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Original Research Article

Acute and sub-chronic toxicity of Liberin, an anti-diabetic polyherbal formulation in rats

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ABSTRACT

Background: The polyherbal formulation (PHF) liberin, is known to exert anti-hyperglycemic effects in type 2 diabetes mellitus. Hence, it is important to study the safety profile of PHF in the current study through acute and chronic toxicity evaluation.
Objectives: This research aims to assess the acute and sub-chronic toxicity of PHF in rats.
Materials and methods: PHF was administered once orally (1000 mg/kg body weight), and the rats (male and female) were monitored for toxicity signs for a 14-day period. For a 28-day chronic toxicity study, rats were daily administered with PHF dose of 500 mg/kg and 1000 mg/kg body weight. Rats were followed up for mortality, weight changes, and other morbidities. Further haematological, biochemical, and histopathological changes were assessed.
Results: No death related to treatment or toxicity signs were recorded in the acute single-dose administration group. The results showed that the PHF was tolerated well up to a dose of 1000 mg/kg body weight. Even at the high dose of 1000 mg/kg body weight, sub-chronic tests did not show any significant difference between the dosed and normal groups. No significant changes were seen in the histopathological analysis of the liver, spleen, and kidney as well as haematological and biochemical parameters in acute, sub-chronic and satellite groups following the administration of PHF.
Conclusion: The results confirmed that there was no adverse effect of this PHF at the maximum dose of 1000 mg/kg body weight in Wistar rats. Further, no adverse delayed effects related to PHF were observed in the satellite group. Therefore, this PHF appears safe for therapeutic purposes in the Ayurvedic medicinal system.

1. Introduction

The medicinal herbs are integrated into different formulations available in the form of tablets, capsules, powders, and *Kashaya*. In many countries, even today, medicinal herbs are being used in the form of drugs or home remedies for the treatment and management of various diseases [1]. Due to a rising interest towards the pharmacological use of herbal formulations in treating chronic diseases, extensive research is being conducted to generate scientific evidence pertaining to the efficacy and toxicity of various widely used anecdotal formulations, resulting in the generation of newer less toxic/non-toxic pharmacological alternatives and adjuvants encouraging holistic approach to disease management [2].

Abbreviations: PHF, Polyherbal formulation; T2DM, type 2 diabetes mellitus.
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Due to the complex chemical compositions of medicinal plants, herbal medications can cause mild to severe adverse effects. Therefore, establishing the safety profile of medicinal plants using proper scientific toxicity protocols that are controlled and verified is essential [3]. Insufficient scientific evidence to support the efficacy of herbal formulations for the management of any disease may lead to both harmful and ineffective results [4]. The results of toxicology studies can bring to light, the harmful effects of herbal drugs, the toxic/lethal dosages, and the therapeutic indices [5]. Toxicity studies specially experimented in rodents are useful for identifying the appropriate dose and to determine the toxic effect on organs at the completion of treatment [6]. Rats and mice are the most used animals in biomedical research. Wistar rats are preferred over mice for most of the toxicology and drug efficacy studies because their bigger size makes handling, sampling, and executing procedures simpler. The abundance of data gathered over the years has led to a greater understanding of the physiological reactions and pathways in rats than in other species. Moreover, the rat resembles human biology in similar way compared to other species [7]. Also, since rats are easier to feed and smaller in size than other complex mammals, such as primates, they are a more convenient option for many researchers. Furthermore, albino Wistar strain's genetic foundation is similar to that of humans and represents the human model, leading researchers to prefer rat model for the majority of their research. Despite these advantages, finding accurate data, comparing it to data from earlier studies that employed rat models, and coming to firm findings that might apply to future studies are some of the limitations of using a rat model in these domains [8].

The World Health Organization (WHO) recommends the use of herbal formulations as an emerging therapeutic strategy for various diseases [9]. Type 2 diabetes mellitus (T2DM) is a major public health problem, and proper management of this complex disease is crucial to prevent diabetic complications (retinopathy, nephropathy, atherosclerotic heart disease, neuropathy etc.) [10]. Antidiabetic polyherbal formulation (APH) may be a viable option for managing T2DM patients due to its lesser adverse effects, and the multifaceted systemic benefits exerted by the synergistic action of the herbs [11].

The increasing popularity of herbal pharmaceuticals for the treatment of a variety of ailments has led to the consideration of herbal drugs as an alternative option to treat diabetes and diabetic complications [12]. According to literature evidence, 1200 blooming plants contain phytoconstituents with anti-diabetic activity as nature's remedial approach to diabetes management. The active ingredient in these herbal extracts lowers blood glucose levels or have antioxidant properties, proving to be useful in treating diabetes and its complications. These therapeutic herbal plants contain flavonoids, phenolics, glycosides, sterols, triterpenoids, alkaloids, and other active ingredients [13]. Herbal formulations have been used in the management of T2DM, and many diabetic patients have been routinely using it and have reported as effective in management of diabetes and its complications [14].

The PHF studied here contains 7 ingredients which are known to be exhibit anti-diabetic properties. The ingredients are *Emblica officianalis, Allium sativum, Commiphora mukul, Terminalia arjuna, Withania somnifera, Centella asiatica,* and *Glycyrrhiza glabra*. As a part of this PHF component *Emblica officinalis* exerts its anti-hyperglycemic effect by acting on pancreatic beta-cells that causes a rise in beta-cell size and number, boosts antioxidant status, lowers blood sugar levels, enhances serum insulin levels, and changes beta-cell morphology and morphometry. Additionally, they reduce advanced glycated end products, inhibit -glucosidase, scavenge free radicals, boost antioxidants, modify adipokines, and inhibit gluconeogenesis [15]. *A. sativum* known as garlic, exhibits its anti-diabetic activity by improving insulin sensitivity. Also, the anti-oxidative property of garlic might be another reason for its anti-hyperglycemic effect. In addition, garlic can decrease blood glucose via diminution glucose absorption from intestine. Garlic may also induce a protective/regenerative effect on pancreatic beta cells by increasing the number of pancreatic islet cells [16]. It is well documented that *C. mukul* lowers hyperglycemia, hypertriglyceridemia, and insulin resistance. It may also normalize blood sugar levels by reducing the activity of glycolytic enzymes in the liver and muscle and by reversing decreased utilization of glucose for energy production in diabetics. Additionally, *C. mukul* increases pyruvate kinase activity in the liver and muscle while decreasing hexokinase and phosphofructokinase activity [17]. By stimulating hepatic antioxidant enzymes, *C. mukul* helps to regulate diabetes, aberrant lipid profiles, and oxidative stress [18]. *T. arjuna* demonstrated a reduction in cholesterol, gluconeogenesis, and ketogenesis as well as body weight. It also prevents the oxidation of hepatic lipids in diabetic rats by lowering the production of free radicals and improving glutathione levels in the liver. The findings demonstrated that *T. arjuna's* antihyperglycemic effect was accompanied by an improvement in nonenzymatic antioxidant defense. Through the stimulation of the cellular antioxidant system, *T. arjuna* may exert its antihyperglycemic impact [19]. The pancreatic release of insulin from the islets of Langerhan's cells may be increased by *W. somnifera* extract or may function as an insulin substitute. Higher levels of hepatic glycogen show that it also accelerates glycogenesis [20]. It is possible that the *C. asiatica* may act by potentiating the pancreatic secretion or increasing the glucose uptake. It enhances antioxidant defenses, increasing the activity of superoxide dismutase, glutathione peroxidase, and catalase [21]. In addition to enhancing GLUT-1 transport from the intracellular location to the plasma membrane, *G. glabra* improves insulin-mediated glucose elimination in muscles. By influencing peroxisome proliferation activated receptors, *G. glabra* also controls the expression of a number of genes crucial to glucose metabolism [22].

The PHF used in this study is an anti-diabetic formulation, the components of which has been shown to have anti-hyperglycaemic or anti-lipidemic properties, however, to make the medication more effective, a toxicity study offers more information regarding safety. It is necessary to determine the safety profiles of various herbs and their dose specific additive effects when they are combined into one formulation. Thus, the current study was planned to determine whether a 28-day oral administration of the PHF in acute and sub-chronic toxicity group is safe, as well as to identify any dose dependent associated toxicities of the formulation.

2. Materials and methods

2.1. Material

The PHF capsule (liberin) was supplied by Clinfound Clinical Research Services (P) Ltd, Kerala, India. All other reagents employed were obtained from Coral Clinical Systems (Uttarakhand, India), and Medsource Ozone Biomedicals Pvt. Ltd (Haryana, India). Wistar rats with the facility of standard food, water, and metallic cages were provided by the Central Animal Research facility, Manipal, India. Each PHF capsule contained 7 ingredients *E. officianalis* (75 mg), *A. sativum* (70 mg), *C. mukul* (65 mg), *T. arjuna* (70 mg), *W. somnifera* (75 mg), *C. asiatica* (75 mg), and *G. glabra* (70 mg).

2.2. Experimental animals

The animals used in this study were maintained in Central Animal Research facility, Manipal under standard conditions and the study protocol was priorly approved by Institutional Animal Ethics

Committee (IAEC/KMC/20/2021). The total of 36 Wistar rats including male and female aged 4 and 6 months and weighing about 150–250 gm were housed in the cage measuring around 16.5 inch in length and 11 inch in width with 3 rats in each cage to avoid any distress due to overcrowding. All rats were provided with food supplied by VRK Nutritional solutions and water ad-libitum and maintained under 12:12 h cycle of darkness and light. A standard environment was maintained with a temperature of 22 ± 2 °C and a relative humidity of 40–60%. Six rats (three males and three females) were used for acute oral toxicity, and 30 rats (15 males and 15 females) were used in sub-chronic oral toxicity. 30 animals were randomly divided into five groups (sub-chronic and satellite group), each with six rats and an equal number of males and females. The rats of the same gender were kept apart in the same cage on the rack. Of 30 rats of sub-chronic group, 18 rats were given repeated doses for 28 days and 12 rats were grouped as the satellite group. Prior to the study, all animals were given five days to acclimatize, made to fast previous night before the dosage, and the body weights of the rats were measured prior to the experiment.

2.3. Acute toxicity

In this study, acute oral toxicity testing was performed in healthy Wistar rats in accordance with guidelines provided by the Organization for Economic Cooperation and Development (OECD 2008) [23]. The rats were weighed before the dose was administered. The first animal was given a dose of 2000 mg/kg PHF in the limit test as per the experimental procedure. In the main test the animals were grouped into two, with 3 rats in each group ($n = 6$; three males and three females). Group1 (Control, males) did not receive any treatment; Group 2 (females) received a single dose of PHF (1000 mg/kg). In the subsequent observations, signs of toxicity, death, and behavioural changes (agitation, convulsions, ataxia, paralysis, tremors, fasciculation, vocalizations, and abnormal locomotion) were observed for the first 30 min, the first hour, then the second hour, the fourth hour, and eighth hour, and finally periodically for the next 24 h for 14 days. For 14 days following single dose, each experimental animal was monitored daily for changes in general behaviour, body weight, and mortality. At the end of the experimental period, all animals were weighed, sacrificed, and the organs were removed for histopathology study. The euthanasia of the rats was done using high dose of ketamine.

2.4. Sub-chronic oral toxicity

The study was performed according to the protocols described by OECD Guideline 407. 30 rats of both sexes were used in this study and were divided into five groups of six rats in each ($n = 6$; three males and three females) and their weights were noted. Before the dosing, rats were managed carefully and investigated for any unusual appearance and behavior. The PHF capsule which was in powdered form was dissolved in the water and administered orally once a day for 28 consecutive days. Group1 (Control rats) received no dosage; Group 2 received the PHF dose of 500 mg/kg; and Group 3 received the PHF dose of 1000 mg/kg. The rats were monitored daily during the experiment for any clinical signs of toxicity. The weight of rats was recorded and documented every week till the end of the procedure. At the end of the 28th day, the animals were made to fast overnight, with access to the water. The rats were then anesthetized, blood samples were collected using capillary tubes from retro-orbital sinus for haematological and biochemical studies. After blood collection, the rats were sacrificed and organs such as liver, kidneys, and spleen were collected.

2.5. Satellite group

The satellite group rats after 28-day daily dosage were observed for next 14 days for any clinical toxicity of the drug and then euthanized at the end of 42nd day. Capillary tubes were used to collect blood samples via retro-orbital puncture for haematological and biochemical analyses. Followed by the blood collection, the rats were sacrificed, and liver, kidney, and spleen were collected for histopathology study.

2.6. Hematological and biochemical analysis

EDTA tubes were used to collect whole blood samples. White blood cell count (WBC), haemoglobin, haematocrit (HCT), mean corpuscular volume (MCV), platelets, mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), and mean platelet volume (MPV) were assessed using automated analyser ERMA- PCE-210 VET. For the biochemical analysis of blood samples, anticoagulant-free tubes were used. The samples were centrifuged for 10 min at 3000 rpm to separate the serum and stored at −20 °C till further use. Alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine, albumin were analysed using coral clinical system kit (Tulip diagnostics) in semi-automated - Erba Chem 5x Clinical Chemistry Analyzer (TransAsia).

2.7. Histological examination

Using graded alcohol, the kidney, liver, and spleen were serially dehydrated after being removed, preserved in 10% formalin (pH 7.0) for 24 h, and then removed. For microscopic inspection, tissue samples were fixed in paraffin wax, cut into five micrometer-thin slices, and stained with haematoxylin and eosin. The images of section were taken using a Microscope Olympus BX43 under 40X magnification.

2.8. Statistical analysis

The mean and SD are used to express all values. One-way analysis of variance (ANOVA) was used for the statistical analysis in EZR software (Version 1.54), and Tukey's test as a post hoc analysis. It was deemed statistically significant when the result was $p \leq 0.05$.

3. Results

3.1. Effect of PHF on body weight changes in Wistar rats

At the end of the study, on 28th and 42nd day, the changes in the body weight of rats in various groups were compared (Tables 1 and 2). There was insignificant difference in control and PHF 500 mg/kg and 1000 mg/kg body weight in sub-chronic group. Further, the body weights did not show any variations between the weeks in all three groups. However, there were significant changes between control and PHF 1000 mg/kg body weight in satellite group.

3.2. Acute oral toxicity study

With a higher dose of PHF (2000 mg/kg) in the limit test, no deaths of rats were observed. Neither the control nor the PHF treated animals in acute oral toxicity group died or developed signs

Table 1
Body weight of sub-chronic group rats from control and PHF administered for 28 days.

Weeks	Sex	Weight in grams			P value
		Control (No dose)	PHF		
			500 mg/kg body weight	1000 mg/kg body weight	
Baseline	M (n = 3)	199.10 ± 38.87	218.33 ± 22.54	255.33 ± 17.24	
	F (n = 3)	205.50 ± 9.19	211.33 ± 11.84	215.11 ± 3.00	
	T (n = 6)	201.30 ± 22.60	214.80 ± 16.54	235.13 ± 24.70	P = 0.058
Week 1	M (n = 3)	204.33 ± 32.31	213.33 ± 22.3	250 ± 21.79	
	F (n = 3)	208.00 ± 10.81	207.13 ± 7.20	215.50 ± 8.66	
	T (n = 6)	206.10 ± 21.60	210.1 ± 15.20	232.50 ± 24.21	P = 0.091
Week 2	M (n = 3)	211.66 ± 25.65	215.66 ± 24.37	247.10 ± 18.24	
	F (n = 3)	211.33 ± 20.25	206.66 ± 16.07	214.33 ± 14.36	
	T (n = 6)	211.51 ± 20.60	211.10 ± 19.12	230.60 ± 23.12	P = 0.218
Week 3	M (n = 3)	206.66 ± 25.4	218.33 ± 25.65	242.10 ± 7.54	
	F (n = 3)	215.33 ± 16.16	203.33 ± 12.58	212.40 ± 15.71	
	T (n = 6)	211.20 ± 19.60	210.80 ± 19.80	227.80 ± 19.74	P = 0.295
Week 4	M (n = 3)	212.40 ± 17.52	212.33 ± 33.23	227.66 ± 15.94	
	F (n = 3)	232.66 ± 17.03	196.66 ± 6.65	213.33 ± 10.06	
	T (n = 6)	222.34 ± 19.10	204.52 ± 23.10	220.5 ± 14.24	P = 0.240

Values expressed as mean ± SD. Significance with One way ANOVA analysis is evaluated as $p \leq 0.05$. Note: F – female, M– male, T – total.

of toxicity such as neurological or behavioural changes throughout the study. Body weight changes, hematological and biochemical parameters between the groups showed no statistical significance (Table 3). In addition, neither group had gross pathological abnormalities and consequently, the LD50 value for PHF is observed to be greater than 2000 mg/kg body weight.

3.3. Sub-chronic oral toxicity study

Both sexes of rats that were given oral doses of 500 mg/kg and 1000 mg/kg for 28 days in the sub-chronic and satellite group showed no evidence of treatment-related toxicity or mortality. Hematological parameters such as haemoglobin, red blood cells, white blood cells, platelet count, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, mean platelet volume and red cell indices in experimental group rats were found to be substantially within the clinical range (Tables 4 and 5). No significant difference in serum biochemical profile such as alkaline phosphatase, creatinine, alanine transferase, and albumin (Tables 4 and 5) were recorded between control and treated sub-chronic and satellite group. In the present study, we performed histopathological examinations in control and treatment group for liver, spleen, and kidney and the report revealed no abnormalities (Figs. 1–3). The bone marrow aspiration smear in control, 500 mg/kg body weight, 1000 mg/kg body weight in sub-chronic group and 1000 mg/kg body weight in satellite group showed the normal cellularity and active haematopoiesis.

Table 2
Body weight of satellite group rats from control and PHF administered for 28 days and follow up of 14 days.

Weeks	Sex	Weight in grams		P Value
		Control (No treatment)	PHF (1000 mg/kg body wt)	
Baseline	M (n = 3)	207.66 ± 18.14	205.66 ± 22.94	
	F (n = 3)	208.18 ± 8.18	206.30 ± 7.21	
	T (n = 6)	207.80 ± 12.52	205.84 ± 15.24	P = 0.809
Week 1	M (n = 3)	207.33 ± 14.15	209.33 ± 25.32	
	F (n = 3)	213.66 ± 8.08	200.33 ± 6.82	
	T (n = 6)	210.52 ± 10.82	204.84 ± 17.30	P = 0.512
Week 2	M (n = 3)	213.33 ± 11.37	206.33 ± 23.02	
	F (n = 3)	213.40 ± 11.15	196.22 ± 11.13	
	T (n = 6)	213.35 ± 10.10	201.10 ± 17.10	P = 0.165
Week 3	M (n = 3)	218.30 ± 11.26	199.66 ± 28.29	
	F (n = 3)	225.20 ± 4.58	198.10 ± 10.58	
	T (n = 6)	221.52 ± 8.50	198.81 ± 19.10	P = 0.024*
Week 4	M (n = 3)	222.12 ± 5.29	205.33 ± 19.85	
	F (n = 3)	236.66 ± 2.88	198.33 ± 9.71	
	T (n = 6)	229.30 ± 8.80	201.82 ± 14.40	P = 0.002*
Week 5	M (n = 3)	224.66 ± 8.08	207 ± 13.11	
	F (n = 3)	245.00 ± 8.88	201.33 ± 6.35	
	T (n = 6)	234.80 ± 13.40	204.14 ± 9.70	P = 0.001*
Week 6	M (n = 3)	223.33 ± 12.58	199.33 ± 15.27	
	F (n = 3)	258.33 ± 18.92	205.66 ± 4.04	
	T (n = 6)	240.80 ± 23.90	198.52 ± 10	P = 0.006*

Values expressed as mean ± SD. Significance with One way ANOVA analysis is evaluated as $p \leq 0.05$. Note: F – female, M– male, T – total.

Table 3
Haematological and biochemical parameters assessed in Wistar rats orally administered with 1000 mg/kg of PHF for acute toxicity at 14 days after administration.

Parameters	Control (n = 3)	PHF (n = 3)	P value
Haematological changes			
WBC ($10^3/\mu L$)	7.63 ± 1.41	8.60 ± 0.43	P = 0.322
RBC ($10^6/\mu L$)	10.35 ± 0.22	9.33 ± 0.71	P = 0.078
Hemoglobin (g/dl)	15.33 ± 0.25	14.73 ± 0.76	P = 0.266
HCT (%)	47.73 ± 0.23	46.03 ± 2.25	P = 0.264
MCV (fL)	46.06 ± 1.16	45.63 ± 1.95	P = 0.758
MCH (pg)	14.76 ± 0.20	13.83 ± 0.87	P = 0.146
MCHC (g/dl)	32.06 ± 0.55	31.46 ± 1.74	P = 0.601
MPV (fL)	5.86 ± 0.05	6.20 ± 0.55	P = 0.361
RDW (%)	16.70 ± 0.10	16.40 ± 1.32	P = 0.715
Platelets ($10^3/\mu L$)	673.60 ± 50.46	673.30 ± 109.50	P = 0.996
Biochemical changes			
Alanine Transaminase (IU/l)	41.80 ± 8.00	57.80 ± 15.10	P = 0.181
Alkaline Phosphatase (IU/l)	94.80 ± 21.70	151.60 ± 48.40	P = 0.138
Albumin (g/dl)	6.03 ± 2.10	6.80 ± 1.90	P = 0.648
Creatinine (mg/dl)	0.50 ± 0.20	0.63 ± 0.30	P = 0.561

Values expressed as mean ± SD. Significance with one way ANOVA analysis is evaluated as $p \leq 0.05$. Note: WBC – white blood cell count, HCT – haematocrit, MCV – mean corpuscular volume, MCH – mean corpuscular haemoglobin, MCHC – mean corpuscular haemoglobin concentration, RDW – red cell distribution width, and MPV – mean platelet volume.

Table 4
Haematological and biochemical parameters assessed in Wistar rats orally administered with 500 mg/kg and 1000 mg/kg of PHF in sub-chronic toxicity for 28 days after administration.

Parameters	Sex/n	Control	PHF 500 mg/kg	PHF 1000 mg/kg	P Value
Hematological changes					
WBC (10^3/uL)	M (n = 3)	8.34 ± 3.64	8.90 ± 3.21	7.71 ± 0.78	
	F (n = 3)	8.76 ± 1.77	10.44 ± 1.85	11.61 ± 4.96	
	T (n = 6)	8.53 ± 2.57	9.68 ± 2.49	9.65 ± 3.83	P = 0.760
RBC (10^6/uL)	M (n = 3)	11.01 ± 1.72	12.32 ± 1.21	11.17 ± 1.77	
	F (n = 3)	12.71 ± 0.18	9.82 ± 0.22	13.35 ± 0.71	
	T (n = 6)	11.86 ± 1.44	11.07 ± 1.57	12.26 ± 1.70	P = 0.436
Hemoglobin (gram%)	M (n = 3)	15.80 ± 2.91	18.76 ± 2.72	17.10 ± 3.45	
	F (n = 3)	19.86 ± 0.70	14.53 ± 0.37	20.23 ± 1.65	
	T (n = 6)	17.83 ± 2.92	16.65 ± 2.89	18.66 ± 2.97	P = 0.504
HCT (%)	M (n = 3)	54.66 ± 7.75	58.13 ± 7.22	54.90 ± 9.69	
	F (n = 3)	61.63 ± 2.84	53.73 ± 12.56	64.80 ± 2.81	
	T (n = 6)	58.15 ± 6.46	52.61 ± 7.63	60.81 ± 7.71	P = 0.170
MCV (fL)	M (n = 3)	49.70 ± 1.31	47.06 ± 1.51	50.83 ± 0.58	
	F (n = 3)	48.36 ± 1.55	47.20 ± 0.98	48.5 ± 1.15	
	T (n = 6)	49.03 ± 1.48	47.16 ± 1.14	49.66 ± 1.51	P = 0.172
MCH (pg)	M (n = 3)	14.31 ± 0.50	15.13 ± 0.73	15.16 ± 0.70	
	F (n = 3)	15.40 ± 0.36	14.76 ± 0.11	15.06 ± 0.41	
	T (n = 6)	14.85 ± 0.71	14.95 ± 0.51	15.11 ± 0.52	P = 0.738
MCHC (g/dl)	M (n = 3)	28.76 ± 1.80	32.20 ± 1.05	29.90 ± 1.21	
	F (n = 3)	31.06 ± 2.81	30.86 ± 0.15	31.13 ± 1.51	
	T (n = 6)	30.50 ± 2.35	31.53 ± 0.99	30.51 ± 1.40	P = 0.493
MPV (fL)	M (n = 3)	6.31 ± 0.45	6.36 ± 0.68	5.86 ± 0.37	
	F (n = 3)	6.16 ± 0.56	5.83 ± 0.05	6.16 ± 0.86	
	T (n = 6)	6.23 ± 0.46	6.10 ± 0.52	6.28 ± 0.64	P = 0.838
RDW (%)	M (n = 3)	16.16 ± 0.55	16.23 ± 0.11	15.83 ± 0.15	
	F (n = 3)	17.90 ± 0.10	16.41 ± 0.20	17.16 ± 0.25	
	T (n = 6)	17.03 ± 1.01	16.31 ± 0.17	16.50 ± 0.75	P = 0.247
Platelets (10^3/uL)	M (n = 3)	673.10 ± 116.60	511.30 ± 94.29	560.60 ± 92.64	
	F (n = 3)	507.33 ± 67.10	643.30 ± 75.79	554.31 ± 56.60	
	T (n = 6)	590.16 ± 124.30	610.66 ± 129.10	557.51 ± 68.76	P = 0.710
Biochemical changes					
Alanine transaminase (IU/l)	M (n = 3)	52.26 ± 7.58	64.91 ± 19.01	44.90 ± 14.40	
	F (n = 3)	50.13 ± 12.01	35.20 ± 20.10	46.11 ± 24.20	
	T (n = 6)	51.62 ± 21.30	50.10 ± 23.91	45.52 ± 20.02	P = 0.879
Alkaline phosphatase (IU/l)	M (n = 3)	176.40 ± 71.82	207.90 ± 38.41	229.10 ± 27.24	
	F (n = 3)	151.52 ± 67.10	171.90 ± 12.26	152.73 ± 21.80	
	T (n = 6)	163.90 ± 69.10	189.95 ± 32.2	190.91 ± 44.30	P = 0.901
Albumin (g/dl)	M (n = 3)	6.22 ± 2.10	4.33 ± 1.12	3.80 ± 0.61	
	F (n = 3)	4.12 ± 3.12	4.81 ± 1.50	5.52 ± 1.63	
	T (n = 6)	5.01 ± 2.80	4.60 ± 1.22	4.68 ± 1.42	P = 0.929
Creatinine (mg/dl)	M (n = 3)	0.78 ± 0.40	0.76 ± 0.32	0.72 ± 0.50	
	F (n = 3)	0.51 ± 0.32	0.56 ± 0.30	0.82 ± 0.43	
	T (n = 6)	0.65 ± 0.40	0.66 ± 0.21	0.75 ± 0.42	P = 0.890

Values expressed as mean ± SD. Significance with one way ANOVA analysis is evaluated as $p \leq 0.05$. Note: Note: F – female, M – male, T – total, WBC – white blood cell count, HCT – haematocrit, MCV – mean corpuscular volume, MCH – mean corpuscular haemoglobin, MCHC – mean corpuscular haemoglobin concentration, RDW – red cell distribution width, and MPV – mean platelet volume.

4. Discussion

Herbal drugs have gained popularity as a potential alternative to traditional allopathic pharmacological therapy [24]. However, to ensure that a plant-based medicine is safe to be used, the effects of historical applications of medicinal plants and their active components on humans and animals, as well as evidence obtained from standard drug toxicity testing experiments, should be considered. To assess the efficacy and safety of these herbal medications, as well as to define the active ingredients of herbal products, a variety of established screening procedures are used [25]. Drug safety studies include three important arms; assessment of clinical signs of adverse reactions/toxicities, evaluation of toxicity indicators in the circulating body fluids and histopathological assessment for tissue specific damages. In the present study undertaken to assess the safety profile of the PHF, in accordance with OECD guideline 423, the limit test at the highest starting dose level (2000 mg/kg body weight) was performed based on information indicating that the test material is low or non-toxic and immortal (OECD, 2001) and there were no signs of toxicity or mortality observed at this dose. OECD guideline 407 was followed in Wistar rats to determine oral toxicity over the course of 28 days toxicity study [23]. During the study, mortality and morbidity were recorded twice daily owing to the key role of clinical signs in toxicological testing. There is no significant difference in body weight changes in sub-chronic group treated with PHF for 28 days. However, there was significant difference in body weight in satellite group, and this observed significance might be due to the lower number of animals in each arm, i.e., a small sample size. Further, it is important to note that the animals in the

Table 5
Haematological and biochemical parameters assessed in Wistar rats orally administered with 1000 mg/kg of PHF in satellite group toxicity for 28 days and follow up of 14 days.

Parameters	Sex/n	Control	PHF 1000 mg/kg	P Value
Hematological changes				
WBC (10^3/uL)	M (n = 3)	7.33 ± 3.04	7.4 ± 1.67	
	F (n = 3)	6.53 ± 0.60	6.73 ± 2.32	
	T (n = 6)	6.93 ± 2.01	7.06 ± 1.84	P = 0.907
RBC (10^6/uL)	M (n = 3)	10.33 ± 0.28	10.23 ± 0.26	
	F (n = 3)	10.69 ± 0.49	10.40 ± 1.43	
	T (n = 6)	10.51 ± 0.41	10.32 ± 0.93	P = 0.660
Hemoglobin (gram%)	M (n = 3)	15.10 ± 0.75	14.76 ± 0.68	
	F (n = 3)	15.50 ± 0.30	15.4 ± 2.08	
	T (n = 6)	15.25 ± 0.58	15.08 ± 1.42	P = 0.797
HCT (%)	M (n = 3)	48.33 ± 0.97	47.00 ± 2.28	
	F (n = 3)	40.03 ± 1.95	48.90 ± 6.1	
	T (n = 6)	48.68 ± 1.43	47.95 ± 4.25	P = 0.697
MCV (fL)	M (n = 3)	46.73 ± 0.37	45.83 ± 1.51	
	F (n = 3)	45.83 ± 0.25	45.33 ± 2.59	
	T (n = 6)	46.28 ± 0.57	46.43 ± 1.29	P = 0.801
MCH (pg)	M (n = 3)	14.20 ± 0.86	14.33 ± 0.3	
	F (n = 3)	14.13 ± 0.25	14.73 ± 0.35	
	T (n = 6)	14.46 ± 0.36	14.53 ± 0.36	P = 0.758
MCHC (g/dl)	M (n = 3)	30.93 ± 1.00	31.4 ± 0.95	
	F (n = 3)	31.56 ± 0.65	31.43 ± 0.45	
	T (n = 6)	31.25 ± 0.83	31.41 ± 0.66	P = 0.711
MPV (fL)	M (n = 3)	6.41 ± 0.34	5.76 ± 0.05	
	F (n = 3)	6.30 ± 0.45	6.76 ± 0.55	
	T (n = 6)	6.35 ± 0.36	6.26 ± 0.65	P = 0.790
RDW (%)	M (n = 3)	15.30 ± 0.43	16.66 ± 0.23	
	F (n = 3)	17.36 ± 0.25	16.76 ± 0.55	
	T (n = 6)	16.33 ± 1.17	16.71 ± 0.38	P = 0.465
Platelets (10^3/uL)	M (n = 3)	679.60 ± 22.5	630.30 ± 72.15	
	F (n = 3)	656.00 ± 49.15	535.00 ± 281.10	
	T (n = 6)	667.80 ± 36.56	582.61 ± 190.80	P = 0.308
Biochemical changes				
Alanine transaminase (IU/l)	M (n = 3)	47.83 ± 12.87	48.56 ± 8.05	
	F (n = 3)	55.83 ± 19.70	45.66 ± 19.91	
	T (n = 6)	51.81 ± 15.52	47.1 ± 13.63	P = 0.589
Alkaline phosphatase (IU/l)	M (n = 3)	85.45 ± 37.01	126.2 ± 30.90	
	F (n = 3)	154.20 ± 57.41	106.62 ± 21.72	
	T (n = 6)	132.6 ± 43.30	116.4 ± 26.23	P = 0.712
Albumin (g/dl)	M (n = 3)	6.03 ± 0.15	5.23 ± 2.41	
	F (n = 3)	4.23 ± 0.75	4.83 ± 1.60	
	T (n = 6)	5.11 ± 1.11	5.03 ± 1.84	P = 0.902
Creatinine (mg/dl)	M (n = 3)	0.61 ± 0.51	0.40 ± 0.21	
	F (n = 3)	0.84 ± 0.42	0.70 ± 0.41	
	T (n = 6)	0.73 ± 0.40	0.55 ± 0.34	P = 0.471

Values expressed as mean ± SD. Significance with One way ANOVA analysis is evaluated as $p \leq 0.05$. Note: F – female, M– male, T – total, WBC – white blood cell count, HCT – haematocrit, MCV – mean corpuscular volume, MCH – mean corpuscular haemoglobin, MCHC – mean corpuscular haemoglobin concentration, RDW – red cell distribution width, and MPV – mean platelet volume.

control arm are showing a higher variance (SD) when compared to the satellite group (which showed a very narrow SD) which could be responsible for the statistical significance observed here. Drug toxicity is largely determined by assessments of haematological and biochemical fluctuations in response to drug administration. The haemopoietic system is considered to be a sensitive index for pathological conditions and is a primary target for toxic chemicals. Blood is a non-invasive body fluid of choice which is directly under the control of haemopoietic system alterations, and it serves as the main transport medium for many drugs and xenobiotics in the body, also blood components are effortlessly exposed to substantial concentrations of toxic compounds. Circulating blood parameters are a true reflection of metabolomic and process derangements and serve as an excellent prognostic indicator for toxicity, therefore assessment of blood parameters is considered appropriate for drug toxicity risk assessment [26]. No changes were recorded in blood cell production or haematological parameters after treatment with PHF in this study, indicating that this drug did not adversely affect blood cells. Albumin and alanine transaminase are useful biomarkers for predicting drug toxicity and indicators of liver function. These markers are elevated in response to damage in liver parenchymal cells, indicating their outflow into the bloodstream. In this study, the level of these markers did not change, proving that this PHF had no effect on liver function or metabolism and did not show any drug related liver toxicities.

Many studies were performed to determine the toxicity level of antidiabetic formulation in rats [27,28]. As described earlier, the

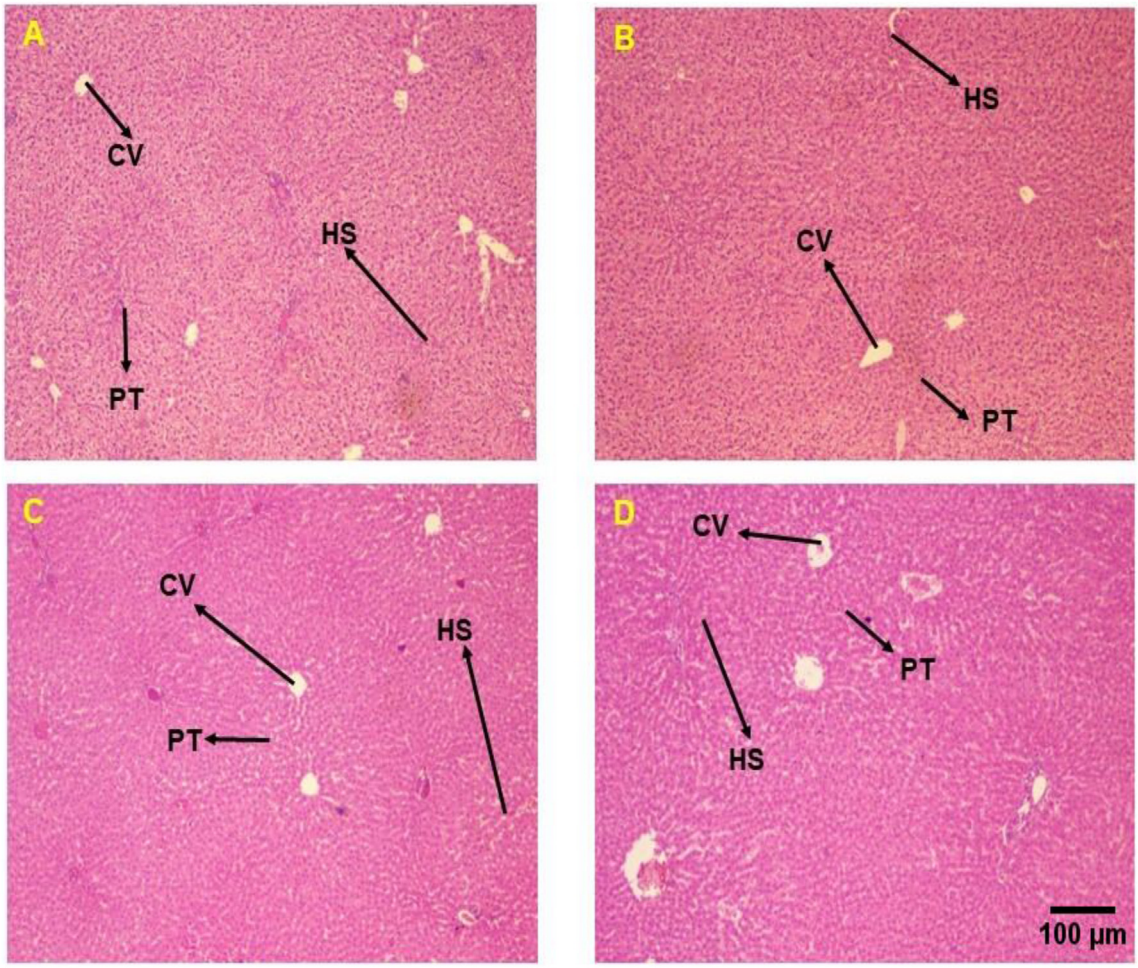

Fig. 1. Histopathological microphotograph of liver of control (A), 500 mg/kg body weight liberin treated sub-chronic group for 28 days (B), 1000 mg/kg body weight liberin dosed sub-chronic group for 28 days (C), and 1000 mg/kg body weight liberin treated satellite group for 28 days and 14 days follow-up (D) showing normal morphology of hepatic cells and veins. Note: CV – Central Vein, HS – Hepatic Sinusoids, PT – Portal Triad.

study PHF consist of *E. officianalis, A. sativum, C. mukul, T. arjuna, W. somnifera, C. asiatica,* and *G. glabra.* Though there are no previous studies assessing the safety profile of this PHF, various studies have explored the safety profile of the individual component extracts in animal as well as human models and their findings also reiterate the safety of individual components present in the PHF. The principal component of this PHF is *E. officianalis.* This fruit has been used in Ayurveda as rejuvenating herb since ancient times and has innumerable healing properties. In view of its safety evaluation, the fruit of *E. officianalis* has been reported to be safe up to a dose of 3 g/kg in an acute toxicity study involving Swiss albino rats [29]. *A. sativum*, an important Allium species, consumed globally and a medicinal remedy for many diseases, is an integral ingredient of this PHF [30]. It is used for decades as a diuretic, antihypertensive, antiseptic, and treatment for viral infections. Studies have also demonstrated the antioxidant, insecticidal, and antimicrobial properties of this compound [31]. At a dosage of 300 mg/kg, *A. sativum* may be relatively safe as an oral remedy and could be explored as a treatment for various disease. Ayurveda uses *C. mukul* to treat hyperlipemia, diabetes, atherosclerosis, and other inflammatory conditions such as arthritis. Human toxicity to *Guggul* extract has been evaluated for its widespread use as a dietary supplement, as well as its metabolic and hormone-stabilizing properties. A study of *C. mukul* in acute and chronic doses did not exhibit any adverse or toxic effect at the doses 2700 mg/kg body weight [32]. Bhawani et al. recently reported that after 28 days of treatment with *T. arjuna* capsules, there was no significance in the body weights between the normal and treated groups of 93 patients with dilated cardiomyopathy [33]. Additionally, the study reported that no mortality or obvious or histopathological abnormalities were noted. The PHF also includes an extract of *W. somnifera,* and numerous studies have verified the non-clinical safety profiles of extracts prepared from the roots of this plant. An acute toxicity profile study involving administration of a standardised

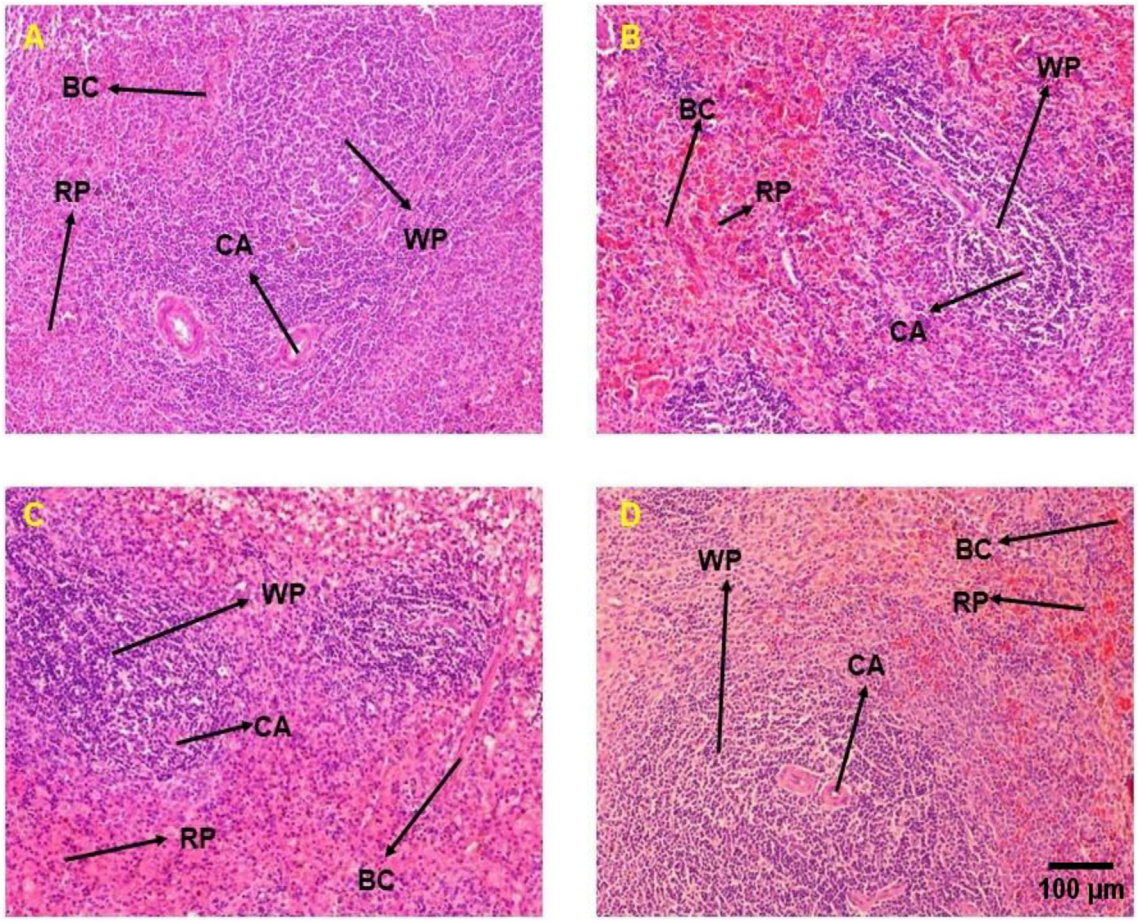

Fig. 2. Histopathology examination of a section of spleen of control (A), 500 mg/kg body weight liberin treated sub-chronic group for 28 days (B), 1000 mg/kg body weight liberin dosed sub-chronic group for 28 days (C), and 1000 mg/kg body weight treated satellite group for 28 days and 14 days follow-up (D) showing normal histology. Note: BC – Billroth Cord, CA – Central Artery, RP – Red Pulp, WP – White Pulp.

root extract of *W. somnifera* to female Wistar rats at dosages of 500, 1000, and 2000 mg/kg reported that the LD50 was higher than 2000 mg/kg body weight, and no toxic, behavioural, or obvious organ abnormalities were seen [34]. A few researchers have studied the level of toxicity of *C. asiatica* extract; however, no significant unfavourable effects have been reported. The oral administration of *C. asiatica* standard extract to human volunteers in doses of 250 and 500 mg revealed that both single and multiple doses were well tolerated [35]. No evidence of a fatal effect or mutagenic potential of the plant extract was found in their study. Since *G. glabra* has no biologically meaningful toxicity after a single oral treatment to female Sprague Dawley rats, the median fatal dose of the plant can be estimated to be > 5000 mg/kg body weight. Further, it has been demonstrated that the component is safe after 90 days of continuous oral administration at tested doses, including the maximum dose of 1000 mg/kg body weight in rats [36].

The main limitation of this study is the absence of a comprehensive profile of liver and kidney function test. The present study as well as the previous evidence on individual components of this PHF show that there were no treatment-related changes in the renal or liver function, indicating that the PHF is non-toxic. The above biochemical and haematological findings are supported by histopathological evidence. Gross as well as histological evaluations of all the investigated organs showed no alterations or abnormalities. Further, the study also aids in establishing that PHF is a safe and non-toxic formulation and will not cause delayed toxicity, since there were no indicators of toxicity regarding haematology, clinical chemistry, gross, and histopathological investigations in the satellite group.

The majority of herbal medications on the market today have not gone through the drug approval procedure to prove their efficacy and safety. The selection, preparation, and application of herbal formulation can be guided by the thousands of years of traditional use. The future studies using same arduous procedure of scientific and clinical validation must be used to demonstrate the safety and efficacy of a therapeutic product for it to be recognised as an acceptable alternative to contemporary medicine.

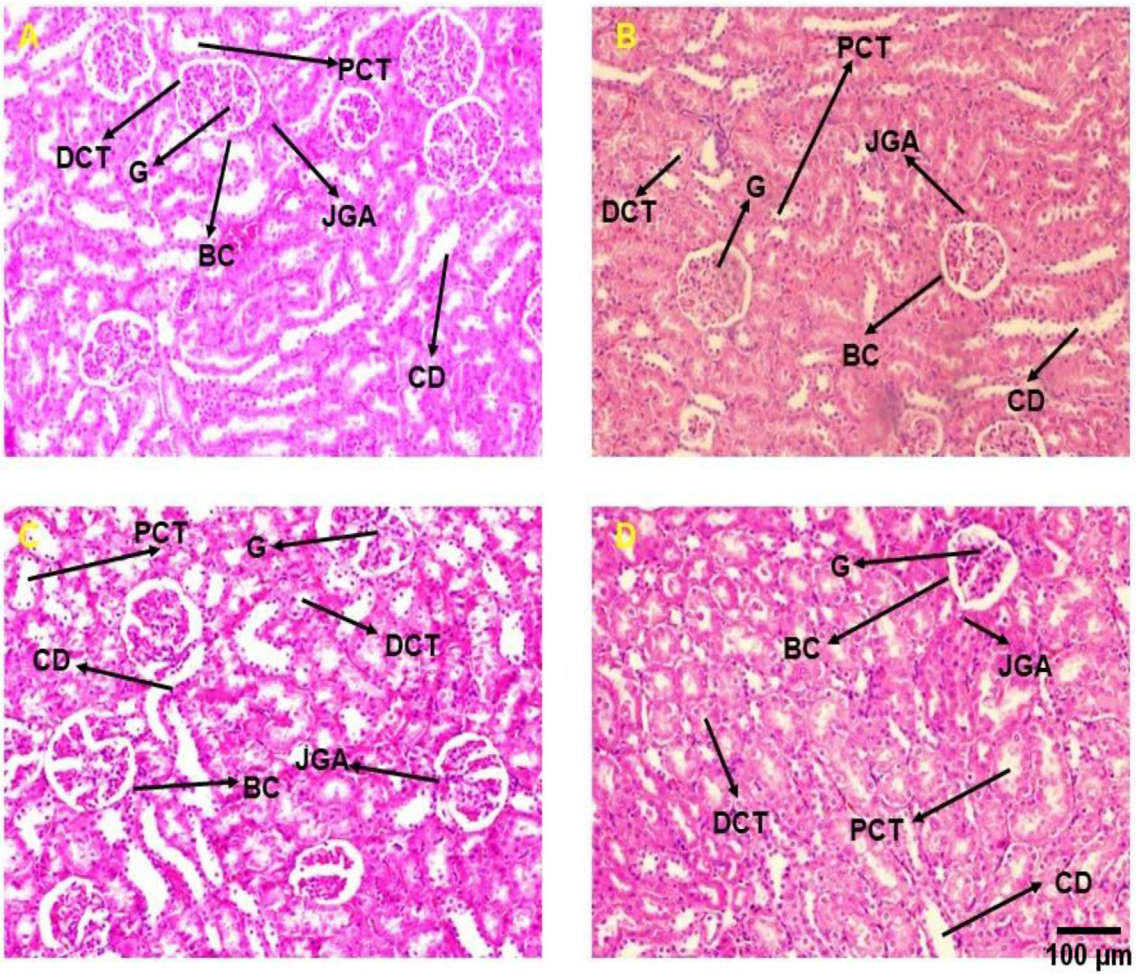

Fig. 3. Photomicrograph of a section of kidney of control (A), 500 mg/kg body weight liberin treated sub-chronic group for 28 days (B), 1000 mg/kg body weight liberin dosed sub-chronic group for 28 days (C), and 1000 mg/kg body weight treated satellite group for 28 days and 14 days follow-up (D) showing normal histology. Note: G – glomerulus, DCT – Distal Convoluted Tubule, PCT – Proximal Convoluted Tubule, JGA – Juxta Glomerular Apparatus, BC – Bowman's Capsule, CD – Collecting duct.

5. Conclusion

The safety evaluation of PHF (liberin) in rats is observed to be safe and non-toxic. Furthermore, it is reasonable to draw the conclusion that PHF is well tolerated up to a dose of 1000 mg/kg body weight administered daily for 28 days. Many Ayurveda medicine in the form of formulations has proved to be safe and highly efficient in the management of diabetes, and these can be used in clinical settings to lessen patients' dependence on synthetic hypoglycaemic medications, hence lowering the risk of long terms adverse drug reactions and drug non-compliance related complications. According to the findings of this study, the PHF may be administered for the effective management of T2DM.

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Author contributions

Renuka Suvarna: Conceptualization, Methodology, Analysis, Data curation, Writing – original draft. Revathi P Shenoy: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – review & editing. Varashree Bolar Suryakanth: Mehtodology, Validation, Writing – review & editing. Pugazhandhi Bakthavatchalam: Analysis, Data curation, Writing – original draft. Guruprasad Kalthur: Conceptualization, Methodology, Validation, Writing – review & editing. Deepak Nayak M: Validation, Review. M Mukhyaprana Prabhu: Validation, Writing-review & editing. Basavaraj S Hadapad: Validation, Review, Writing – original draft.

Declaration of competing interest

The authors declare that there is no conflict of interest in this study.

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