

# Amelioration of Hepato-Renal Toxicity by a Flavonoid-Rich Fraction of *Hibiscus rosa-sinenses* (Leaves and Flowers) in Male Rabbits Intoxicated by Cadmium

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## Abstract

This study was initiated to anticipate the potential protective effects of flavonoid-rich fractions of *Hibiscus rosa-sinenses* flowers (HF) and leaves (HL) on cadmium (Cd)-induced toxicity in adult albino rabbits. Cd, being a prominent environmental pollutant, poses a threat for its widespread range of toxic expressions. A dramatic increase in total phenolics, flavonoids, and antioxidant capacity has been observed in flavonoid-rich fractions of HF and HL as compared to their crude extracts, suggesting for the rationale for their use in this study. Thirty-six male rabbits were indiscriminately allocated into six groups, including control, Cd (6.1 mg Cd/kg/day), HF and HL (200 mg HF & HL /Kg/day), Cd+HF, and Cd+HL (receiving the same doses of Cd, HF, and HL) groups. Treatment (p.o) for 60 days resulted in improvement of weight gain in Cd+HF and Cd+HL groups in comparison with the Cd group. Treatment with Cd+HF showed better results than Cd+HL by significantly decreasing ( $p < 0.05$ ) transaminases (AST, ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), and creatinine and urea levels, while total bilirubin and albumin were increased on the 15<sup>th</sup>, 30<sup>th</sup>, 45<sup>th</sup>, and 60<sup>th</sup> days in a progressive manner when compared with the Cd receiving group. Likewise, the Cd+HF group showed better activities than Cd+HL by significantly increasing ( $p < 0.05$ ) superoxide dismutase, catalase, and vitamins C and E, while LPO and Cd concentrations were significantly decreased in the liver and kidney tissues when compared with Cd-intoxicated bucks. Values of these parameters in only HF and HL-receiving groups were near the control. The histopathological

studies for these tissues have also sustained the above-mentioned data. Thus, these outcomes suggest that the flavonoid-rich fraction of HF may act as a potent hepatic and renal protective agent than HL against Cd-induced toxicity in animal models.

**Keywords:** cadmium toxicity, antioxidant, transaminases, hepato-renal toxicity, histopathological studies

## Introduction

It is apparent that increasingly anthropogenic activities have modified the cycle of heavy metals and metalloids globally, including toxic elements like Cd. Thus a plentiful prospect for exposure to Cd is possible in and outside the workplace [1]. Concentrations of Cd in an adjacent environment is cumulative owing to its miscellaneous sources and very long biological half-life ranging from 10 to 30 years. Cd in biological systems is dispensable and it is known to be toxic even at low concentrations [2-3]. Cadmium exposure (acute and chronic) in plants, animals, and humans may cause harm to various organs, including the brain, gastro-intestinal tract, liver, lungs, and testes. Many related disorders include renal dysfunction, hepatotoxicity, anemia, osteoporosis, DNA damage, neurodegenerative diseases, and immunosuppressive and proinflammatory effects [3-6]. A number of mechanisms have been proposed to explain the damage induced by Cd, but it is not yet well understood, although some experimental evidence can determine that deleterious effects of cadmium are due to oxidative stress by depletion of the endogenous antioxidants that serve as a premier source of protection against free radicals and other oxidative stressors. Conventionally, ethno medicines are being used widely in the sub-continent and elsewhere generally owing to their low cost, easy accessibility, and apparently reduced side effects [3]. The mechanism of action of these herbal remedies is diverse in many respects from that of the synthetic drugs/pure compounds. This effect may be regarded as polyvalent action and understood as an additive.

The *H. rosa-sinensis* L. (family Malvaceae) evergreen medicinal shrub is commonly used in conventional medicine (red-flowered variety) as an antidiabetic and for gastrointestinal disorders, and for its cardioprotective, anticarcinogenic, antioxidative, renoprotective, hepatoprotective, neuroprotective, and anti-inflammatory properties, and for managing cognitive disorders [7-11]. The phytochemicals reported so far in *Hibiscus rosa sinensis* L. include alkaloids, amino acids, anthocyanins, carbohydrate, flavonoids, glycosides, phytosterols, polyphenols, and vitamins [7, 12-13]. The promising protective activity of *H. rosa-sinensis* flavonoid-rich fractions, leaves, and flowers against Cd-induced renal and hepatotoxicity has not been reported so far. Therefore, this study aims to evaluate the potential defensive effects of the *H. rosa-sinensis* by using the Cd-induced sub-chronic oxidative hepato-renal injury model in male rabbits.

## Materials and Methods

### Collection, Identification and Extraction of Plant Material

Fresh leaves and mature flowers of *Hibiscus rosa-sinensis* Linn (red-flowered variety) were collected from the Botanical Garden of the University of Punjab, Lahore, during July-August 2016. Identification and authentication were performed by the Department of botany, GC University, Lahore. The plant material was also duly identified by Prof. Dr. Abdul Nasir Khalid, the in-charge herbarium and senior taxonomist for the Department of Botany at the University of the Punjab, Lahore-Pakistan. Fresh leaves and mature flowers were washed with distilled water, air-dried, and finely ground and extracted with ethanol (1:20 w/v) using an ultrasonic bath for six hrs at room temp. After filtration through Wattman filter paper No. 1 it was evaporated to dryness at 40°C using a rotary evaporator (Edolph Rotary, Germany) at PCSIR Laboratories Complex, Lahore. Extraction was carried out in triplicate to increase the percentage yield of extracts. Then crude extracts of flowers and leaves were suspended in double-distilled H<sub>2</sub>O, then partitioned successively with pet-ether and ethyl acetate (EtOAc), and both fractions were evaporated to dryness using a rotary evaporator, then obtained EtOAc fraction was dissolved in ethanol (etOH) and precipitation was achieved, in EtOAc fraction, by treating this fraction with neutral lead acetate. These precipitates were centrifuged, re-suspended in etOH, treated with H<sub>2</sub>S, and filtered to harvest the flavonoid-rich fraction by evaporation according to the method of [14]. These EtOAc fractions were preferably selected, out of crude extracts and pet ether fractions, due to their more phenolic and flavonoid contents and high antioxidant potential. These fractions were retained in glass bottles, covered with aluminum foil, and kept in a refrigerator until further use.

### *In-vitro* Determination of Total Phenolic, Flavonoids and Antioxidant Contents

The total phenolic and flavonoid contents of crude extracts and flavonoid-rich fractions were determined, based on Folin-Ciocalteu's reagent and the aluminum chloride method, respectively, as described by the method of Singleton and [15-16]. Results of total phenolic and flavonoid contents of crude extracts and flavonoids rich fractions were stated as mg gallic acid equivalents (GAE) and quercetin equivalents (QE) per gram of dry

weight of sample. Total antioxidant capacity (TAOC) was determined using the phospho-molybdenum method by Prieto et al. [17], and stated as mg BHT equivalents (BHTE) per gram of sample.

### Identifying Polyphenolic Compounds

The flavonoid-rich fraction was analyzed by thin layer chromatography (TLC) for the presence of gallic acid using a mobile phase comprised of toluene:ethyl acetate:formic acid:methanol (3:3:0.8:0.2 v/v), quercetin using toluene:ethyl acetate:formic acid (5:4:1 v/v), and rutin using ethyl acetate:n-butanol:formic acid:water (5:3:1:1 v/v) at room temperature (25±2°C) as described as [14, 18]

### Animals

Male albino rabbits (*Oryctolagus cuniculus*), aged between 22-28 weeks and weighing between 1.8-2.4 Kg, bred in the animal house of the Pakistan Council of Scientific and Industrial Research, Lahore-Pakistan were used in this study. Rabbits were offered a routine diet and free access to fresh drinking water. Animals were kept in individual cages and were handled carefully under standard conditions of 12h dark light cycle in temperature 24±3°C and relative humidity of 50-60%. Before commencing this experiment, all animals were injected with 0.1mL/Kg body weight ivermectin (s.c.) [19] to eradicate endo and ecto-parasites, followed by a week of adjustment period. This treatment, with ivermectin, will help to minimize the interference of parasites in the course of the study. The study protocols were approved (dated 18/06/2014) by the Advanced Studies and Research Board of Punjab University, Pakistan. Experimental protocols were in agreement with the NIH guidelines for the care and use of laboratory animals. Measurements of live body weights were recorded carefully, on electronic balance, after every 10 days up to 60 days.

### Selection of Cadmium and *H. rosa sinensis* Dosage

The 1/10<sup>th</sup> dose of LD<sub>50</sub> of Cd (as CdCl<sub>2</sub>), determined previously as 61.34 mg/Kg body weight [20], corresponding to 6.1 mg/Kg body weight, was selected to induce toxicity in male albino rabbits. In order to assess the pertinent dose of ethyl acetate fractions of *H. rosa sinensis* leaves and flowers, three different concentrations, i.e., 50, 100, and 200 mg/Kg body wt. per day, were given via gastric lavage for 15 days. Various biochemical parameters like creatinine, ALP, ALT, AST, and urea were estimated in serum (data not shown). Results of these diagnostic factors recommend that 200 mg/Kg body wt. per day was a suitable dose to further conduct this study for a period of 60 days.

### Experimental

The experimental design has been made in agreement with the investigational design of Milton Prabu [1]. Six rabbits were apportioned randomly into six groups. These groups were as follows:

1. Control: non-treated animals only received vehicle (distilled water).
2. Received only leaf extract (HL) at a dose of 200 mg/Kg/day.
3. Received only flower extract (HF) at a dose of 200 mg/Kg/day.
4. Received cadmium in normal saline at a dose of 6.1 mg/Kg/day.
5. Rabbits received 6.1mg/Kg/day cadmium and 200 mg/Kg/day flower extract (HF).
6. Rabbits received 6.1mg/Kg/day cadmium and 200 mg/Kg/day leaf extract (HL).

This experimental study was conducted for 60 days, representing a chronic model according to Bae, et al. [21]. All the doses were administered per oral.

### Blood and Tissue Sampling

The day before the initiation of the experiment and after every fifteenth day, 5 ml of blood was collected from the marginal ear vein of each rabbit from all six groups. Samples were divided into two aliquots, one of 2 ml in EDTA containing a tube for CBC (data not shown) and the other of 3 ml in plain dry vacutainer to separate serum after centrifugation (1,000 rpm, 10 min.) for selected biochemical analysis. On the 60<sup>th</sup> day, we dissected all rabbits from each group to remove liver, kidneys, heart, lungs, brain, stomach, testis, small intestine, skeletal muscle, and the femur bone for analysis of cadmium residue (data not shown). Both kidneys and liver were rinsed with cold normal saline, dried, and weighed individually. Small portions of the left kidney and caudal part of the liver were preserved for histopathological examinations and about one gram of each tissue was minced and homogenized (10 times the weight of tissue) in appropriate buffer (pH 7.4) and cold centrifuged (2,000 g for 10 min). The resulting clear supernatant was collected for further estimations.

### Estimating Markers of Hepatic and Renal Damage

The serum hepatic and serum renal markers such as alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT), creatinine, urea, albumin, total protein (TP), and total bilirubin were tested using chemistry analyzer (Microlab-300, Merck) according to the standard procedures by using diagnostic kits from Sigma diagnostics (Pvt.) Limited, Pakistan. At the start and after every

10<sup>th</sup> day of experiment, the urine was collected in metabolic cages on a 24-hr basis for each animal from all groups. Levels of creatinine and urea in urine were assessed by spectrophotometric methods using commercial Sigma diagnostic kits, Pakistan.

#### Determination of Lipid Peroxidation and Cadmium Contents in Tissues

Lipid peroxidation (LPO) in both kidney and liver tissues was assessed by the thiobarbituric acid reacting substance (TBARS) method of Placer et al. [22] and was stated in terms of the malondialdehyde (MDA) content, which served as the standard of 1,1,3,3-tetraethoxypropane. Samples of tissues, assayed for MDA, were added with 1.0 milli-molar BHT for the purpose of preventing artefactual lipid peroxidation in the course of the boiling step. The kidney and liver samples were oven-dried, weighed, and digested with nitric acid and hypochloric acid to estimate the residual level of Cd by AAS (Perkin Elmer, Analyst 800). A calibration curve was constructed with 1, 2, and 3 ppm standards of Cd (Perkin Elmer). For certainty of results bovine liver (NIST: 1577b) was used as certified material.

#### Determining Antioxidant Enzymes, Vitamin C and E in Tissues

The activity of superoxide dismutase (SOD) and catalase (CAT; EC 1.11.1.6) was measured in tissue homogenates by the method of [23-24] and using a spectrophotometer at 560 nm and 240 nm, respectively. Ascorbic acid and  $\alpha$ -tocopherol (Vitamins C and E) were assayed in homogenates of kidney and liver according to the methods of [25-26].

#### Histopathological Studies

A small portion of left kidney and caudal part of the liver were fixed for 24 hrs in buffered formalin (10%), then washed under tap water for 12 hrs, dehydrated by passing through different aqueous ethanol mixtures, being cleaned

in xylene, and embedded in soft white paraffin. Thin tissue sections (5-6  $\mu$ m thick) were prepared by rotary microtome and staining was done with haematoxylin and eosin dye with neutral DPX medium for microscopic observations.

#### Statistical Analysis

All the data were stated as mean values with standard deviation ( $\pm$ S.D.) for six rabbits in all groups. The statistical significance was calculated by using two factors of completely randomized design (CRD) using SPSS (version 16.0), and the individual comparisons were obtained by least significant difference (LSD). Values were considered statistically significant when  $p < 0.05$ .

### Results and Discussion

#### Determination of Total Phenolics, Flavonoids, and Antioxidant Contents

Bioactive compounds were extracted from leaves and flowers in Aq. methanolic crude extracts, fractions of petroleum ether, and ethyl acetate obtained from sequential partitioning of crude extracts. This study showed variable amounts of extraction yields (g/100 g of dry weight), TPC (mg GAE/100g), TFC (mg QE/100g), and TAOC (mg AAE/g) among various fractions of both crude extracts. Yield, TPC, TFC, and TAOC of ethyl acetate fraction of flowers were higher, i.e., 72.36 $\pm$ 2.6, 90.5 $\pm$ 4.8, 42.2 $\pm$ 2.1, and 77.8 $\pm$ 1.7 than ethyl acetate fraction of leaves, i.e., 68.36 $\pm$ 2.6, 78.5 $\pm$ 4.8, 33.2 $\pm$ 2.1, and 69.8 $\pm$ 1.7, respectively, whereas Pet. ether fractions have showed the lowest values when compared to ethyl acetate fractions of both extracts as shown in Table 1. Our findings are in near agreement with [10, 27-29].

The dissimilarities in TPC, TFC, and TAOC values may be attributed to the use of different solvents for fractionation. It has been designated in numerous studies that higher levels of polyphenols may be associated with the use of more polar solvents for extraction [30]. Flavonoids and phenolics are produced as natural secondary

Table 1. Percentage yields, total phenolics, flavonoids, and antioxidant capacity of *H. rosa-sinensis* L. flowers and leaf extracts.

Parts used	Extract/ Fraction	Percentage yields (g/100g DW)	TPC (mg GAE/g DW)	TFC (mg QE/g DW)	TAOC (mg BHTE/g DW)
Flower	Aq. MeOH	19.31 $\pm$ 2.1	51.30 $\pm$ 2.5	46.51 $\pm$ 1.5	37 $\pm$ 1.9
	Pet. Ether	13.48 $\pm$ 0.9	7.8 $\pm$ 0.7	3.2 $\pm$ 0.4	8.2 $\pm$ 0.3
	EtOAc	72.36 $\pm$ 2.8	90.56 $\pm$ 5.1	62.21 $\pm$ 2.2	77 $\pm$ 2.9
Leaves	Aq. MeOH	15 $\pm$ 2.2	39.64 $\pm$ 1.5	38.90 $\pm$ 1.2	32 $\pm$ 1.6
	Pet. Ether	14.28 $\pm$ 0.8	4.3 $\pm$ 0.5	2.8 $\pm$ 0.3	6.5 $\pm$ 0.2
	EtOAc	66.16 $\pm$ 2.6	78 $\pm$ 3.6	53 $\pm$ 1.8	69 $\pm$ 3.2

\*weight of fraction/weight of crude extract 100

Data are presented as means  $\pm$  standard deviation (n = 3); GAE: Gallic acid equivalents; QE: quercetin equivalents; BHTE: butylated hydroxy toluene equivalents; DW: dry weight; Aq. MeOH: aqueous methanol; EtOAc: ethyl acetate

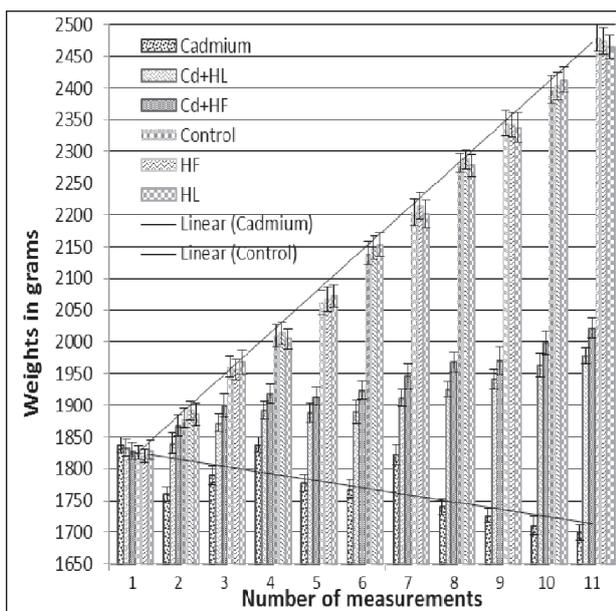


Fig. 1. Variations of body weights in albino rabbits treated sub-chronically with Cd, Cd+HL, Cd+HF, control, HF, and HL from the start to day 60.

metabolites and possess rich antioxidant properties. These are capable of interacting and scavenging free radicals, which primarily impair cells and may result in the genesis of various diseases. Our results clearly illustrate that phenolic and flavonoid compounds like quercetin and rutin (identified by TLC) are most probably the major contributors to the observed higher antioxidant properties and subsequently result in the prevention of hepato-renal tissues of Cd-intoxicated rabbits.

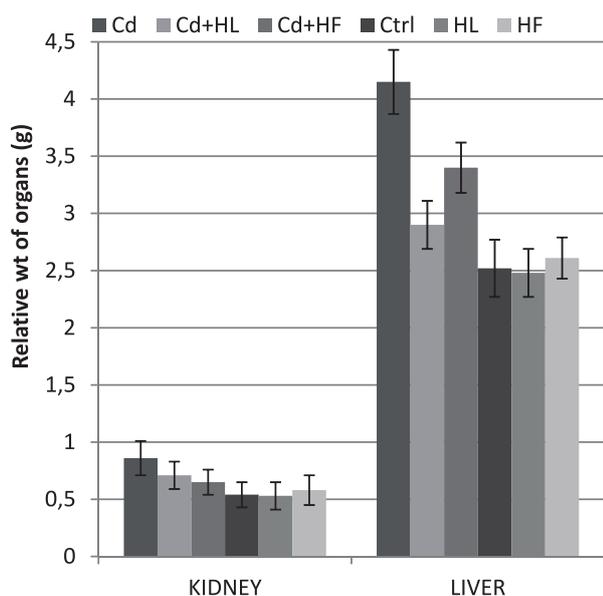


Fig. 2. Comparison of relative weight (g) in liver and kidney of male albino rabbits on day 60 of the experiment (mean  $\pm$  S.D, n = 6).

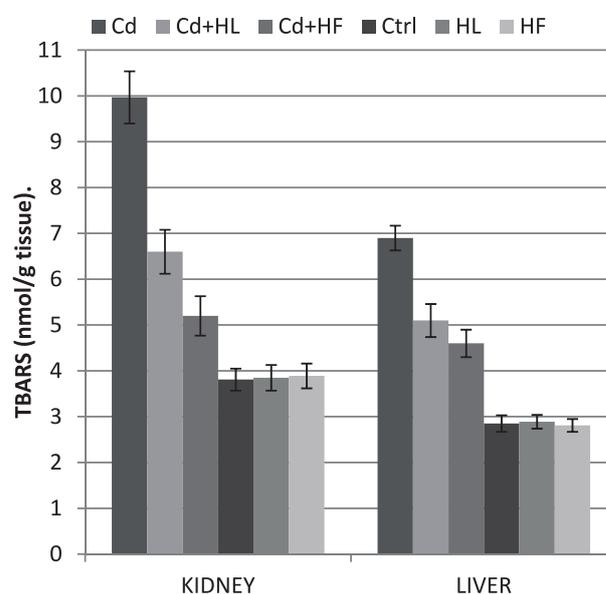
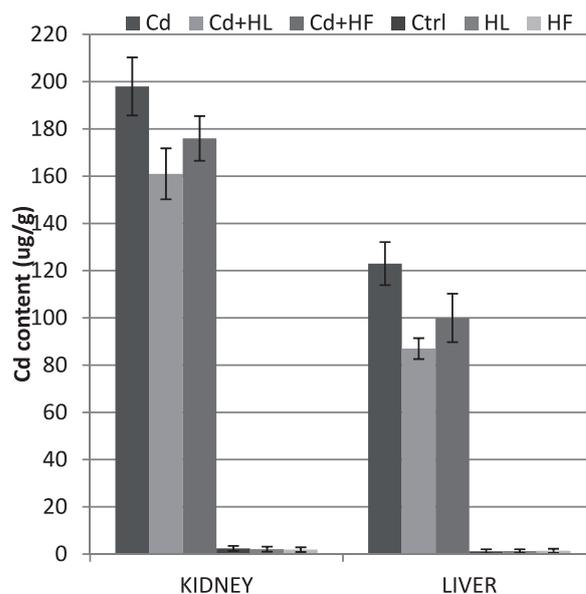


Fig. 3. Comparison of Cd content ( $\mu\text{g/g}$  tissue), and LPO content (nmol/g tissue) in liver and kidney of male albino rabbits on day 60 of the experiment (mean  $\pm$  S.D, n = 6).

### Changes in Body Weights of Rabbits

Fig. 1. shows the variation in body weight among control, Cd-, Cd+HF-, Cd+HL-, HF-, and HL- treated animals during 60 days of this study. Weights were measured carefully on an electronic balance and were recorded after every six days until day 60. The Cd-treated group showed a decreasing trend in gaining weight from start to the end of the study as compared to control as suggested by [31-32]. These results also revealed that the decreased weight gain due to Cd exposure existed even with the quantity of food consumption not being disturbed. No change in food consumption habit of animals was observed in the Cd-receiving group. This indicates that this effect of Cd was intervened by

Table 2. Values of serum hepatic markers in male albino rabbits at the start and on days 15, 30, 45, and 60 of the experiment (mean  $\pm$  S.E.M., n = 6).

Treatment	Days				
	0	15	30	45	60
ALT (IU/L)					
Cd	52 $\pm$ 4.87I	82 $\pm$ 6.03F	136 $\pm$ 6.85C	193 $\pm$ 7.53B	218 $\pm$ 7.09A
Cd + HF	51 $\pm$ 4.34I	60 $\pm$ 4.82HI	75 $\pm$ 6.54FG	99 $\pm$ 8.02E	128 $\pm$ 6.23C
Cd + HL	50 $\pm$ 5.09I	68 $\pm$ 5.93GH	84 $\pm$ 6.72F	113 $\pm$ 7.92D	143 $\pm$ 7.32G
HF	51 $\pm$ 4.79I	53 $\pm$ 5.93I	52 $\pm$ 5.18I	50 $\pm$ 5.40I	55 $\pm$ 4.52I
HL	54 $\pm$ 5.02I	52 $\pm$ 4.94I	50 $\pm$ 4.86I	53 $\pm$ 4.86I	51 $\pm$ 5.37I
Control	53 $\pm$ 5.22I	54 $\pm$ 4.47I	51 $\pm$ 5.37I	50 $\pm$ 4.38I	52 $\pm$ 4.05I
AST (IU/L)					
Cd	69 $\pm$ 4.56I	104 $\pm$ 6.90G	161 $\pm$ 7.90DE	219 $\pm$ 7.38B	249 $\pm$ 7.64A
Cd + HF	72 $\pm$ 4.90I	88 $\pm$ 6.47H	125 $\pm$ 7.56F	153 $\pm$ 8.10E	171 $\pm$ 8.81CD
Cd + HL	71 $\pm$ 3.99I	92 $\pm$ 6.80GH	131 $\pm$ 8.04F	165 $\pm$ 8.49DE	183 $\pm$ 7.62C
HF	69 $\pm$ 4.90I	70 $\pm$ 5.18I	71 $\pm$ 4.86I	74 $\pm$ 4.86I	72 $\pm$ 5.00I
HL	68 $\pm$ 4.47I	69 $\pm$ 4.90I	73 $\pm$ 5.29I	70 $\pm$ 4.23I	71 $\pm$ 5.18I
Control	70 $\pm$ 4.24I	69 $\pm$ 4.43I	74 $\pm$ 6.20I	72 $\pm$ 4.69I	73 $\pm$ 4.98I
ALP (IU/L)					
Cd	114 $\pm$ 4.63IJ	151 $\pm$ 6.87G	203 $\pm$ 7.16E	264 $\pm$ 8.07B	287 $\pm$ 8.32A
Cd + HF	111 $\pm$ 4.83J	127 $\pm$ 6.23HI	161 $\pm$ 7.07G	194 $\pm$ 7.92E	219 $\pm$ 7.16D
Cd + HL	113 $\pm$ 3.73J	134 $\pm$ 7.07H	174 $\pm$ 7.51F	204 $\pm$ 8.46E	232 $\pm$ 8.27C
HF	110 $\pm$ 4.37J	114 $\pm$ 5.16IJ	112 $\pm$ 4.77J	111 $\pm$ 3.83J	113 $\pm$ 4.28J
HL	115 $\pm$ 4.02IJ	111 $\pm$ 4.36J	113 $\pm$ 4.28J	110 $\pm$ 3.08J	114 $\pm$ 3.72IJ
Control	112 $\pm$ 4.72J	110 $\pm$ 3.88J	115 $\pm$ 4.58IJ	109 $\pm$ 5.08J	111 $\pm$ 5.34J
GGT (IU/L)					
Cd	2.1 $\pm$ 0.26FG	2.84 $\pm$ 0.49DG	4.29 $\pm$ 0.77B	5.82 $\pm$ 0.78A	6.59 $\pm$ 0.90A
Cd + HF	2.04 $\pm$ 0.18FG	2.23 $\pm$ 0.30EFG	2.93 $\pm$ 0.62DEF	3.80 $\pm$ 0.87BCD	4.31 $\pm$ 0.82B
Cd + HL	2.07 $\pm$ 0.21FG	2.37 $\pm$ 0.41EFG	3.13 $\pm$ 0.68CDE	4.08 $\pm$ 0.73BC	4.68 $\pm$ 0.77B
HF	2.10 $\pm$ 0.23FG	2.07 $\pm$ 0.24FG	2.09 $\pm$ 0.19FG	1.96 $\pm$ 0.20FG	1.92 $\pm$ 0.24FG
HL	2.10 $\pm$ 0.29FG	2.14 $\pm$ 0.27FG	1.99 $\pm$ 0.19FG	2.02 $\pm$ 0.17FG	2.12 $\pm$ 0.27FG
Control	2.07 $\pm$ 0.19FG	1.98 $\pm$ 0.16FG	2.1 $\pm$ 0.14FG	2.08 $\pm$ 0.21FG	1.94 $\pm$ 0.20FG
Total Bilirubin (mg/dL)					
Cd	0.22 $\pm$ 0.03I	0.57 $\pm$ 0.05EF	0.94 $\pm$ 0.08D	1.49 $\pm$ 0.10B	1.71 $\pm$ 0.21A
Cd + HF	0.23 $\pm$ 0.02I	0.39 $\pm$ 0.04GH	0.64 $\pm$ 0.05E	0.90 $\pm$ 0.09D	1.20 $\pm$ 0.15C
Cd + HL	0.24 $\pm$ 0.02HI	0.42 $\pm$ 0.03FG	0.72 $\pm$ 0.06E	0.99 $\pm$ 0.08D	1.31 $\pm$ 0.17C
HF	0.22 $\pm$ 0.02I	0.23 $\pm$ 0.2I	0.26 $\pm$ 0.03HI	0.22 $\pm$ 0.03I	0.25 $\pm$ 0.03HI
HL	0.23 $\pm$ 0.03I	0.24 $\pm$ 0.03HI	0.22 $\pm$ 0.03I	0.26 $\pm$ 0.03HI	0.24 $\pm$ 0.02HI
Control	0.26 $\pm$ 0.03HI	0.23 $\pm$ 0.02I	0.25 $\pm$ 0.03HI	0.24 $\pm$ 0.03HI	0.22 $\pm$ 0.04I

Means sharing similar letter in a row or column (for each parameter) are statistically non-significant ( $P > 0.05$ ).

enhancing metabolism rather than disturbing feeding mechanism. However, the incremental trend in gaining weight for Cd+HF and Cd+HL was seen when compared with the Cd-treated group. The administration of HF and HL to Cd-intoxicated rabbits was capable of protecting against the lowered body weight gain and overall health of rabbits owing to the presence of polyphenols and flavonoids, mainly due to their antioxidant potential that may have led to counteract the damage caused by Cd. The relative weight of kidney and liver showed significant increases in the Cd-receiving group when compared to control as suggested by [33] and as shown in Fig. 2. Values of HF and HL-treated animals were in agreement with the control group and did not show significant variations in weight gain and relative organ weight (kidney, liver) as shown in Figs 1 and 2.

### Status of Serum Hepatic Marker Enzymes

The present study was conducted to assess the potential protective effects of *H. rosa sinensis* on liver toxicity due to oxidative stress caused by Cd in rabbits for 60 days. Table 2 shows the serum enzyme and total bilirubin of the control, Cd, Cd+HF, Cd+HL, HF, and HL-treated adult male rabbits groups. Administration of oral CdCl<sub>2</sub> resulted in abnormal values of hepatic functions, in a progressive manner, from day 0 to days 15, 30, 45 and 60 of the experiment. This incremental trend was found to be significant ( $p < 0.05$ ) when compared with animals of the control group. In the Cd-treated group, values of serum transaminases such as ALT and AST were increased from 52 to 82, 137, 193, and 218, and from 69 to 104, 161, 219, and 249 IU/L, respectively. An increment in the levels of ALP and GGT from 114 to 151, 203, 264, and 287, and from 2.13 to 2.84, 4.29, 5.82, and 6.59 IU/L was observed, respectively. Total bilirubin was also augmented from 0.20 to 0.57, 0.94, 1.47, and 1.71 mg/dL. Values of all above-mentioned parameters have shown a significant incremental trend ( $p < 0.05$ ) in Cd-intoxicated rabbits when compared to the control group. Our findings are in close agreement with [34-35] that Cd intoxication significantly raise total bilirubin and liver enzymes in serum. The administration of the ethyl acetate fraction of *Hibiscus* flowers (200 mg/kg/day) plus Cd (6.1 mg/kg/day) greatly reduced these levels in *Hibiscus* leaves (200 mg/kg/day) plus Cd (6.1 mg/kg/day) at days 15, 30, 45, and 60 when compared only to the Cd-receiving group. In Cd+HF levels of liver, enzymes such as ALT and AST decreased from 218 to 130 and from 249 to 171 IU/L, whereas in the Cd+HL group ALT and AST were decreased from 218 to 141 and from 249 to 183 IU/L at day 60, respectively, when compared to the Cd-receiving group. The levels of ALP and GGT in Cd+HF decreased from 287 to 219 and from 6.59 to 4.24 IU/L, whereas in the Cd+HL group ALP and GGT decreased from 287 to 232 and from 6.59 to 4.68 IU/L at day 60, respectively, when compared to the Cd-receiving group. Total bilirubin also decreased from 1.71 to 1.20 mg/dL in Cd+HF, whereas in the Cd+HL group it was decreased from 1.71 to 1.31 mg/dL at day 60

when compared to the Cd-receiving group. Levels of liver enzymes and bilirubin persisted at satisfactory levels in HF and HL groups when compared with the control group as shown in Table 2.

The elevated levels of hepatic enzymes in serum may indicate their leakage from hepatic cells into the bloodstream, thus damaging the hepatic histo-architecture. Higher levels of transaminases and alkaline phosphatase are the decisive factors for identifying liver injury in Cd-intoxicated rabbits as described by [35]. Serum GGT, an enzyme bound to membrane, is the prominent indicator of cellular and tissue injury by lethal substances, and its level is also significantly increased in the Cd-intoxicated group of rabbits. In this study it raised levels of markers of liver injury, recommending that Cd has caused functional and structural injury to the cellular membranes. It has also augmented the membrane permeability that results in the leakage of liver enzymes into the bloodstream. Total bilirubin was also increased in Cd-treated animals, suggesting that augmented levels of serum bilirubin is also a strong marker of liver dysfunction. Hepatic damage caused by oxidative stress [29, 36] can be protected either by employing strong antioxidants that inhibit the generation of free radicals. Several outcomes of a number of studies have also ascertained the protective effects of plant extracts on Cd-induced hepatotoxicity [1, 37-39].

### Status of Urine and Serum Renal Marker Enzymes and Serum Proteins

Table 3 shows the urinary and serum renal enzymes, serum albumin, and total proteins of control, Cd-, Cd+HF-, Cd+HL-, HF-, and HL-treated adult male rabbit groups. Administering oral Cd caused unusually elevated renal functions in a progressive manner from day 0 to days 15, 30, 45, and 60 of the experiment. The levels of serum creatinine and urea in the Cd-intoxicated group were increased from 1.62 to 2.5, 3.2, 4.5, and 5.42, and from 36 to 51, 67, 79, and 99 mg/dL, respectively, representing a significant incremental trend ( $p < 0.05$ ) in the Cd-treated group when compared to the control group as described by [34]. The administration of ethyl acetate fraction of *Hibiscus* flowers (200 mg/kg/day) plus Cd (6.1 mg/kg/day) has greatly reduced these levels compared to the fraction of *Hibiscus* leaves (200 mg/kg/day) plus Cd (6.1 mg/kg/day) at days 15, 30, 45, and 60 when compared to only the Cd-receiving group. In Cd+HF levels of serum, renal markers such as creatinine and urea were decreased from 5.42 to 2.65 and from 99 to 69 mg/dL, whereas in the Cd+HL group these values were decreased from 5.42 to 2.95 and from 99 to 72 mg/dL at day 60 respectively when compared to the Cd-treated group. The values of urinary creatinine and urea were decreased in the Cd-intoxicated group from 3.1 to 2.2, 1.4, 1.24, and 1.08, and from 134 to 107, 87, 71, and 58 mg/dL, respectively, representing a significant decreasing trend ( $p < 0.05$ ) in the Cd-induced group when compared to the control group as suggested by [40]. The administration of ethyl acetate fraction of *Hibiscus*

Table 3. Values of albumin, creatinine, total proteins and urea (serum) and creatinine, and urea (in urine) in male albino rabbits at the start and on days 15, 30, 45, and 60 of the experiment (mean  $\pm$  S.D, n= 6).

Treatment Groups	Days				
	0	15	30	45	60
Serum Albumin (mg/dL)					
Cd	3.77 $\pm$ 0.26A	3.1 $\pm$ 0.22D	2.2 $\pm$ 0.15G	1.6 $\pm$ 0.14H	1.1 $\pm$ 0.09I
Cd+HF	3.7 $\pm$ 0.30A	3.2 $\pm$ 0.24BCD	2.9 $\pm$ 0.14DE	2.5 $\pm$ 0.19EFG	2.2 $\pm$ 0.21G
Cd+HL	3.75 $\pm$ 0.27A	3.13 $\pm$ 0.22CD	2.7 $\pm$ 0.15DEF	2.3 $\pm$ 0.16FG	2.03 $\pm$ 0.20GH
HF	3.8 $\pm$ 0.21A	3.64 $\pm$ 0.26AB	3.65 $\pm$ 0.21AB	3.72 $\pm$ 0.25A	3.70 $\pm$ 0.26A
HL	3.68 $\pm$ 0.25AB	3.7 $\pm$ 0.29A	3.8 $\pm$ 0.26A	3.65 $\pm$ 0.26AB	3.78 $\pm$ 0.29A
Control	3.63 $\pm$ 0.23AB	3.75 $\pm$ 0.26A	3.69 $\pm$ 0.22AB	3.7 $\pm$ 0.24A	3.8 $\pm$ 0.30A
Serum Total proteins (mg/dL)					
Cd	7.5 $\pm$ 0.51A	6.5 $\pm$ 0.33BCD	5.3 $\pm$ 0.29FG	4.1 $\pm$ 0.26H	2.9 $\pm$ 0.18I
Cd+HF	7.45 $\pm$ 0.39A	7.1 $\pm$ 0.49AB	6.5 $\pm$ 0.36BCD	6.0 $\pm$ 0.3DEF	5.2 $\pm$ 0.21FG
Cd+HL	7.55 $\pm$ 0.36A	6.9 $\pm$ 0.24ABC	6.2 $\pm$ 0.32CDE	5.6 $\pm$ 0.29EFG	4.9 $\pm$ 0.24GH
HF	7.42 $\pm$ 0.44A	7.38 $\pm$ 0.46A	7.45 $\pm$ 0.52A	7.4 $\pm$ 0.46A	7.53 $\pm$ 0.37A
HL	7.47 $\pm$ 0.40A	7.43 $\pm$ 0.39A	7.53 $\pm$ 0.46A	7.48 $\pm$ 0.45A	7.5 $\pm$ 0.41A
Control	7.3 $\pm$ 0.47AB	7.4 $\pm$ 0.41A	7.5 $\pm$ 0.57A	7.35 $\pm$ 0.53AB	7.6 $\pm$ 0.41A
Serum Creatinine (mg/dL)					
Cd	1.6 $\pm$ 0.24IJ	2.5 $\pm$ 0.28DEF	3.2 $\pm$ 0.34C	4.5 $\pm$ 0.30B	5.45 $\pm$ 0.34A
Cd+HF	1.55 $\pm$ 0.23IJ	1.74 $\pm$ 0.22HIJ	2.08 $\pm$ 0.27FI	2.32 $\pm$ 0.26EFG	2.77 $\pm$ 0.31CDE
Cd+HL	1.58 $\pm$ 0.17IJ	1.90 $\pm$ 0.28GJ	2.22 $\pm$ 0.21FGH	2.51 $\pm$ 0.19DEF	2.95 $\pm$ 0.30CD
HF	1.6 $\pm$ 0.28IJ	1.55 $\pm$ 0.23IJ	1.58 $\pm$ 0.19IJ	1.62 $\pm$ 0.21IJ	1.52 $\pm$ 0.20J
HL	1.62 $\pm$ 0.25IJ	1.56 $\pm$ 0.22IJ	1.57 $\pm$ 0.21IJ	1.65 $\pm$ 0.29IJ	1.54 $\pm$ 0.21J
Control	1.53 $\pm$ 0.22IJ	1.58 $\pm$ 0.29IJ	1.6 $\pm$ 0.18IJ	1.59 $\pm$ 0.17IJ	1.62 $\pm$ 0.12IJ
Serum Urea (mg/dL)					
Cd	36 $\pm$ 2.83IJ	51 $\pm$ 3.46FG	67 $\pm$ 4.34CD	79 $\pm$ 4.24B	99 $\pm$ 4.47A
Cd+HF	35.5 $\pm$ 2.88IJ	42 $\pm$ 3.03HI	51 $\pm$ 3.58FG	60 $\pm$ 3.22DE	69 $\pm$ 3.90C
Cd+HL	37 $\pm$ 2.83HIJ	44 $\pm$ 2.61GH	56 $\pm$ 3.41EF	66 $\pm$ 3.35CD	72 $\pm$ 3.74BC
HF	37 $\pm$ 3.10HIJ	36.5 $\pm$ 2.88IJ	33.5 $\pm$ 3.2IJ	33 $\pm$ 3.41J	37 $\pm$ 3.29HIJ
HL	36.6 $\pm$ 2.66IJ	37 $\pm$ 3.41HIJ	35 $\pm$ 3.35IJ	34 $\pm$ 3.41J	36.5 $\pm$ 2.88IJ
Control	35 $\pm$ 3.35IJ	35.5 $\pm$ 2.59IJ	37 $\pm$ 3.58HIJ	34.5 $\pm$ 3.39J	36 $\pm$ 3.16IJ
Creatinine in urine (mg/dL)					
Cd	3.1 $\pm$ 0.26A	2.4 $\pm$ 0.19B	1.8 $\pm$ 0.17CD	1.4 $\pm$ 0.14DE	1.08 $\pm$ 0.19E
Cd+HF	3.05 $\pm$ 0.24A	2.3 $\pm$ 0.25B	2.1 $\pm$ 0.11BC	1.8 $\pm$ 0.19CD	1.73 $\pm$ 0.16CD
Cd+HL	3 $\pm$ 0.24A	2.1 $\pm$ 0.14BC	1.8 $\pm$ 0.19CD	1.6 $\pm$ 0.21D	1.68 $\pm$ 0.14CD
HF	2.9 $\pm$ 0.26A	3.1 $\pm$ 0.21A	3.08 $\pm$ 0.27A	2.91 $\pm$ 0.21A	2.98 $\pm$ 0.23A
HL	2.93 $\pm$ 0.21A	3.05 $\pm$ 0.24A	3.0 $\pm$ 0.25A	2.92 $\pm$ 0.24A	2.96 $\pm$ 0.20A
Control	3.05 $\pm$ 0.18A	3 $\pm$ 0.24A	3.1 $\pm$ 0.28A	3.05 $\pm$ 0.21A	2.95 $\pm$ 0.27A

Table 3. Continued.

Urea in urine (mg/dL)					
Cd	135± 9.4A	107±8.34CF	87±7.95GHI	71±7.56IJ	58±6.81J
Cd+HF	134± 9.72A	123±9.51ABC	110±8.46CDE	98±7.21DH	89±7.75FI
Cd+HL	133± 9.94A	114±8.88BCD	104±8.74DG	92±7.92EH	84±7.67HI
HF	135±9.36A	136±9.27A	134±8.76A	132±9.13AB	135.5±9.07A
HL	138±8.76A	134±9.92A	132±7.55AB	136±8.29A	137±8.22A
Control	132±8.94AB	137±9.90A	135±9.06A	133±9.61A	139±9.32A

Means sharing similar letters in a row or column (for each parameter) are statistically non-significant ( $P>0.05$ ).

flowers plus Cd greatly improved these levels over fractions of *Hibiscus* leaves plus Cd at days 15, 30, 45, and 60 when compared to only the Cd-receiving group. In Cd+HF levels of serum, renal markers such as creatinine and urea were increased from 1.08 to 1.74 and from 58 to 89 mg/dL, whereas in the Cd+HL group these values were

increased from 1.08 to 1.68 and from 58 to 85 mg/dL at day 60 when compared to the Cd-treated group. Serum levels of albumin and total proteins were also decreased by the Cd-intoxicated group from 3.8 to 3.1, 2.2, 1.6, and 1.1, and from 7.4 to 6.5, 5.3, 4.1, and 2.9 mg/dL, respectively, representing a significant decreasing trend ( $p<0.05$ ) in the

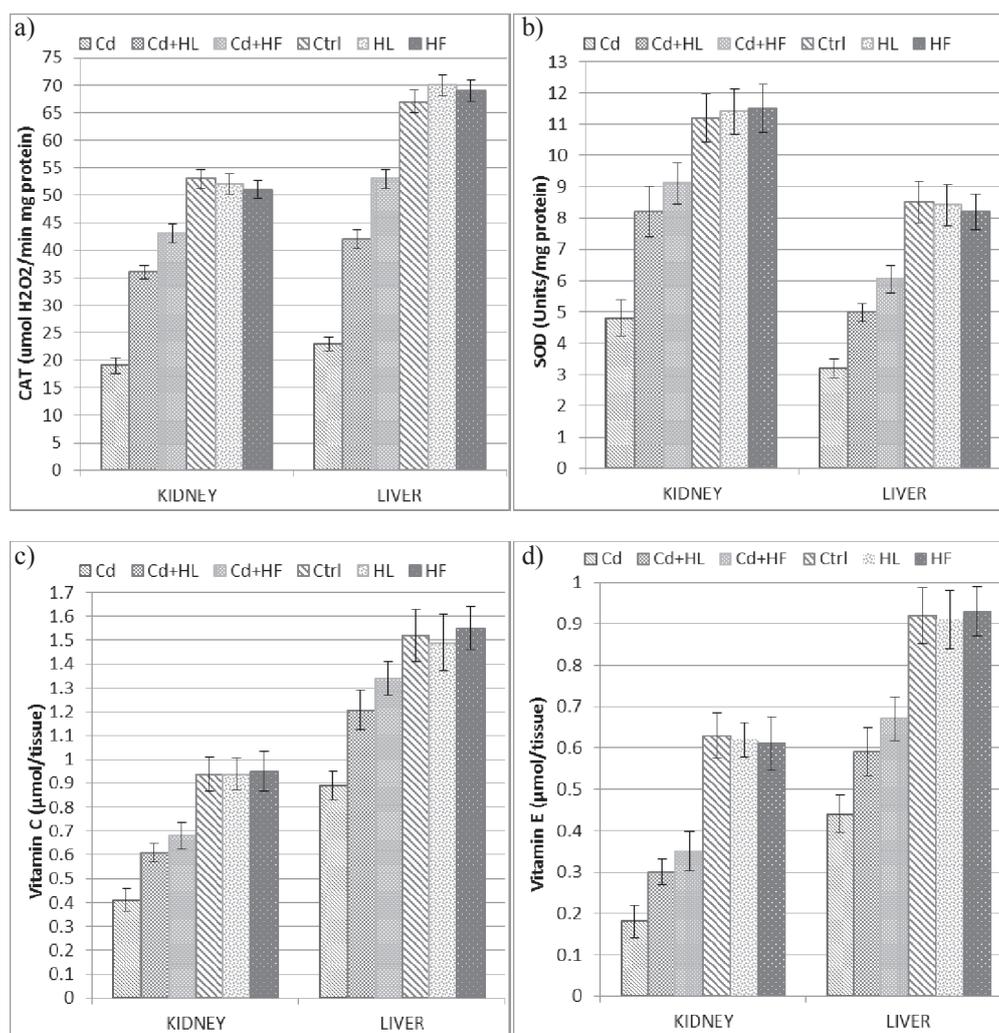


Fig. 4. a) Catalase (CAT,  $\mu\text{mol H}_2\text{O}_2/\text{min mg protein}$ ), b) superoxide dismutase (SOD, units/mg protein), c) vitamin C (ascorbic acid,  $\mu\text{mol/mg tissue}$ ), d) and vitamin E (alpha tocopherol,  $\mu\text{mol/mg tissue}$ ) in liver and kidney of male albino rabbits on day 60 of the experiment (mean  $\pm$  S.D, n= 6).

Cd-induced group when compared to the control group, contrary to the results of [41], who revealed an increment in serum albumin in Cd-intoxicated rats. But our findings are in agreement with the results of [37]. Cd plus HF and Cd plus HL significantly restored these levels when compared to the Cd-receiving group. Levels of serum and urine functional enzymes and serum proteins remained at satisfactory levels in HF and HL when compared to the control group as shown in Table 3.

Continued exposure of Cd may result in damage to various organs and systems, primarily the renal tissue via accumulation in the proximal tubule, initiating generalized re-absorptive dysfunctions. It also creates a great number of non-specific toxicological issues resulting in a decrease of renal cells via apoptosis or necrosis. However, it is also proposed that early stages of Cd-induced proximal tubular destruction may implicate much more specific alterations in cell-cell adhesions, autophagy responses, and signaling pathways in cells, which happen well before the inception of necrotic or apoptotic damage [42]. Renal tissues are more vulnerable to damage because of greater perfusion and a large number of combinations are excreted by renal tubular cells. Urea is the major metabolic product obtained from protein breakdown that contains nitrogen. It is well-known that Cd impedes the merger of amino acids into proteins, leading to augmented levels of urea. Creatinine is a waste product of creatine phosphate by muscle metabolism and it usually signifies impaired renal function or renal disease. As the kidneys become impaired, the creatinine level in serum will rise due to poor creatinine clearance by the kidneys. Thus, urea and creatinine levels both in serum

and urine samples were considered indicators of renal functioning, as hyperuricemia is a renal prognostic factor. The administration of flower and leaf fractions of *H. rosa sinensis*, possessing reasonable antioxidant potential [36], may have protected the renal functions from Cd-induced toxicity by significantly restoring the serum and urine urea and creatinine levels. The present study supports the findings of [43]. Various results suggest that plant extracts may prevent nephrotoxicity by suppressing the oxidative stress caused by Cd [31, 37, 44-46].

#### Determining Cadmium and LPO Contents in Tissues

Fig. 3 shows the Cd and LPO contents in liver and kidney of control, Cd-, Cd+HF-, Cd+HL-, HF-, and HL-treated groups of male adult albino rabbits. Oral CdCl<sub>2</sub> has led to an increase in Cd and LPO contents in renal and hepatic tissues. A significant decline in these contents was detected for Cd plus HF and Cd plus HL in kidney and liver samples when these were compared to the control group. Accumulation of Cd in liver is a notorious consequence of Cd exposure and it is documented to produce hepatic oxidative damage by increasing peroxidation of membranous lipids, a deleterious process absolutely executed by free radicals. Although free radicals are not generated by Cd directly, many radicals like superoxide, nitric oxide and hydroxyl are generated indirectly, thus producing harm consistent with oxidative stresses [47], whereas Cd-MT (cadmium-metallothionein) complex made in hepatic cells is released slowly by

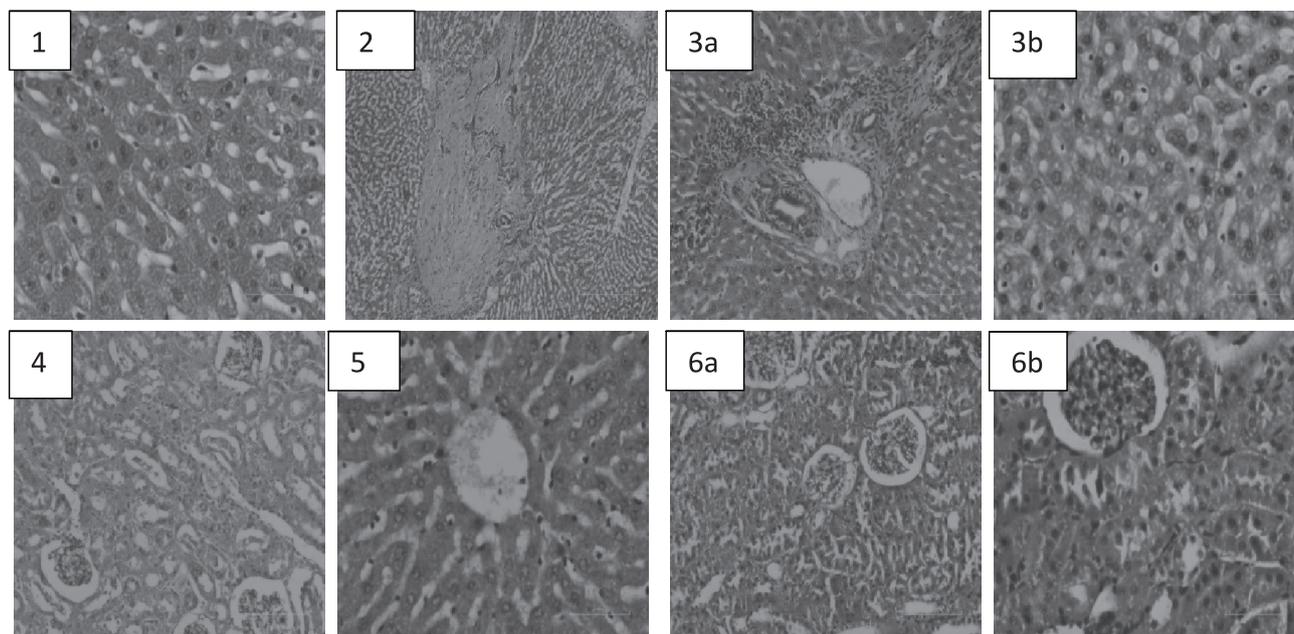


Fig. 5. Microphotographs of: 1) control group rabbit liver showing normal architecture of hepatic cells (400x), 2) CdCl<sub>2</sub>-intoxicated rabbit liver with increased sinusoidal space and hepatocytes atrophy (400x), 3a) CdCl<sub>2</sub>+HL-treated rabbit's liver with near normal cells of liver with few mononuclear cells infiltration (200x), 3b) CdCl<sub>2</sub>+HF-treated rabbit's liver with almost normal cells of liver (400x), 4) control group rabbit's kidney showing normal architecture of renal cells (200x), 5b) CdCl<sub>2</sub>-intoxicated rabbit's kidney showing cellular swelling and glomerular atrophy (400x), 6a) CdCl<sub>2</sub>+HL-treated rabbit with some normal renal tubular cells and some tubules show swelling (200x), and 6b) CdCl<sub>2</sub>+HF-treated rabbit with near normal renal cells (400x).

liver cells and circulates to the kidney tissue. This Cd-MT complex is dissolved in renal cells, releasing free Cd that is absorbed by proximal tubules. If renal-MT detoxification and defense systems are incredulous, free Cd can cause injury to the renal proximal and distal tubules [48], whereas elevated levels of LPO in liver and kidney of Cd-induced rabbits may indicate augmented oxidative stress. MDA levels, being an index of oxidative stress, may enhance peroxidation of membraneous lipids, mechanisms involved in damaging tissues, by changing the antioxidant defense system of tissues [49].

#### Determination of Antioxidant Enzymes and Vitamins C and E in Tissues

Fig. 4 shows the enzymatic anti-oxidants, i.e., SOD and CAT and non-enzymatic anti-oxidants, i.e., vitamins C and E in kidney and liver homogenates of control, Cd-, Cd+HF-, Cd+HL-, HF-, and HL-treated groups of adult albino rabbits, respectively. The administration of oral CdCl<sub>2</sub> has caused abnormal hepatic and renal functions in a progressive manner from days 0 to 15, 30, 45, and 60 of the experiment. It resulted in a decline of the levels of SOD and CAT for the Cd-treated group from 11.2 to 4.8 and 53 to 19 for kidney, and from 8.5 to 3.2 and 67 to 23 for liver homogenates, respectively, when compared to the control group. Values of vitamin C and E for the Cd-treated group were decreased from 0.94 to 0.41 and 0.63 to 0.18 for kidney, and from 1.52 to 0.89 and 0.92 to 0.44 for liver homogenate, respectively, when compared to the control group. Cd plus HF and Cd plus HL has resulted in significant improvement of these values when compared to the Cd-receiving group. Levels of these enzymatic (SOD, CAT) and non-enzymatic (vitamins C and E) antioxidants in tissue homogenates persisted at acceptable levels in HF and HL when compared to control rabbits. Enzymatic antioxidants such as SOD and CAT may establish a supportive and defensive group against reactive oxygen species (ROS). Superoxide dismutase, being a metallo-protein, may catalyze the dismutation of superoxide radicals. Catalase, being a heme protein, may catalyze the reduction of H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O and O<sub>2</sub>, thus defending the cells from oxidative destruction by H<sub>2</sub>O<sub>2</sub> and OH. The activity of SOD may be decreased either owing to the opposed effect of Cd with Cu and Zn, which are considered to be significant metals due to its activity or inactivation of superoxide dismutase by lipid peroxidation induced by Cd. It has been reported that the decline in CAT activity by Cd might reflect the reduced absorption of Fe, a vital trace metal that is essential for the activity of CAT [34]. Our results are in accordance with [34, 50], who also showed that SOD and CAT activities are decreased by Cd intoxication. The deficiency in the antioxidant defensive system may be considered as an acute event in Cd-induced hepato-renal toxicity. Cd exposure may be characterized by the depletion of non-enzymatic antioxidants (vitamin C and E) in tissues and in the bloodstream. These are the main non-enzymatic antioxidants that show synergetic effects in scavenging

reactive oxygen species or free radicals. Our findings are in accordance with [51-52], who showed that the level of vitamin C and vitamin E are significantly reduced during Cd-intoxication, leading to the development of Cd-induced hepatic and renal impairment. The administration of plant extracts in Cd-intoxicated rabbits may protect the enzymatic and non-enzymatic antioxidants by means of either their metal chelation activity or by their antioxidant activities that might reduce the exploitation of these antioxidants, thus reinstating their levels to near normal as described by [53].

#### Histopathological Studies

Changes in histopathological patterns in kidneys and liver tissues were assessed in such a manner that a histopathologist, responsible for interpretation, was kept uninformed of the treatment protocols. Studies of liver tissues in the Cd-treated group revealed that per oral Cd intoxication may have produced severe hepatic damage, including inflammation of the central vein, sinusoidal dilation, inflammatory cell infiltration, necrosis, portal inflammation, and fibrosis when compared to the control group. These changes were reduced in the liver of HF plus Cd and HL plus Cd rabbits. While studies of kidney tissues in the Cd-treated group revealed that intoxication with orally administered Cd has caused severe renal damage that includes damage to proximal and distal tubules, hydropic swelling, and degeneration of tubular epithelium when compared to control. Reversal of these changes was observed in HF+Cd and HL+Cd rabbits. The histo-architecture of liver and kidney was almost normal in HF and HL rabbits as shown in Fig. 5.

#### Conclusion

Biochemical markers of liver and kidney function were decreased significantly, whereas albumin and total protein were increased significantly at days 15, 30, 45, and 60 in Cd+HF than Cd+HL when compared to the Cd-induced group. Moreover, SOD, CAT, and vitamins C and E in kidney and liver tissues were increased significantly, whereas Cd content and LPO were decreased more in Cd+HF than Cd+HL when compared to Cd-induced rabbits. The histopathological studies for kidney and liver have also supported the idea that Cd+HF has lessened the Cd toxicity in both tissues more than in Cd+HL by reversing histo-architecture of said tissues close to normal, thus suggesting that ethyl acetate fractions of *Hibiscus rosa sinensis* may act as natural protective agents against Cd toxicity, mainly due to the presence of polyphenols and antioxidant activity.

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