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Original Article

Acute and sub-acute toxicity study of anti-obesity herbal granules in Sprague Dawley rats

Estudo de toxicidade aguda e subaguda de grânulos de ervas antiobesidade em ratos Sprague Dawley

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Abstract

Toxicological studies are essential for developing novel medications in pharmaceutical industries including ayurvedic preparation. Hence, the present study is aimed to evaluate acute and 28-days repeated dose oral toxicity of anti-obesity polyherbal granules (PHG) in Sprague Dawley rats by OECD guidelines No 425 and 407, respectively. In an acute oral toxicity study, a single dose of 2 g/kg PHG was administered to rats and mortality, body weight, and clinical observations were noted for fourteen days. However, in the subacute oral toxicity study, the PHG was administered orally at doses of 0.3, 0.5 and 1 g/kg daily for 28 days to rats. Food intake and body weight were recorded weekly. On the 29th day, rats were sacrificed and subjected to haematological, biochemical, urine, necropsy, and histopathological analysis. In an acute oral toxicity study, no treatment-related, mortality, behavioral changes, and toxicity were found throughout fourteen days. Likewise, in the sub-acute toxicity study, no mortality and toxic effects were found in haematology, biochemical, urine, necropsy and histopathological analysis in rats for 28 days of treatment with PHG. Based on these results, the LD50 of PHG was found to be greater than 2 g/kg and the no-observed-adverse-effect level (NOAEL) of PHG for rats was found to be 0.5 g/kg/day. Thus, anti-obesity polyherbal granules showed a good safety profile in animal studies and can be considered an important agent for the clinical management of obesity.

Keywords: anti-obesity herbal granule, acute oral toxicity, 28-day sub-acute toxicity, repeated dose toxicity studies, *Achyranthes aspera*, *Phaseolus vulgaris*, *Camellia sinensis*, *Vitis- vinifera*, *Salacia reticulate*.

Resumo

Estudos toxicológicos são essenciais para o desenvolvimento de novos medicamentos nas indústrias farmacêuticas, incluindo a preparação aiurvédica. Assim, o presente estudo tem como objetivo avaliar a toxicidade oral aguda e de dose repetida de 28 dias de grânulos de polierva (PHG) antiobesidade em ratos Sprague Dawley pelas diretrizes da OCDE nº 425 e 407, respectivamente. Em um estudo de toxicidade oral aguda, uma dose única de 2 g/kg de PHG foi administrada a ratos, e mortalidade, peso corporal e observações clínicas foram observadas por 14 dias. No entanto, no estudo de toxicidade oral subaguda, o PHG foi administrado oralmente em doses de 0,3, 0,5 e 1 g/kg diariamente por 28 dias em ratos. A ingestão alimentar e o peso corporal foram registrados semanalmente. No 29º dia, os ratos foram sacrificados e submetidos a análises hematológicas, bioquímicas, de urina, necropsia e histopatológica. Em um estudo de toxicidade oral aguda, nenhuma mortalidade, alterações comportamentais e toxicidade relacionadas ao tratamento foram encontradas ao longo de 14 dias. Da mesma forma, no estudo de toxicidade subaguda, não foram encontrados mortalidade e efeitos tóxicos em análises hematológicas, bioquímicas, de urina, necropsia e histopatológica em ratos durante 28 dias de tratamento com PHG. Com base nesses resultados, verificou-se que a DL50 de PHG era superior a 2 g/kg e o nível de efeitos adversos não observados (NOAEL) de PHG para ratos foi de 0,5 g/kg/dia. Assim, os grânulos poliervais antiobesidade apresentaram um bom perfil de segurança em estudos com animais e podem ser considerados um importante agente para o manejo clínico da obesidade.

Palavras-chave: grânulo de ervas antiobesidade, toxicidade oral aguda, toxicidade subaguda de 28 dias, estudos de toxicidade de dose repetida, *Achyranthes aspera*, *Phaseolus vulgaris*, *Camellia sinensis*, *Vitis-vinifera*, *Salacia reticulada*.

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1. Introduction

Toxicological studies at the preclinical stage are a mandatory step to go through for every developed drug in the pharmaceutical industry (Andrade et al., 2016). These studies when performed in a suitable animal model make it possible to identify the toxic potential of drugs. Toxicological studies include acute, sub-acute, and chronic exposure to drugs, which provide scientific evidence of the drug safety spectrum as well as the drug's toxic response to targeted organs of the body (Parasuraman, 2011). The laboratory animals used in these studies are generally of low cost, easy to maintain, and provide accurate and reliable results. The use of herbal medicines is expanding every year in most countries due to a flawed belief that ayurvedic preparation is free from adverse effects without considering cases reported in Pharmacovigilance (Leenaars et al., 2019). According to the World Health Organization estimate, 80% of the population in many Asian and African countries are dependent on herbal medication (WHO, 2013). Herbal medicines are employed in treating several diseases like arthritis, cardiovascular disorders, diabetes, obesity, liver, and renal disease (Ishtiaq et al., 2017). Considering the health and safety of the population taking herbal preparations, it is essential to conduct toxicity studies of herbal preparations. The toxicity studies can be conducted in animal models and can be extrapolated to humans with some qualitative or quantitative differences (Leenaars et al., 2019). The use of laboratory animals such as rodents is relatively inexpensive and easy to maintain. Further, it is possible to screen large numbers of rodents over a wide range of doses and thus increasing the chance of detecting adverse events.

The Ayurvedic literature, Sarangdhar Samhita', emphasizes the idea of polyherbal for enhancing the therapeutic efficacy of herbs (Parasuraman et al., 2014; Karole et al., 2019; Dev et al., 2019; Shan et al., 2020). The required therapeutic effect can often be achieved by thoughtfully combining various plants in specific ratios (Parasuraman, 2011). For example, in our previous study, we selected various plants to treat obesity and the list of these plants along with their potential antiobesity targets are mentioned in Table S1-1. The anti-obesity polyherbal granules (PHG) consisted of an aqueous extract of *Camellia sinensis*, an ethanolic extract of *Achyranthes aspera*, an aqueous extract of *Salacia reticulate*, an aqueous extract of *Phaseolus vulgaris* and an aqueous extract of *Vitis- vinifera*. Our group has formulated and characterized ready-to-drink PHG and further evaluated its therapeutic efficacy in high-fat-diet-(HFD) induced obese rats. We reported that 0.3 g/kg granules significantly lowered the body and adipose tissue weight and plasma TC and TG levels in comparison to the HFD control group (Patel et al., 2020). This study was intended to assess acute and sub-acute toxicity of anti-obesity PHG granules in Sprague Dawley rats. This includes testing every important biochemical, cellular, and physiological endpoint parameter, which helps in determining any possible toxic effect produced by the formulation.

2. Material and Methods

2.1. Preparation of experimental animals

Sprague Dawley rats of weight 150-200 gm were procured from Jay Research foundation, Vapi, India. The female rats were nulliparous and non-pregnant. The rats were allowed for acclimatization periods of 1 week. A proper cycle of 12 h day and 12 h night was maintained with animals. The animal house was maintained at 20-25 °C and 55% relative humidity. A proper diet and water supply were provided to the animal. After the acclimatization period, animals were grouped for toxicity studies as per OECD guidelines. All animal experiments were carried out by CPCSEA guidelines for animal experiments.

2.2. Acute oral toxicity study

This study was performed as per the protocol suggested in OECD guideline 425 (2008) (OECD, 2008a; Lee et al., 2015; Lakshmi et al., 2017; Lima et al., 2017). For, the acute toxicity study of PHG, a limit test was performed (2000 mg/kg p.o. as a single dose) as shown in Figure 1. After dose administration, animals were kept in close observation for the initial 30 minutes, and then periodic observations were taken after 4 hours and after every 24 hours for 14 days. After no mortality was registered in the first 48 hours, four additional animals were administered with the same dose (2000 mg/ kg). The weight of animals, clinical observations based on their behavioral, neuronal and other abnormalities were noted for each animal for 14 days. On the 15th day, the animal's weight was recorded and then anaesthetized and euthanized for the collection of blood samples and vital organs. Blood samples were examined for haematology and biochemistry analysis. Urine was collected from all surviving animals before the sacrifice using metabolic cages and subjected to urine analysis. The vital organs were removed, weighed and preserved for histopathological evaluation.

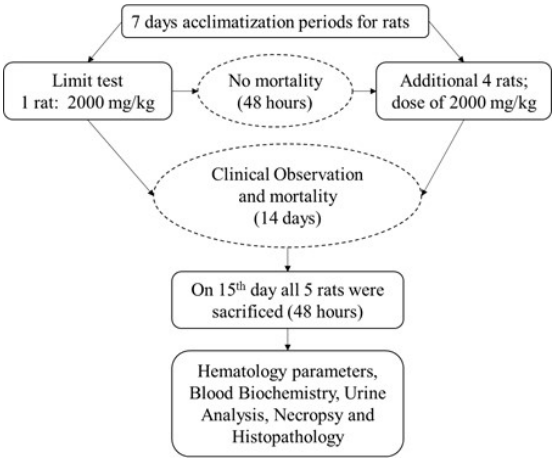

Figure 1. Flow chart showing experimental design of acute oral toxicity study.

2.3. Sub-acute oral toxicity study

The sub-acute oral toxicity study was performed as per the OECD guidelines 407 (2008). The experimental design is shown in Figure 2 (OECD, 2008b; Lee et al., 2015; Lakshmi et al., 2017; Lima et al., 2017). This study included four groups with 10 animals in each group (five males and five females). Group-I was intended as a control group, while group-II, III, and IV were treated with oral dosing of 0.3 g/kg, 0.5 g/kg and 1 g/kg of PHG, respectively. The dosing was scheduled seven days a week continuously for 28 days.

2.3.1. Clinical signs and mortality

Rats were observed once daily for morbidity and mortality for 28 days. Clinical signs such as posture, gait, fur, skin, eye/pupil, mucous, respiration, response to handling, convulsions, stereotypy and bizarre movements were observed.

2.3.2. Body weight and feed consumption

Individual animal weight and feed consumption were measured weekly during the dosing periods. The food intake was measured in the unit of g/day/rat. During the experiment body weight of rats was determined by digital weighing balance (weekly) (Lima et al., 2017).

2.3.3. Hematological and biochemistry analysis

On the 29th day, animals were weighed, anaesthetized, and euthanized for collection of blood samples and vital organs. Blood samples were examined for haematology and biochemistry analysis. Haematological parameters were determined using ABX Micros 60 haematology analyzer (Horiba Medical, France). Hb (g%), RBC (x 10^6/cmm), Rt (%), PCV (%), MCV(Fl), MCH (pg), MCHC (%), Platelets(x10^3/cmm), WBC: (x 10^3/cmm), N: Neutrophil (%), L: Lymphocyte (%), E: Eosinophils (%), M: Monocyte (%), B: Basophil (%). The following biochemical parameters such as total protein (g/dL), alanine aminotransferase (IU/L), aspartate aminotransferase (IU/L), alkaline phosphatase (IU/L), blood glucose (mg/dL), cholesterol (mg/dL), triglyceride (mg/dL), blood urea nitrogen (mg/dL), bilirubin (mg/dL), creatinine (mg/dL), albumin (g/dL), sodium (mmol/L), potassium (mmol/L), phosphorous (mg/dL) and calcium (mg/dL) were studied using Accurex AT 112 biochemical analyzer (Accurex Biomedical Pvt. Ltd, India).

2.3.4. Urine analysis

Urine was collected from all surviving animals before sacrifice using metabolic cages. The following parameters were studied using the appropriate methodology. Volume (24 hours output in ml), color, appearance, pH, specific gravity, glucose, ketones, proteins, bilirubin, urobilinogen, nitrite, occult blood and microscopy (P: Pus cell, E: Epithelial cell, C: Casts, R: Red blood cell (Erythrocyte), Cr (T): Crystals)

2.3.5. Necropsy, organ collection and histopathological examination

In the end, all surviving rats were weighed and sacrificed using the carbon dioxide asphyxiation method. The animals were subjected to gross necropsies such as inspection of the outer body surface, cranial, abdominal and thoracic cavity and their contents. Gross examination of collected organs and tissues (viz. liver, kidneys, heart, aorta, brain, lungs, esophagus, stomach, duodenum, jejunum, ileum, colon, rectum, eyes, skin, skeletal muscle, thyroids, thymus, spleen, adrenals, mesenteric lymph node, urinary bladder, uterus, mammary gland, epididymis, testis, ovaries, spinal cord, middle ear, parathyroid, pancreas, and trachea). The organs, from males and females, were collected, weighed and preserved. The absolute organ weight was recorded for liver, kidney, heart, brain, spleen, adrenal glands, lungs and testis/ovaries and the corresponding relative body to organ weight ratio was determined. Organs were preserved in 10% formalin for histopathology evaluation (Lima et al., 2017).

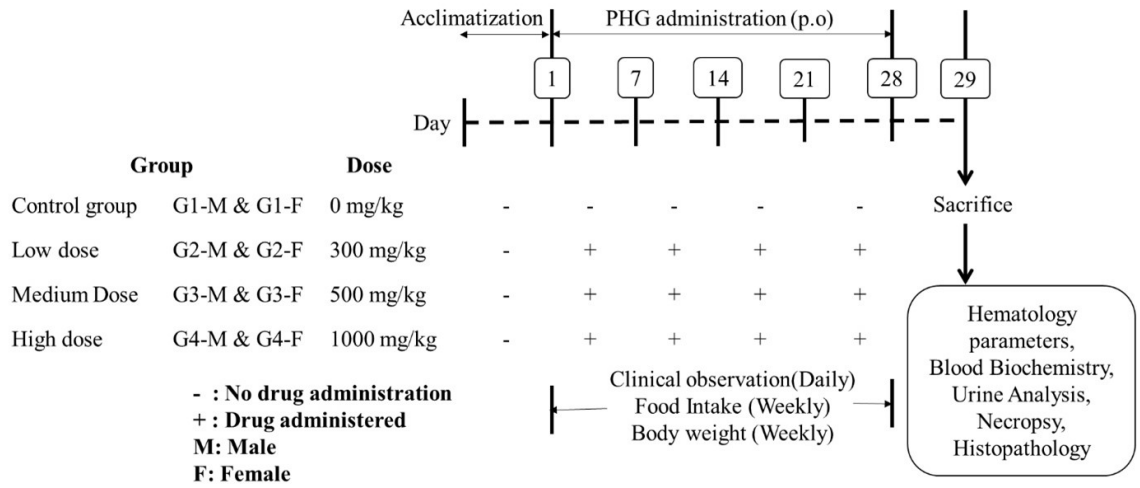

Figure 2. Flow chart showing experimental design of sub-acute oral toxicity study.

2.4. Statistical analysis

The results were subjected to one-way ANOVA followed by Dunnett: compare all columns vs control-column. All the statistical analyses were performed using the GraphPad InStat program. P-value < 0.05 was considered to be significant. The results were expressed as mean ± standard deviation (SD).

3. Results

The selection of OECD guidelines helped in employing the limited number of animals in our study.

3.1. Acute oral toxicity study

No treatment-related, mortality or behavioral changes or toxicity were observed throughout 14 days after the oral administration of a single dose of 2 g/kg herbal granules. During clinical observation, fur, skin, eyes, mucous membrane, gait, posture and respiration appeared normal. No lacrimation, clonic or tonic movement, salivation, piloerection, diarrhea, stereotype and bizarre behaviors were observed. This indicates that the LD50 value of PHG is greater than 2 g/kg. Hence, 0.3, 0.5 and 1 g/kg, doses of PHG were selected for the oral sub-acute toxicity study.

3.2. Sub-acute oral toxicity study

3.2.1. Clinical signs and mortality

The effect of PHG on clinical signs and mortality is presented in Table 1. Control animals (male and female) were free of intoxicating signs for 28 days. Male and female animals exhibited a slight decrease in activity immediately after treatment with PHG. Whereas male animals treated with 1 g/kg PHG showed mild diarrhea on the 28th day. No treatment-related, mortality was recorded in rats for 28 days of oral treatment at PHG doses of 0.3 g/kg, 0.5 g/kg and 1 g/kg.

3.2.2. Body weight and food consumption

Both male and female rats from Group II & III exhibited significant dose-dependent weight loss compared to the control group between 14 to 28 days after initiation of the study ($P < 0.05$) (Figure 3). The food consumption measurements in Table 2 did not reveal a significant change in food consumption in rats treated with PHG compared to the control group.

3.2.3. Haematological analysis

Table 3 represents the haematological parameters. Haematological parameters, like haemoglobin, total RBCs, hematocrit, PCV, red blood cell indices, total and differential WBC count, platelet count, bleeding time and clotting time in PHG administrated animals were not significantly different from control animals.

3.2.4. Biochemical analysis

The effect of PHG on biochemical parameters is depicted in Table 4. In the low dose administered group, all biochemical parameters studied were found to be within the normal biological limits. The PHG at all three-dose levels did not affect serum total protein, albumin, globulin and electrolytes (sodium, potassium, calcium, and phosphorus). Hepatic indicator profiles such as ALT, AST, and ALP were not significantly higher under the influence of 0.5 g/kg and 1 g/kg PHG. The kidney function parameters, like BUN, and creatinine level were slightly higher in male and female rats treated with 0.5 g/kg and 1 g/kg of PHG compared to the control group. Besides, a dose-dependent decrease in triglyceride levels were found in 0.5 g/kg and 1 g/kg PHG treated groups compared to the control group.

3.2.5. Urine analysis

The details of urine analysis in male and female rats are presented in Table 5. Urine analysis of control and treated animals revealed no abnormality attributable to the PHG treatment.

3.2.6. Necropsy and absolute organ weights

The gross pathological examination revealed mild leukocytic infiltration and aspiration in the lungs and discoloration with occasional cholestasis in the liver.

Table 1. Clinical sign and mortality of rats treated with different doses of PHG for 28 days.

Group No	Dose (mg/kg)	Total No. of Animals	Observed Signs	Period of signs in days	Mortality
			Male		
G1-M	0	5	Nil	28	0/5
G2-M	300	5	Nil	28	0/5
G3-M	500	5	Nil	28	0/5
G4-M	1000	5	Nil	28	0/5
			Female		
G1-F	0	5	Nil	28	0/5
G2-F	300	5	Nil	28	0/5
G3-F	500	5	Nil	28	0/5
G4-F	1000	5	Nil	28	0/5

Enlargement with a very mild hemorrhage in the kidneys were observed at 1 g/kg PHG treatment (Table S1-2). On the other hand, treatment with 0.3 g/kg and 0.5 g/kg PHG showed no abnormality when compared with the control group. The mean absolute (Table 6) and relative (Table 7) weights of liver, kidneys, brain, lungs, adrenal, spleen, uterus and testes/ ovaries of rats were not significantly different in control and PHG-treated groups. The data indicated no toxic effect of herbal granules.

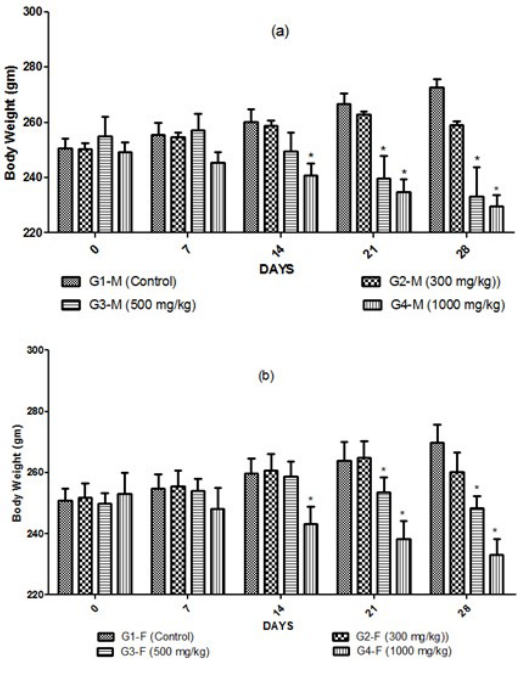

Figure 3. Initial and weekly mean body weight of (a) male rats and (b) female rats, in the sub-acute dose of control, 300, 500 and 1000 mg/kg of PHG, for 28 days. Values were expressed as the mean ± SD (n=5). *Significantly different from the control group ($p < 0.05$).

3.2.7. Histopathology

Histopathological studies were performed on the liver, kidney, spleen, heart, and lungs of both the male and female rats. A summary of histopathological observations is given in Table S2-2.1 to 2-8. Histopathological examination of different tissues of animals treated with PHG did not find any structural alterations due to the treatment.

4. Discussion

Obesity is a well-known nutritional disorder and it is well-recognized as a contributor to many diseases (Klein et al., 2002; Shah et al., 2021). It is a condition where the body starts converting excessive free energy into loose adipose tissue (Luo and Liu, 2016). There are multifactorial reasons, especially genetics, diet, and the presence of disease, which can lead to the accumulation of adipose tissue in the body (Curioni et al., 2019). Obesity is a proven root cause of many health-related issues and even affects the physical, mental and emotional health of patients (Laverack, 2018). According to the WHO statistics in 2016, around 650 million adults were in the obese category out of 1.9 billion adults aged 18 years and older (Bhupathiraju and Hu, 2016). Allopathic medicine is used widely in treating obesity, however, it can produce adverse effects in patients and can even result in a new disorder in the people undergoing treatment. Today, people are opting for ayurvedic preparations to avoid the adverse effects posed by allopathic treatment. We had previously formulated an anti-obesity ayurvedic formulation by combining five types of plant extracts that are proven to have anti-obesity effects. Combining the extracts can have a synergistic effect on weight reduction which in turn could result in speedy recovery in patients with fewer or no side effects. The formulation includes an aqueous extract of *Camellia sinensis*, an ethanolic extract of *Achyranthes aspera*, an aqueous extract of *Salacia reticulate*, an aqueous extract of *Phaseolus vulgaris* and an aqueous extract of *Vitis- vinifera*. The preliminary evaluation of extract and herbal granules

Table 2. Food consumption (g/animal) of rats treated with different doses of PHG for 28 days.

Group No	Dose (mg/kg)	Group mean food consumption (g/animal)				
		0 Days	7 Days	14 Days	21 Days	28 Days
			Male			
G1-M	0	27.74 ± 0.80	28.34 ± 0.75	30.66 ± 0.53	32.54 ± 0.56	33.98 ± 0.53
G2-M	300	27.88 ± 0.89#	28.62 ± 0.99#	29.26 ± 1.00#	30.42 ± 0.72#	29.26 ± 0.50#
G3-M	500	27.66 ± 1.00#	29.18 ± 0.75#	31.50 ± 0.62#	30.38 ± 0.90#	29.28 ± 0.44#
G4-M	1000	27.58 ± 0.85#	28.04 ± 1.00#	27.36 ± 1.00#	26.76 ± 0.95#	25.82 ± 1.29#
			Female			
G1-F	0	28.26 ± 0.73	28.64 ± 0.73	29.76 ± 0.80	31.78 ± 0.51	33.00 ± 0.42
G2-F	300	27.40 ± 0.49#	28.50 ± 0.58#	29.62 ± 0.70#	31.36 ± 0.43#	30.40 ± 0.74#
G3-F	500	27.00 ± 0.74#	29.22 ± 0.73#	30.58 ± 0.62#	29.92 ± 0.51#	29.08 ± 0.36#
G4-F	1000	27.66 ± 0.69#	27.40 ± 0.79#	26.84 ± 0.65#	26.24 ± 0.73#	25.54 ± 0.53#

Values were expressed as the mean ± SD (n=5). #Significantly different from the control group ($p > 0.05$).

Table 3. Hematological profile of rats treated with different doses of PHG for 28 days.

Name of Test	Unit	Control	300 mg/ kg	500 mg/ kg	1000 mg/ kg
Male					
Hemoglobin	(g%)	17.00 ± 0.88	16.02 ± 1.09#	15.88 ± 0.47#	15.68 ± 0.33#
Total RBC	(x 10^6 /cmm)	7.90 ± 0.35	7.38 ± 0.37#	7.16 ± 0.30#	6.80 ± 0.35#
Rt.	(%)	2.40 ± 0.08	2.46 ± 0.04#	2.44 ± 0.08#	2.49 ± 0.06#
PCV	(%)	54.80 ± 2.14	53.48 ± 1.80#	53.18 ± 1.57#	53.24 ± 1.74#
MCV	(Fl)	69.48 ± 3.66	72.63 ± 4.58#	74.32 ± 2.06#	78.54 ± 6.07#
MCH	(pg)	21.56 ± 1.49	21.76 ± 1.96#	22.22 ± 1.30#	23.12 ± 1.55#
MCHC	(%)	31.07 ± 1.99	29.95 ± 1.62#	29.88 ± 1.34#	29.48 ± 1.10#
Platelets	(x10^3 /cmm)	1.46 ± 0.08	1.53 ± 0.03#	1.53 ± 0.04#	1.52 ± 0.04#
WBC	(x 10^3 /cmm)	6.25 ± 0.63	5.88 ± 0.36#	5.32 ± 0.44#	5.27 ± 0.23#
Neutrophil	(%)	34.60 ± 1.02	34.40 ± 1.14#	34.80 ± 1.48#	37.60 ± 0.89#
Lymphocyte	(%)	57.80 ± 1.47	57.80 ± 1.48#	58.40 ± 1.52#	58.60 ± 0.55#
Eosinophil	(%)	0.96 ± 0.34	1.10 ± 0.28#	1.12 ± 0.29#	1.06 ± 0.21#
Monocyte	(%)	6.26 ± 0.39	6.40 ± 0.60#	6.02 ± 0.54#	6.06 ± 0.50#
Basophil	(%)	0.44 ± 0.24	0.50 ± 0.31#	0.46 ± 0.19#	0.48± 0.22#
Bleeding time	(min)	2.66 ± 0.22	2.51 ± 0.07#	2.46 ± 0.07#	2.47 ± 0.06#
Coagulation time	(min)	5.72 ± 0.34	5.82 ± 0.33#	5.72 ± 0.31#	5.51 ± 0.21#
Prothrombin time	(min)	15.90 ± 0.30	15.38 ± 0.47#	15.64 ± 0.15#	15.92 ± 0.37#
APTT	(sec)	14.72 ± 0.36	15.04 ± 0.38#	14.78 ± 0.53#	14.90 ± 0.51#
Female:					
Hemoglobin	(g%)	15.68 ± 0.37	15.72 ± 0.66#	15.82 ± 0.50#	15.92 ± 0.54#
Total RBC	(x 10^6 /cmm)	7.66 ± 0.38	7.12 ± 0.28#	7.24 ± 0.43#	7.12 ± 0.28#
Rt.	(%)	2.45 ± 0.11	2.42 ± 0.25#	2.50 ± 0.16#	2.41 ± 0.07#
PCV	(%)	53.60 ± 2.70	51.20 ± 2.59#	54.00 ± 1.58#	53.90 ± 1.14#
MCV	(Fl)	70.17 ± 5.96	71.96 ± 3.88#	74.86 ± 6.04#	75.82 ± 3.92#
MCH	(pg)	20.52 ± 1.23	22.13 ± 1.71#	21.93 ± 1.68#	22.40 ± 1.54#
MCHC	(%)	29.32 ± 1.82	30.80 ± 2.54#	29.30 ± 0.71#	29.55 ± 1.16#
Platelets	(x10^3 /cmm)	1.49 ± 0.06	1.44 ± 0.03#	1.51 ± 0.02#	1.50 ± 0.02#
WBC	(x 10^3 /cmm)	5.89 ± 0.56	5.54 ± 0.66#	5.84 ± 0.20#	5.97 ± 0.50#
Neutrophil	(%)	34.60 ± 1.14	35.60 ± 1.52#	36.00 ± 2.12#	36.90 ± 1.92#
Lymphocyte	(%)	58.40 ± 1.52	57.40 ± 2.07#	56.20 ± 1.79#	55.40 ± 2.30#
Eosinophil	(%)	1.12 ± 0.37	1.02 ± 0.19v	1.12 ± 0.29#	1.00 ± 0.25#
Monocyte	(%)	5.64 ± 0.40	5.62 ± 0.16#	6.44 ± 0.68#	6.32 ± 0.62#
Basophil	(%)	0.52 ± 0.22	0.44 ± 0.19#	0.44 ± 0.30#	0.56 ± 0.18#
Bleeding time	(min)	2.74 ± 0.23	2.88 ± 0.37#	2.49 ± 0.04#	2.47 ± 0.06#
Coagulation time	(min)	5.54 ± 0.36	5.88 ± 0.38#	5.96 ± 0.36#	5.88 ± 0.50#
Prothrombin time	(min)	15.98 ± 0.48	15.92 ± 0.33#	15.48 ± 0.47#	15.08 ± 0.39#
APTT	(sec)	14.74 ± 0.34	14.80 ± 0.34#	14.98 ± 0.33#	15.04 ± 0.45#

Values were expressed as the mean ± SD (n=5). #Significantly different from the control group ($p > 0.05$).

resulted in satisfactory results. Individual toxicology study of each extract is reported in the literature to a certain extent, however, the toxicity of combination in the form PHG remains unknown (Patel et al., 2020). In this study, we performed acute and 28-day repeated dose toxicity in Sprague Dawley rats.

In acute toxicity studies, we did not find any clinical changes produced in animal physiological and psychological

Table 4. Biochemical analysis of rats treated with different doses of PHG for 28 days.

Name of Test	Unit	Control	300 mg/ kg	500 mg/ kg	1000 mg/ kg
Male					
Total Protein	(g/dL)	67.20 ± 3.70	65.80 ± 4.32#	66.20 ± 3.90#	69.80 ± 3.27#
ALT	(IU/L)	34.00 ± 3.67	36.80 ± 3.27#	37.40 ± 1.52#	37.60 ± 2.30#
AST	(IU/L)	92.00 ± 12.59	96.80 ± 8.73#	102.40 ± 4.39#	100.20 ± 7.29#
ALP	(IU/L)	117.00 ± 10.10	113.60 ± 15.08#	121.40 ± 11.08#	125.20 ± 6.98#
Blood Glucose	(mg/dL)	98.80 ± 7.85	101.40 ± 9.91#	93.60 ± 10.83#	97.20 ± 7.40#
Cholesterol	(mg/dL)	103.00 ± 10.42	98.20 ± 5.81#	96.80 ± 4.60#	95.40 ± 5.94#
Triglyceride	(mg/dL)	201.60 ± 13.24	196.60 ± 7.70#	185.00 ± 16.64*	189.00 ± 12.53*
BUN	(mg/dL)	17.50 ± 1.06	17.54 ± 0.71#	17.40 ± 0.66#	17.18 ± 0.38#
Bilirubin	(mg/dL)	0.34 ± 0.07	0.34 ± 0.04#	0.34 ± 0.03#	0.33 ± 0.03#
Creatinine	(mg/dL)	0.63 ± 0.05	0.64 ± 0.05#	0.67 ± 0.03#	0.70 ± 0.03#
Albumin	(g/dL)	33.20 ± 2.59	36.00 ± 2.92#	38.80 ± 2.59#	38.00 ± 2.74#
Sodium	(mmol/L)	153.60 ± 4.04	151.80 ± 3.56#	151.60 ± 3.78#	155.60 ± 3.97#
Potassium	(mmol/L)	6.98 ± 0.19	7.04 ± 0.19#	6.84 ± 0.30#	7.14 ± 0.23#
Phosphorous	(mg/dL)	6.98 ± 0.37	7.14 ± 0.23#	7.12 ± 0.19#	7.00 ± 0.24#
Calcium	(mg/dL)	12.72 ± 0.47	12.98 ± 0.51#	13.02 ± 0.33#	13.16 ± 0.46#
Female					
Total Protein	(g/dL)	67.80 ± 3.83	68.20 ± 2.86#	66.60 ± 3.71#	65.80 ± 3.11#
ALT	(IU/L)	37.60 ± 2.07	37.00 ± 1.58#	35.80 ± 2.77#	38.20 ± 2.17#
AST	(IU/L)	101.60 ± 6.80	107.00 ± 9.22#	100.40 ± 12.46#	105.20 ± 5.26#
ALP	(IU/L)	111.20 ± 4.21	118.00 ± 11.47#	119.00 ± 15.03#	121.60 ± 11.06#
Blood Glucose	(mg/dL)	104.20 ± 5.26	101.00 ± 10.15#	101.40 ± 4.10#	105.80 ± 7.22#
Cholesterol	(mg/dL)	102.00 ± 5.52	99.80 ± 6.69#	96.60 ± 4.93#	92.20 ± 8.96#
Triglyceride	(mg/dL)	201.40 ± 12.99	197.80 ± 9.58#	180.80 ± 10.78*	180.00 ± 7.62*
BUN	(mg/dL)	16.96 ± 0.45	17.54 ± 0.68#	16.88 ± 0.81#	17.26 ± 0.75#
Bilirubin	(mg/dL)	0.33 ± 0.04	0.32 ± 0.04#	0.32 ± 0.04#	0.37 ± 0.03#
Creatinine	(mg/dL)	0.62 ± 0.04	0.69 ± 0.04#	0.67 ± 0.03#	0.69 ± 0.05#
Albumin	(g/dL)	34.60 ± 1.14	39.80 ± 1.48#	38.40 ± 2.97#	37.40 ± 2.79#
Sodium	(mmol/L)	154.00 ± 3.54	151.00 ± 2.74#	149.60 ± 1.82#	153.00 ± 3.16#
Potassium	(mmol/L)	7.00 ± 0.16	6.76 ± 0.21#	6.72 ± 0.20#	6.92 ± 0.35#
Phosphorous	(mg/dL)	7.04 ± 0.24	7.04 ± 0.19#	6.96 ± 0.13#	7.08 ± 0.13#
Calcium	(mg/dL)	12.68 ± 0.30	13.26 ± 0.35#	12.84 ± 0.52#	12.90 ± 0.27#

Values were expressed as the mean ± SD (n=5). *Significantly different from the control group ($p < 0.05$). #Significantly different from the control group ($p > 0.05$).

behavior. Hence, LD50 of PHG is considered to be greater than 2000mg/kg. While in the subacute toxicity study there is a decrease in the bodyweight of animals, no mortality was observed. All haematological and renal function parameters were found to be in the normal range. In the lipid profile, a decrease in triglyceride level was noted. This might be due to the aqueous extract of *Phaseolus vulgaris,* which was reported to alter lipid profile when exposed in subacute toxicity studies (Chokshi, 2006). The increase in total bilirubin level in female rat serum was noted during the hepatic function test. This could be again attributed to *Phaseolus vulgaris* as reported by Chokshi (2006). All other hepatic function test parameters were found to be in the normal range. In a gross pathological study, we found that there is an increase in the weight of the liver and spleen, which may be due to the presence of *Achyranthes aspera* (Rani et al., 2012; Reddy and Kamble, 2014). The increase in liver weight may also result due to the presence of the aqueous extract of *Vitis- vinifera* as noted earlier (Fiume et al., 2014; Zhang et al., 2017). Rest all organs like the heart, kidney, and lungs were found to be in the normal weight. In histopathological studies, we found

Table 5. Urine analysis of rats treated with different doses of PHG for 28 days.

Name of Test	Control	300 mg/ kg	500 mg/ kg	1000 mg/ kg
Male				
Volume (24 hours output)	14.20 ± 2.59	10.00 ± 1.58#	14.00 ± 1.58#	14.20 ± 1.48#
Color	Pale yellow	Pale yellow to dark yellow	Pale yellow to dark yellow	Pale yellow to dark yellow
Appearance	Clear	Clear	Clear to turbid	Clear to turbid
pH	7.58 ± 0.28	7.50 ± 0.21#	7.54 ± 0.26#	7.64 ± 0.36#
Sp. Gravity	1.08 ± 0.08	1.10 ± 0.12#	1.15 ± 0.12#	1.19 ± 0.13#
Glucose	0	0	0	0
Ketones	0	0	0	0
Proteins	0	0	0	0
Bilirubin	0	0	0	0
Urobilinogen	0	0	0	0
Nitrite	0	0	0	0
Occult blood	0	0	0	0
P: Pus cell	0	0	0	0
Epithelial cell	0	0	0	0
C: Casts	0	0	0	0
Cr (T): Crystals	0	0	0	0
Female				
Volume (24 hours output)	10.00 ± 1.58	10.40 ± 3.21#	9.20 ± 1.30#	10.20 ± 2.86#
Color	Pale yellow	Pale yellow to dark yellow	Pale yellow to dark yellow	Pale yellow to dark yellow
Appearance	Clear	Clear	Clear to turbid	Clear to turbid
pH	7.26 ± 0.21	7.30 ± 0.16#	7.46 ± 0.30#	7.50 ± 0.30#
Sp. Gravity	1.10 ± 0.07	1.10 ± 0.07#	1.18 ± 0.13#	1.21 ± 0.12#
Glucose	0	0	0	0
Ketones	0	0	0	0
Proteins	0	0	0	0
Bilirubin	0	0	0	0
Urobilinogen	0	0	0	0
Nitrite	0	0	0	0
Occult blood	0	0	0	0
P: Pus cell	0	0	0	0
Epithelial cell	0	0	0	0
C: Casts	0	0	0	0
Cr (T): Crystals	0	0	0	0

Values were expressed as the mean ± SD (n=5). #Significantly different from the control group ($p > 0.05$). Qualitative urine analysis: Absent = 0; Trace = +; Small amount of analyte = ++; Moderate amount of analyte = +++; Large amount of analyte = ++++. Microscopic urine analysis: 0= Not found in field; 1= Few found in some field; 2= Few found in many field; 3= Many found in many field.

Table 6. Absolute organ weight (gm) of rats treated with different doses of PHG for 28 days.

Name of Test	Control	300 mg/ kg	500 mg/ kg	1000 mg/ kg
Male				
Brain	1.36 ± 0.08	1.35 ± 0.09#	1.22 ± 0.13#	1.14 ± 0.13#

Values were expressed as the mean ± SD (n=5). #Significantly different from the control group ($p > 0.05$).

Table 6. Continued...

Name of Test	Control	300 mg/ kg	500 mg/ kg	1000 mg/ kg
Liver	6.52 ± 0.52	6.31 ± 0.51#	5.74 ± 0.65#	5.63 ± 0.49#
Spleen	0.50 ± 0.07	0.51 ± 0.10#	0.47 ± 0.10#	0.47 ± 0.10#
Kidney	1.90 ± 0.15	1.82 ± 0.09#	1.57 ± 0.16#	1.65 ± 0.12#
Adrenal	0.05 ± 0.01	0.05 ± 0.01#	0.05 ± 0.01#	0.05 ± 0.01#
Testis	1.74 ± 0.07	1.60 ± 0.11#	1.46 ± 0.10#	1.43 ± 0.15#
Heart	0.83 ± 0.07	0.79 ± 0.18#	0.73 ± 0.14#	0.68 ± 0.10#
Lungs	1.29 ± 0.19	1.20 ± 0.10#	1.09 ± 0.14#	1.08 ± 0.11#
Female				
Brain	2.01 ± 0.18	1.96 ± 0.12#	1.85 ± 0.14#	1.76 ± 0.11#
Liver	6.47 ± 0.42	6.38 ± 0.46#	6.21 ± 0.47#	5.70 ± 0.67#
Spleen	0.56 ± 0.05	0.56 ± 0.10#	0.55 ± 0.12#	0.49 ± 0.06#
Kidney	1.91 ± 0.23	1.78 ± 0.16#	1.72 ± 0.20#	1.67 ± 0.19#
Adrenal	0.05 ± 0.01	0.05 ± 0.02#	0.05 ± 0.01#	0.04 ± 0.00#
Uterus	0.58 ± 0.05	0.59 ± 0.11#	0.54 ± 0.07#	0.50 ± 0.08#
Ovary	0.06 ± 0.01	0.06 ± 0.01#	0.06 ± 0.01#	0.05 ± 0.01#
Heart	0.94 ± 0.11	0.95 ± 0.10#	0.86 ± 0.11#	0.76 ± 0.09#
Lungs	1.25 ± 0.11	1.26 ± 0.15#	1.17 ± 0.10#	1.07 ± 0.09#

Values were expressed as the mean ± SD (n=5). #Significantly different from the control group ($p > 0.05$).

Table 7. Relative organ weight (%) of rats treated with different doses of PHG for 28 days.

Name of Test	Control	300 mg/ kg	500 mg/ kg	1000 mg/ kg
Male				
Brain	0.50 ± 0.03	0.52 ± 0.03#	0.52 ± 0.07#	0.50 ± 0.06#
Liver	2.39 ± 0.17	2.44 ± 0.19#	2.46 ± 0.23#	2.45 ± 0.18#
Spleen	0.18 ± 0.02	0.20 ± 0.04#	0.20 ± 0.05#	0.22 ± 0.05#
Kidney	0.70 ± 0.05	0.70 ± 0.04#	0.67 ± 0.04#	0.72 ± 0.06#
Adrenal	0.018 ± 0.005	0.019 ± 0.004#	0.020 ± 0.005#	0.020 ± 0.005#
Testis	0.64 ± 0.03	0.62 ± 0.04#	0.63 ± 0.04#	0.62 ± 0.07#
Heart	0.30 ± 0.02	0.31 ± 0.07#	0.31 ± 0.05#	0.30 ± 0.04#
Lungs	0.47 ± 0.07	0.46 ± 0.04#	0.47 ± 0.04#	0.47 ± 0.04#
Female				
Brain	0.74 ± 0.07	0.75 ± 0.05#	0.74 ± 0.05#	0.75 ± 0.05#
Liver	2.40 ± 0.13	2.45 ± 0.18#	2.50 ± 0.19#	2.48 ± 0.26#
Spleen	0.21 ± 0.02	0.22 ± 0.04#	0.22 ± 0.05#	0.23 ± 0.02#
Kidney	0.71 ± 0.09	0.68 ± 0.05#	0.69 ± 0.08#	0.72 ± 0.07#
Adrenal	0.019 ± 0.003	0.020 ± 0.006#	0.021 ± 0.004#	0.020 ± 0.001#
Uterus	0.22 ± 0.02	0.23 ± 0.05#	0.22 ± 0.03#	0.22 ± 0.03#
Ovary	0.023 ± 0.003	0.023 ± 0.003#	0.025 ± 0.002#	0.023 ± 0.002#
Heart	0.35 ± 0.04	0.36 ± 0.04#	0.35 ± 0.05#	0.32 ± 0.04#
Lungs	0.46 ± 0.03	0.49 ± 0.06#	0.47 ± 0.04#	0.45 ± 0.04#

Values were expressed as the mean ± SD (n=5). #Significantly different from the control group ($p > 0.05$).

significant changes in the liver. This could be attributed to the presence of the aqueous extract of *Phaseolus vulgaris* as reported earlier in sub-acute toxicity studies (Barrett and Udani, 2011).

5. Conclusion

In the way of gathering knowledge regarding the toxicity of herbal formulation, we conclude that the LD50 of PHG is >2 g/kg and the no-observed-adverse-effect level (NOAEL) of PHG in both male and female rats is 0.5 g/kg when administered orally for 28 days. Thus, anti-obesity polyherbal granules showed a good safety profile in animal studies and can be considered as an important agent for the clinical management of obesity.

Abbreviations

ALP: Alkaline Phosphatase; ALT: Alanine Aminotransferase; ANOVA: Analysis of variance; APTT: Activated Partial Thromboplastin Clotting Time; AST: Aspartate Aminotransferase; BUN: Blood urea Nitrogen; CPCSEA: Committee for the purpose of control and supervision of experiments on animals; Hb: Hemoglobin; PHG: Herbal granules; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; MCV: Mean Corpuscular Volume; OECD: Organization for Economic Co-operation and Development; PCV: Packed Cell Volume; RBC: Red Blood Corpuscles; Rt.: Reticulocyte; SD: standard deviation; WBC: White Blood Corpuscles; WHO: World Health Organization.

Ethical Approval

IAEC (Institutional Animal Ethics Committee) approved the experimental protocol SSR/IAEC/2017/02.

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Supplementary Material

Supplementary material accompanies this paper.

Table S1-1. Description of herbal plants extracts.
Table S1-2. Summary of necropsy findings.

Table S2-2.1 to 2.8. Histopathology data.

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