidelines prescribed by the Institutional Animal Ethical Committee constituted under the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Experimental Design: A total of 36 rats were divided into 6 groups of 6 rats each.

- **Group I:** served as normal control and received only the vehicle (1 ml, 0.5 % Tween 80; p.o. and Olive oil (1 ml/kg; s.c.) on day 2 and 3.
- **Group II:** received the vehicle (1 ml, 0.5 % Tween 80; p.o. and CCl_4 2 ml/kg (1:1 of CCl_4 in olive oil) s.c. on day 2 & 3.
- **Group III:** received CCl_4 2 ml/kg (1:1 of CCl_4 in olive oil) s.c. on day 2 & 3 and silymarin 100 mg/kg orally (p.o.) for 5 days.
- **Groups IV, V and VI:** were administered the HF at 100, 200, and 400 mg/kg body weight p.o. respectively for 5 days and dose of 2 ml/kg of CCl_4 (1:1 of CCl_4 in olive oil) s.c. on days 2 & 3.

Body Weight Gain: Body weight gain was recorded by subtracting the final bodyweight from the initial body weight.

Blood Sampling: After 5 days of treatments, anesthetized fasted overnight animals were decapitated and blood samples were collected in sterilized plain tubes. Serum was stored at -20 °C for determination of lipid profile and for carrying out further biochemical investigations.

Tissue Sampling: The liver was excised, rinsed with ice phosphate buffer saline, dried by filter paper, and weighed. The liver for each rat was divided into 5 parts; 3 of which were kept at −80 °C until preparation of liver homogenate for assay of markers of oxidative stress, including catalase (CAT), Reduced glutathione activity (GSH), Superoxide dismutase (SOD), and thiobarbituric acid reactive substances (TBARS). The remaining parts were immersed in 10% neutral buffered formalin for histopathological and immunohistochemical examination.

Lipid Profile and Liver Functions: Serum levels of triglycerides (TG), low-density lipoprotein (LDL) cholesterol, total cholesterol (TC), and high-density lipoprotein (HDL) were measured using enzymatic calorimetric kits [19, 21]. The activities of serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and Gamma-glutamyltransferase (γ-GT) were determined using the Hitachi 912 clinical chemistry automatic analyzer (Roche Diagnostic GmbH, Mannheim, Germany).

Assessment of Oxidative Stress: To determine oxidative stress, homogenized liver tissue was treated and subjected to the assay of oxidative stress markers.

Estimation of Catalase (CAT): Catalase (CAT) content in liver homogenate was assayed by the method described previously [22].

Estimation of Superoxide Dismutase (SOD): Superoxide dismutase (SOD) was also assayed by the method described previously [23, 24].

Reduced Glutathione Activity (GSH): GSH activity in liver homogenate was determined at absorbance 412 nm using calorimetric kit

(BioVision, USA) according to the method described elsewhere [25].

Estimation of Thiobarbituric Acid Reactive Substances (TBARS): Thiobarbituric acid reactive substances (TBARS) contents were estimated by a method described previously [26].

Histopathology: Formalin-fixed liver sections were prepared using standard procedures for Hematoxylin and Eosin stain as described elsewhere [27, 29].

Immunohistochemistry of P^{53}: The paraffin-embedded livers were cut into 5 µm sections and mounted on positively charged slides for p53 immunohistochemistry as per the method described by Chang *et al.* [30]. Sections were deparaffinized in xylene and rehydrated with absolute ethanol, followed by water. The sections were incubated with a Primary monoclonal antibody for p^{53} D07 (NCL-L-p^{53}-DO7; Novocastra, Newcastle, UK) at a dilution of 1:200 for 2 h at 25 °C in a humidification chamber. The tissue sections were then subjected to biotinylated polyvalent secondary antibody (Thermo Scientific Co., UK) and co-incubated for 30 min. The reaction was visualized by Metal Enhanced DAB Substrate Working Solution to the tissue and incubated 10 min after washing. Following the washing, a suitable amount of hematoxylin stain was applied as a counterstain to the slide to cover the whole tissue surface. For the quantitative study, the intensity of immunoreactive fragments was used as a measure of cellular activity after deducting background noise. Image J program was used for image analysis, and the total field alongside the immunohistochemical (IHC) stained zones were noted, and the percentage of IHC stained area was calculated using the equation below.

$$\% \text{ IHC stained area} = \text{IHC stained area} / \text{ total area} \times 100$$

Statistical Analysis: The data were expressed as mean ± SD. Statistical differences at $p < 0.05$ between the groups were analyzed by one-way ANOVA followed by Turkey as post hoc using Graph Pad Instat software package. The IC_{50} values were calculated graphically by linear regression analysis. All the analyses were done using Graph Pad Prism (Version 5.01, Graph Pad Software, San Diego, USA).

RESULTS:

Acute Toxicity Studies: The HF did not cause any mortality upto 2000 mg/kg dose level. Hence $1/20^{th}$, $1/10^{th}$, and $1/5^{th}$ of the maximum dose (*i.e.*, 100, 200 and 400 mg/kg, p.o.) were selected for the present study. At dose level of 2000 mg/kg, the HF was non-toxic, and it did not produce any toxic symptoms and lethality.

Determination of Total Phenolic Compounds: The amount of total phenolics in the HF was found to be 227.03 ± 6 mg QE/g extract. The present study did not try to establish any correlation between phenolic content and biological activity.

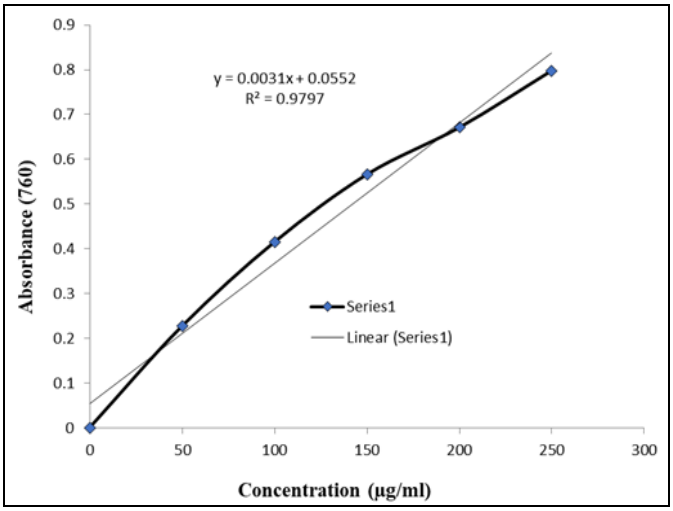


FIG. 1: TOTAL PHENOLIC COMPOUNDS IN HF

In-vitro Antioxidant Activity:

Reducing Power: The reducing power of HF compared to BHT and α-tocopherol is shown in **Fig. 2.** With the increasing concentration, the reducing power of HF was increased. The HF revealed an equivalent reducing power to α-tocopherol, but the reductive capability was lower than BHT.

Total Antioxidant Activity: HF showed effectively and commanding antioxidant activity in terms of peroxidation of linoleic delete it emulsion at a concentration of 250 µg/ml **Fig. 3.** HF showed higher antioxidant activity than α-Tocopherol but lower antioxidant activity than BHA (Butylated hydroxyanisole) at 250 µg/ml concentration. The percentage inhibition of peroxidation in linoleic acid system was found to be 66.98 % for HF. And percentage inhibition of 250 µg/ml concentration of BHA and α-Tocopherol was found as 94.57 and 31.93 %, respectively.

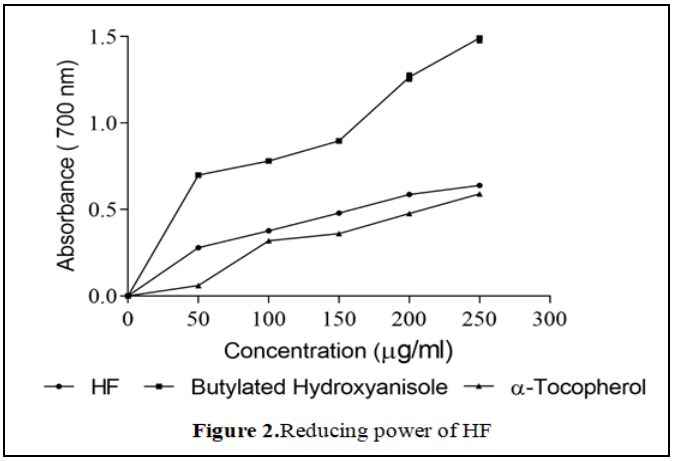


FIG. 2: REDUCING POWER OF HF

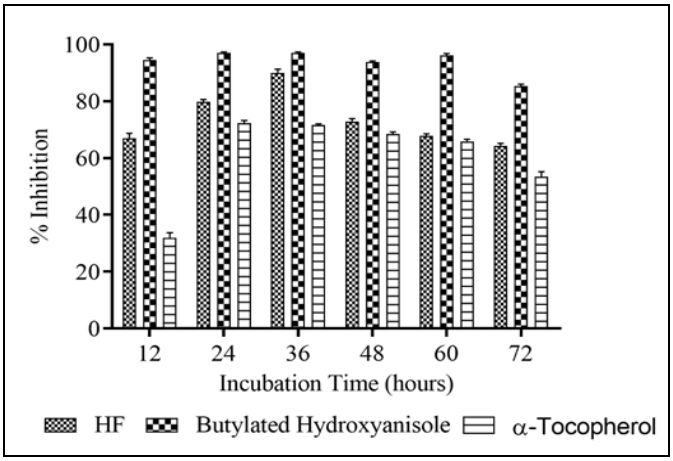


FIG. 3: TOTAL ANTIOXIDANT ACTIVITY IN HF

DPPH Radical Scavenging Activity: Fig. 4. displays the decrease in concentration of DPPH radical due to scavenging ability of HF and standard compound (BHT) at different concentration levels (10-250 µg/ml). An equivalent scavenging ability of HF and BHT on DPPH radical was observed. The percent DPPH scavenging activity of HF and BHT were found to be 95.88 and 97.87 %, respectively at the concentration of 250 µg/ml. The RESULTS showed HF as a strong scavenger of DPPH radical comparable to standard BHT. The IC_{50} values were also calculated for HF and BHA using the equation obtained from linear regression analysis. The IC_{50} values of HFand BHT were found to be 68.37 µg/ml and 70.09 µg/ml, respectively.

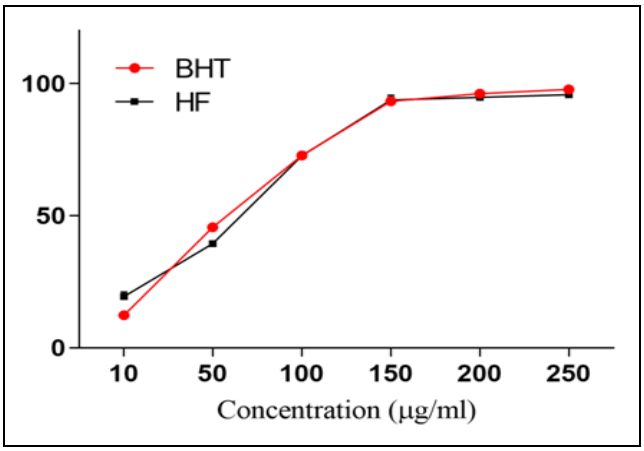
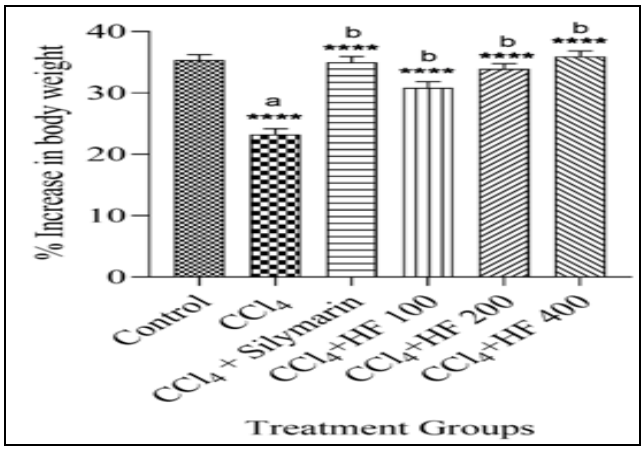


FIG. 4: DPPH RADICAL SCAVENGING ACTIVITY IN HF



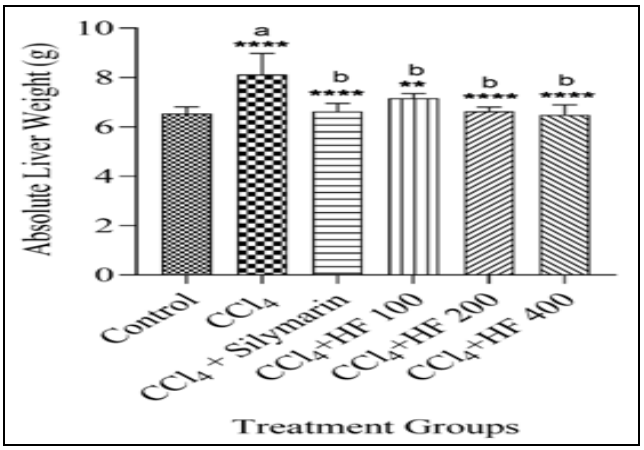


FIG. 5: BODY WEIGHT GAIN AND ABSOLUTE LIVER WEIGHT IN PERCENTAGE. A INDICATE SIGNIFICANCE FROM THE CONTROL GROUP AT P<0.0001 PROBABILITY LEVEL.B INDICATE SIGNIFICANCE FROM THE CCL4 GROUP AT ** P<0.01, ***P<0.0001 PROBABILITY LEVEL.

***In-vivo* Hepatoprotective Activity:**

Body Weight Gain and Liver Weight: Treatment of CCl_4 produced a significant decrease (P<0.0001) in body weight whereas augmented the absolute liver weight (P<0.0001) comparatively to the control group. The increased absolute liver weight by CCl_4 intoxication was found to be significantly (P<0.0001) reduced and restored with HF treatment **Fig. 5.** A dose-dependent restoration in the bodyweight by HF was also observed (P<0.0001).

Lipid Profile and Liver Functions: In the CCl_4 treated rats, the serum cholesterol (TC), triglyceride (TG), and low-density lipoprotein (LDL) levels were increased significantly (P<0.0001), while high-density lipoprotein (HDL) concentration was decreased significantly (P<0.0001) compared to that in control group rats. HF treatment at 100. 200 and 400 mg/kg dose levels alongside CCl_4 improved the lipid profile in treated rats **Table 1**. However, HF at 100 mg/kg dose level did not significantly increase HDL level (Not significant). The hepatoprotective effects of HF on serum biochemical parameters in CCl_4-intoxicated rats are shown in **Table 2**.

Compared to control group animals, rats treated with CCl_4 exhibited a significant upsurge in serum AST, ALT, ALP, and γ-GT levels. Treatment with HF at 100, 200, and 400 mg/kg dose levels showed significant hepatoprotection in terms of restoration of serum AST, ALT, ALP and γ-GT levels compared to the CCl_4 control group (P<0.0001)

TABLE 1: EFFECT OF HF ON LIPIDS PROFILE

	TG (mg/dl)	TC (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Control	7.93±0.45	6.24±0.29	3.89±0.28	2.69±0.32
CCl_4	[a] 11.24±0.54***	[a] 11.33±0.27***	[a] 2.41±0.19**	[a] 8.64±0.18***
Silymarin	[b] 8.11±0.22***	[b] 6.52±0.07***	[b] 3.81±0.52**	[b] 3.06±0.09***
HF 100 + CCl_4	[b] 10.09±0.46**	[b] 9.11±0.09***	[b] 3.29±0.52[ns]	[b] 7.02±0.09***
HF 200 + CCl_4	[b] 8.12±0.47***	[b] 7.24±0.11***	[b] 3.63±0.71**	[b] 5.07±0.08***
HF 400 + CCl_4	[b] 7.89±0.84***	[b] 6.37±0.08***	[b] 3.85±0.74***	[b] 3.11±0.09***

Mean ±SD (n=6 number). A indicate significance from the control group at P<0.0001 probability level. b indicate significance from the CCl_4 group at ** p<0.01, ***P<0.0001 probability level.

TABLE 2: EFFECT OF HF ON HALLMARKS OF HEPATOTOXICITY

	ALT (SGPT) (IU/L)	AST (SGOT) (IU/L)	ALP (IU/L)	γ-GT(nM/min /mg protein)
Control	65.19±2.43	86.32±1.27	188.23±3.73	115.25±2.22
CCl_4	[a] 124.27±3.36***	[a]244.57±3.54***	[a]460.43±6.22***	[a] 164.03±2.22***
Silymarin	[b] 69.51±1.22***	[b] 95.86±1.47***	[b]196.64±2.58***	[b]119.98±3.1***
HF 100 + CCl_4	[b] 98.83±1.46***	[b]157.77±2.79***	[b] 311.68±2.52***	[b]137.88±3.14***
HF 200 + CCl_4	[b] 76.81±1.44***	[b]100.55±2.11***	[b]215.11±2.73***	[b]121.03±3.62***
HF 400 + CCl_4	[b] 68.66±1.74***	[b] 92.66±1.18***	[b]199.66±2.73***	[b]118.23±3.09***

Mean ±SD (n=6 number). A indicate significance from the control group at ***P<0.0001 probability level. b indicate significance from the CCl_4 group at ***P<0.0001 probability level.

Assessment of Oxidative Stress: CCl_4 treatment in rats significantly diminished the activity of CAT, SOD, GSH (P<0.01) whereas augmented TBARS contents (***P<0.0001) in liver samples. The upsurge of lipid peroxidation triggered; decrease in the activities of antioxidant enzymes (CAT and SOD), and glutathione (GSH) contents were distinctly diminished (P<0.01) by administration of HF at all the studied dose levels (100, 200, and 400 mg/kg bw) in CCl_4 intoxicated rats **Table 3.** However, the efficacy of HF at 100 mg/kg bw dose level in terms of CAT enzyme and GSH level restoration was found to be less significant (* p<0.05) as compared to other significant levels **Table 3.**

Liver Histopathology: The protective effect produced by HF against CCl_4 induced heaptotoxicity was further established by histological evaluation. Histopathological observations showed that the normal architecture of the liver was significantly lost in rats treated with CCl_4 with the appearance of vacuolization, fatty changes and necrosis of hepatocytes in the central lobular area **Fig. 6.** Widespread liver damage characterized by moderate to severe necrosis, fibrosis, heaptocellular hydropic degeneration, and mononuclear inflammatory leukocyte infiltrations was observed in the CCl_4 treated group. However, Silymarin and HF treatment noticeably ameliorated the hepatic lesions caused by CCl_4. The fibrotic

tissues were observed as blue colour by Masson's trichrome stain. In normal control animals, the liver sections exhibited normal hepatic cells without any sign of fibrosis (a). The livers of rats that were treated with CCl_4 exhibited widespread buildup of thick fibrotic tissue, producing in the development of continuous fibrotic septa compared to the normal control (b). A little of hepatic fibrosis was observed in the livers of HF-treated rats (d and e). Moreover, HF treatment at 400 mg/kg dose level (f) demonstrated significant protection from the signs of CCl_4 intoxication and produced a more or less normal lobular pattern with short septa of connective tissue, and a mild degree of fatty change and necrosis (score of 1) almost comparable to the control and silymarin treated groups.

TABLE 3: EFFECT OF HF ON MARKERS OF OXIDATIVE STRESS

	CAT (U/min)	SOD (U/mg protein)	GSH (µM /g tissue)	TBARS (nM /min/mg protein)
Control	3.77±0.87	20.6±1.01	2.87±0.02	27.22±1.1
CCl_4	[a] 1.52±0.12**	[a] 9.81±0.78***	[a] 0.41±0.01**	[a] 51.06±1.22***
Silymarin	[b] 3.49±1.32**	[b] 18.77±0.67***	[b] 2.76±2.58**	[b] 25.95±1.33***
HF 100 + CCl_4	[b] 3.24±0.86*	[b] 14.44±2.79***	[b] 2.56±0.05*	[b] 38.92±1.26***
HF 200 + CCl_4	[b] 3.44±0.76**	[b] 17.49±0.47***	[b] 2.68±0.04**	[b] 30.06±1.14***
HF 400 + CCl_4	[b] 3.53±0.91**	[b] 19.97±0.62***	[b] 2.79±0.02**	[b] 26.09±1.12***

Mean ±SD (n=6 number). A indicate significance from the control group at **P<0.01, ***P<0.0001 probability level. b indicate significance from the CCl_4 group at * p<0.05, ** p<0.01, ***P<0.0001 probability level

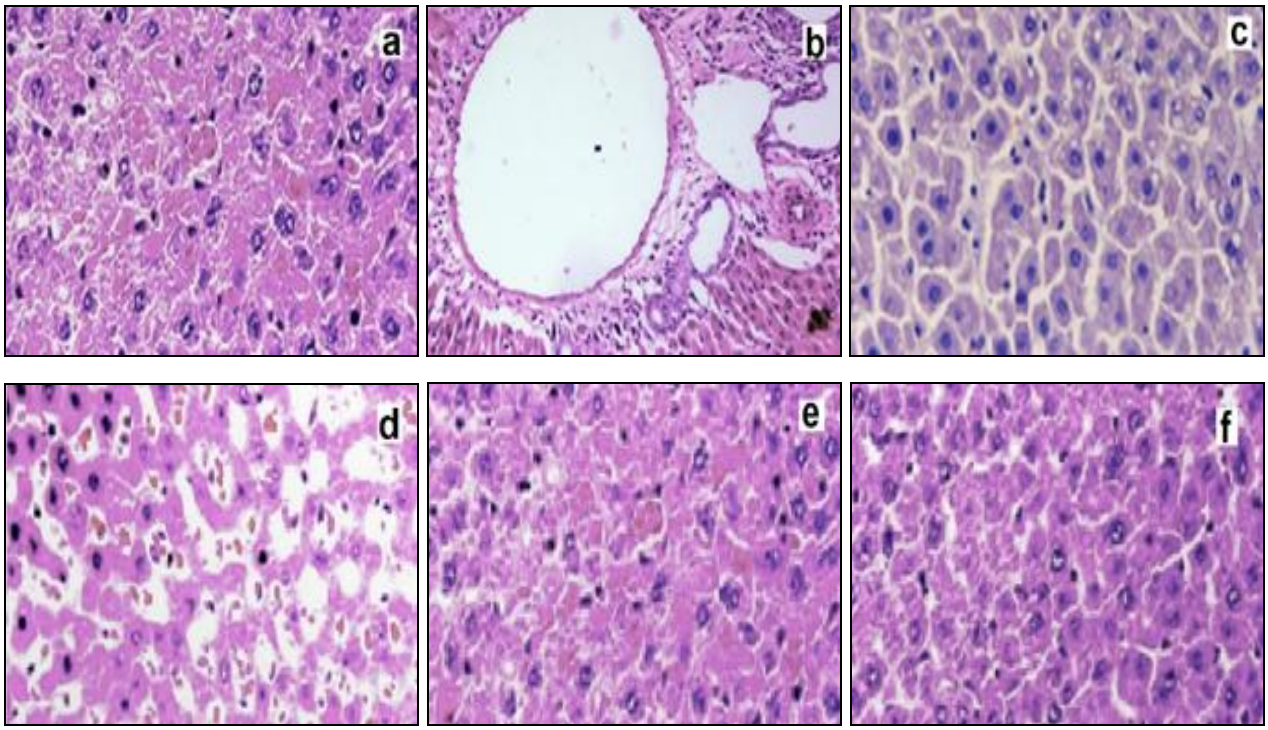
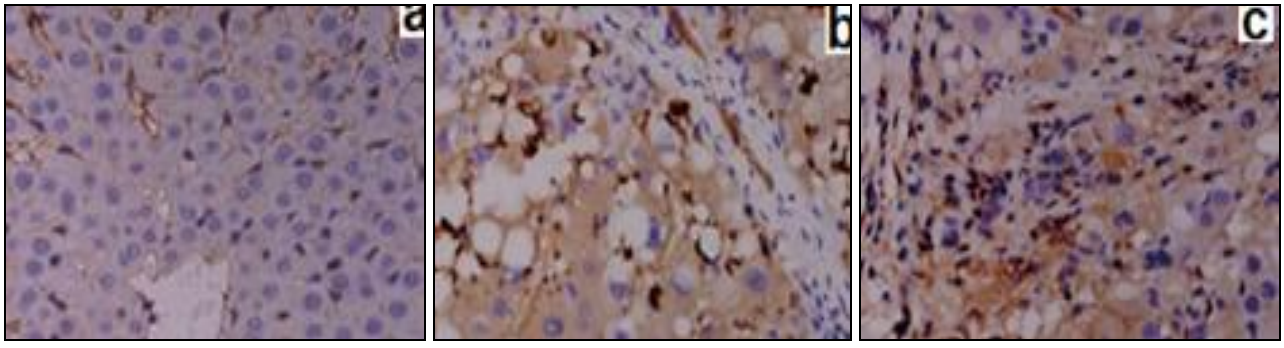


FIG. 6: HISTOPATHOLOGICAL PHOTOMICROGRAPHS OF LIVER SECTIONS. A) NORMAL CONTROL, B) CCL4 CONTROL, C) SILYMARIN TREATED, D) HF 100 MG/KG BW TREATED, E) HF 200 MG/KG BW TREATED, F) HF 400 MG/KG BW TREATED,



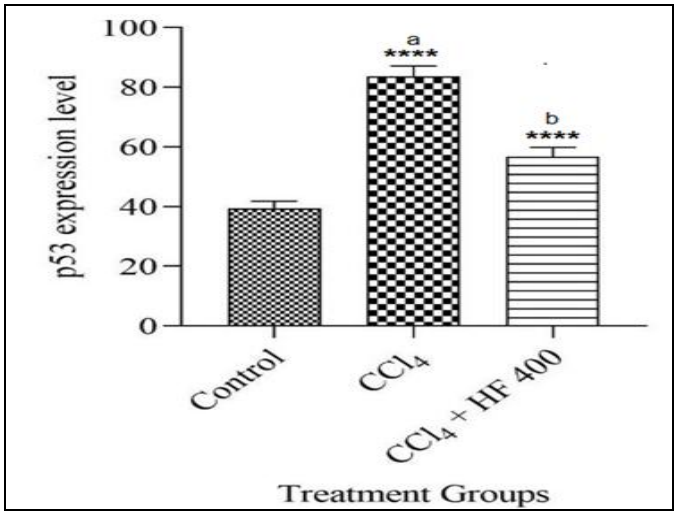


FIG. 7: IMPACT OF HF 400 ON HEPATIC APOPTOSIS INDUCED-CCL4 BY IMMUNOHISTOCHEMISTRY. A POSITIVE EXPRESSION OF P53 WAS FOUND IN CCL4 TREATED (B) COMPARED TO CONTROL (A) RATS. AN IMPROVEMENT WAS SHOWN IN HF 400 TREATED RATS (C), WHERE A LOWER LEVEL OF P53 EXPRESSION WAS OBSERVED. (A P<0.0001 COMPARED TO CONTROL AND B P<0.0001COMPARED TO CCL_4)

Immunohistochemistry of P53: A significantly (P< 0.001) higher expression of p53 was found in CCl_4 treated group (b) compared to control (a) and HF 400 (c). An improvement was shown when HF 400 administrated with CCl_4 (b), **Fig. 7.** The administration of HF 400 in combination with CCl_4 induced a significant (P < 0.05) reduction in p53 expression level **Fig. 7.**

DISCUSSION: The present study demonstrates the heaptoprotective and antioxidant effects of HF against CCl_4–induced liver injury in rats. The liver primarily cleanses toxic chemicals and drugs and becomes the principal target organ for all likely toxic xenobiotics. CCl_4 is a strong hepatotoxin and the most widely utilized chemical agent to induce and explore heaptoprotective activity on numerous experimental animal models. The hepatic impairment and injury experimentally induced by CCl_4 resembles viral hepatitis histologically [31]. CCl_4 is bio transformed to a very active CCl_3 radical in liver by cytochrome P450 enzymes; which in turn reacts with oxygen to generate trichloromethylperoxyl radical ($CCl_3O_2\cdot$), which cause lipid peroxidation of the lipid membranes of the adipose tissue by covalently binding with cellular biological macromolecules including bio membrane structures. There are several biomarkers of lipid peroxidation like peroxide products including Malonaldehyde (MDA), TBARS, *etc*., which can be detected as a hallmark sign of cellular damage or hepatic injury [32]. A plethora of research revealed oxidative stress as a significant pathophysiological modality indicative of cellular damage or injury leading to various disease conditions [33, 35]. In this context, lipid peroxidation is a significant marker of oxidative stress along with other free radical damage that happened in the biochemical cascade. Consequently, the antioxidant potential is observed as one of the significant parameters suggestive of the likely mechanism of HEPA to protection. In necrosis or hepatic injury, serum hepatobiliary enzymes (AST, ALT, ALP, and γ-GT) escape into the circulation, rising serum level of these enzymes [36]. In CCl_4 treated animals, owing to the cellular damage, elevated serum AST, ALT, ALP, and γ-GT levels were observed which is indicative of loss of functional integrity of cell membranes in the liver [36]. This hepatic damage was confirmed by the increased lipid peroxidation induced by CCl_4, indicated by an elevated level of TBARS. In addition, failure of physiological antioxidant defense mechanism was observed by the decreased estimation of CAT, SOD, and GSH in CCl_4 treated animals. Moreover, in hepatic damage, serum levels of cholesterol (TC), triglyceride (TG), and low-density lipoprotein (LDL) were found to be increased significantly in CCl_4 intoxicated rats. However, the high-density lipoprotein (HDL) level was found to be decreased significantly. These alterations were indicative of hepatic damage or hepatotoxicity.

In the present study, antioxidant activity and possible antioxidant mechanism of HF had been explored by assessing various in vitro mechanistic antioxidant assays, including reducing power assay, total antioxidant activity, and DPPH radical scavenging activity. Reducing power assay is one of the most explored and noteworthy measures of antioxidant potential. Primarily ability of bioactive compounds to give hydrogen and electron indicates reducing power [37, 38]. HF exhibited a decent and significant concentration-dependent increase in reducing power. DPPH radical is very prevalent and engrained free radical applied to explore free radical scavenging potential *in-vitro* [39, 40]. HF also demonstrated significant DPPH radicals scavenging

activity. There was also observed a significant inhibition of the degree of lipid peroxidation by HF as advised by the RESULTS of the thiocyanate method in the total antioxidant activity assay. Treatment with HF in different animal groups revealed a significant decrease and restoration in serum AST, ALT, ALP, andγ-GT levels as compared to CCl_4 treated group. The extent of hepatic necrosis and the parallel leakage of hepatic intracellular enzymes were found to be inhibited by stabilizing hepatic cellular membranes by the treatment of HF. The lipid profiles *i.e.,* serum levels of cholesterol (TC), triglyceride (TG), and low-density lipoprotein (LDL) were found to be restored by the treatment with HF. The HDL level was also found to be increased significantly as compared to CCl_4 intoxicated animals. There was observed restoration of the physiological antioxidant defense enzyme system as indicated by the rise in the CAT, SOD, and GSH levels. The decrease in the levels of TBARS in the HF treated animals also indicated a significant heaptoprotection.

These RESULTS clearly revealed the significant heaptoprotective activity of HF as suggested by the various biochemical markers and estimations. Furthermore, the Results were confirmed by histopathological observations, which revealed the protective effect of HF. The effect of CCl_4 and HF (400 mg/kg) on hepatic histology and p53 expression was also studied. CCl_4 treated rats exhibited widespread liver injuries characterized by moderate to severe necrosis, fibrosis, heaptocellular hydropic degeneration and signs of leukocyte infiltrations. Rats treated with HF at 400 mg/kg showed less thick fibrotic tissue, which resulted in less marked annihilation of the liver architecture than the CCl_4 treated group.

Corresponding microscopic examinations, severe hepatic fibrosis induced by CCl_4 was considerably reduced by the administration of HF at 400 mg/kg that was in accordance with the results of the oxidative damage analysis. Expression of p53 was significantly ($P < 0.01$) increased in CCl_4 treated rats than that in the control group. Interestingly, HF treatment at 400 mg/kg dose level markedly ameliorated the hepatic lesions and significantly ($P < 0.05$) decreased p53 expression than CCl_4 treated rats. In the long run, the results of this present study, it can be clinched that the herbal formula has recognized itself as a significant heaptoprotective as well as a significant antioxidant with credible mechanisms.

CONCLUSION: The present research undoubtedly showed the '*in-vitro*' and '*in-vivo*' heaptoprotective as well as antioxidant efficacy of the herbal formula. The herbal formula at all the studied dose levels (100, 200 and 400 mg/kg) demonstrated a significant heaptoprotective activity, well evidenced by the various biochemical markers and histopathological observations.

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