Pralhad Wangikar, Pradhnya Chaudhari, Eshita Sharma, Chhaya Godse, Ashit Vora and Sujit Nair*

Acute and sub-chronic oral GLP toxicity of *Withania somnifera* root extract in Sprague Dawley rats

https://doi.org/10.1515/dmpt-2024-0056
Received July 23, 2024; accepted August 20, 2024;
published online October 2, 2024

Abstract

Objectives: *Withania somnifera* (WS) is a valuable medicinal plant that has been used against several ailments. The medicinal properties of WS are ascribed to existence of secondary metabolites which are in great demand in herbal nutraceutical industry. Despite well-known therapeutic effects of WS, it is necessary to assess preclinical toxicity of WS plant on rats and further explore its potential application against treatment of various disorders in humans. The existing study assessed oral acute and sub-chronic toxicities of WS root extract in Sprague Dawley (SD) rats (male and female) for 14 and 90 days, respectively under OECD-423 and -408 guidelines as well as GLP compliance.

Methods: In acute toxicity, rats of either sex were orally fed a dose of 2,000 mg/kg. In sub-chronic toxicity, animals were orally administered repeated doses of WS root extract at 250, 500, 1,000 mg/kg for 90 days with an additional 14-day recovery period. Two more groups (n=5 animals each) receiving vehicle and 1,000 mg/kg of WS root extract for 90 days were also observed.

Results: In acute toxicity, the results revealed that LD_{50} of WS root extract in SD rats was higher than 2,000 mg/kg. In sub-chronic toxicity, oral administration of extract for 90 days showed no significant toxicological changes in rats. Haematological and serum chemistry markers were found within normal range. Terminal necropsy showed no gross or histopathological outcomes.

Conclusions: The no-observed-adverse-effect level (NOAEL) of WS root extract was 1,000 mg/kg body weight, and safe to use at this dose in rats.

Keywords: *Withania somnifera*; toxicity; OECD guidelines; GLP compliance; no-observed-adverse-effect level (NOAEL); pharmacological activity

Introduction

Nutraceuticals and herbal supplements have been gaining attention in the last few decades due to enhanced awareness of people towards natural products compared to synthetic ones [1]. *Withania somnifera* (WS) is getting popular due to its wide range of therapeutic activities, especially in chronic diseases like neurological disorders, muscle stiffness, insomnia, and as nootropic, immunomodulatory, general tonic in weakness [1–6]. WS has been well recognized as 'Rasayana' in Ayurveda, which nourishes the body at cellular, tissue, organ, and system levels, increases vitality, improves the immune system, preserves health, prevents diseases, and enhances the quality of life in diseased persons, thus, collectively promoting healthy ageing [2, 7, 8].

The diverse class of constituents present in WS (Ashwagandha) comprises steroidal lactones, flavonoids, alkaloids, and tannins. Roots, berries, and aerial parts of WS possess about 40 withanolides, and 12 alkaloids along with several sitoindosides while leaves are rich in different alkaloids such as withanine, pseudowithanine, somniferine etc. [5]. Despite the presence of several alkaloids in leaves and other parts of the WS plant, only roots have been recommended for therapeutic use as well as for internal administration by "Ayurveda", the traditional Indian medicine system [1]. Extracts of WS are generally taken as tablets or capsules once or 3 times a day with no common side effects [9, 10]. Commercial herbal supplements contain 1–5 % withanolides along with withaferin A which have been well-identified for the pharmacological activity of WS, although some of the alkaloids in WS have been recognized as toxic and harmful by the European Food Safety Authority [11].

Each part of WS varies in the concentration of bioactive compounds and has been tested for toxicity studies as a methanolic or ethanolic extract, seed powder, aqueous alcoholic extract, root paste etc. [10–19]. Patel et al. [20] investigated the toxicity of methanolic root extract of WS at higher doses *viz*. 500, 1,000 and 2,000 mg/kg and revealed

***Corresponding author: Dr. Sujit Nair**, MPharm, PhD (USA), Chief Scientific Officer, Phytoveda Pvt. Ltd. Viridis Biopharma Pvt. Ltd., V.N. Purav Marg, Mumbai 400 022, India, E-mail: sujit108@gmail.com. https://orcid.org/0000-0002-7322-7375
Pralhad Wangikar and Pradhnya Chaudhari, PRADO, Preclinical Research and Development Organization Pvt. Ltd., Pune, India
Eshita Sharma, Chhaya Godse and Ashit Vora, Phytoveda Pvt. Ltd., Mumbai, India; and Viridis Biopharma Pvt. Ltd., Mumbai, India

that the 2,000 mg/kg dose was well tolerated *via* oral administration with no adverse effects. The alcoholic extract of WS roots when administered by intraperitoneal (IP) route showed LD_{50} of 1,260 mg/kg in mice; further, subacute toxicity studies did not show any changes in the peripheral blood system or mortality in rats [21]. Balkrishna et al. [13] reported that whole plant extract of WS was safe at the dose level of 1,000 mg/kg in sub-acute toxicity assessment in SD rats for 28 days. Hussein et al. [16] reported that oral subchronic toxicity of aerial plant parts of WS showed LD_{50} of 52 mg/kg in male albino rats for 60 days.

Currently, concern about the potential toxicity of WS has received great attention, thus, drawing the attention of clinicians and nutraceutical industry. Recent studies have reported liver toxicity in some of the marketed formulations of WS (leaf/root/mix of root and leaf extracts) [6, 22–25]. LiverTox database has recently incorporated Ashwagandha with the score 'C' (probable cause of clinically apparent liver injury), based on the clinical reports of liver toxicity caused by some of the marketed products [26]. The Ministry of Ayush, Government of India has published an Advisory refraining from the use of WS leaves. However, a sub-chronic toxicity study has not been performed for the USP-NF standardized root extract of WS as per Organization for Economic Cooperation and Development (OECD) guidelines in Good Laboratory Practice (GLP) conditions. Hence, the current work has been designed to evaluate the oral acute and sub-chronic toxicities of standardized WS root extract (containing not less than 2.5 % w/w withanolides quantified by HPLC-PDA as per USP-NF Ashwagandha root extract monograph) in SD rats in accordance with OECD standards with GLP compliance. Further, acute toxicity was investigated *via* single dose oral administration of 2,000 mg/kg followed by 14 days observation time; whereas sub-chronic toxicity was assessed by oral intake of the repeated varying doses of WS root extract for 90 days in rats along with 14 days recovery period.

Materials and methods

Test conditions

Oral acute and sub-chronic toxicity studies were performed at the test facility of Preclinical Research and Development Organization (PRADO) Pvt. Ltd., Pune, India. The certification of test facility was by the National GLP Compliance Monitoring Authority (NGCMA), Department of Science & Technology, Government of India (GLP/C-127/2018; GLP/C-168/2021). Further, certification was by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA; Registration number: 1723/PO/RcBiBt/S/13/CPCSEA), Ministry of Fisheries, Animal Husbandry and Dairy, Government of India. WS standardized hydro-alcoholic root extract containing 2.69 % total withanolides (aglycone and glycosides) was used for the toxicity studies.

Experimental animals

Rats (Sprague Dawley), male and nulliparous/non-pregnant female, 6–8 weeks old with body weight 150–220 gm were selected for the study. Rats were maintained during acclimatization and throughout the study period at 22 ± 3 °C, and relative humidity (30–70 %), with controlled photoperiod as 12 h light and 12 h dark alternately using an automated system. Filtered drinking water (reverse osmosis water treated with ultraviolet light), and standard rat pellet feed was provided. A maximum of 6 animals (3 M and 3 F) were housed per cage.

Standardization of WS root extract

WS roots were obtained from Madhya Pradesh, India, and authenticated by a taxonomist at Botanical Survey of India, Jodhpur, India and a voucher specimen was deposited (BSI/AZRC/I.12012/Tech/19-20/PI.Id/671). The material was washed, pulverized, and a rigorous examination was performed to determine the content of total withanolides, contaminants, and heavy metals. The powder was extracted using ethanol: water (8:2 v/v) at 60 °C and the extract was further processed to obtain a powder. The extract was analyzed for withanosides and withanolides content using HPLC-PDA and tested for impurities, heavy metals and microbial load [27]. The standardized root extract of WS containing not less than 2.5 % w/w total withanolides is globally marketed by Phytoveda Pvt. Ltd., India and Phytoveda, USA under the brand name LongeFera™.

Preparation of the dosing formulation and administration

The required quantity of WS root extract powder was triturated with a small volume of vehicle (0.1 % CMC) using a mortar and pestle. The stock suspension was obtained *via* the addition of the remaining volume of the vehicle along with continuous stirring. The stock solution was further diluted to get the desired dose of WS root extract. The volume of administration was determined based on the weight of each animal and was administered by an oral gavage needle

Table 1: Animal groups for 90-day repeated oral toxicity study and dose formulation.

Group no.	No. of animals	Group	Treatment	Dose, mg/kg
G1	10 M + 10 F	Control	0.1 % w/v CMC	0
G2	10 M + 10 F	Low	*Withania Somnifera*	250
G3	10 M + 10 F	Mid	(Ashwagandha) root	500
G4	10 M + 10 F	High	extract	1,000
G4-1000-R[a]	5 M + 5 F	High – Recovery		1,000
G1-0-R[a]	5 M + 5 F	Control–Recovery	0.1 % w/v CMC	0

[a]Recovery groups: animals were maintained for observation of reversibility, persistence, or delayed occurrence of adverse effects, for a minimum period of two weeks post-treatment along with the concurrent control.

(16–18 gauge) fitted with a graduated syringe. All animals were fed for approximately 2 h post-administration. The details of the dose for acute and sub-chronic study are given in Table 1.

Toxicity study

Acute toxicity

The study was executed to estimate the acute toxicity dose concentration of WS root extract in SD rats after a single oral administration followed by a 14-day observation period to determine Maximum Tolerated Dose (MTD) and LD_{50} cut-off dose as per the Global Harmonization System (GHS). The limit test was conducted at the oral dose level *viz.* 2,000 mg/kg. As no mortality was observed, further testing of lower dose groups was not performed. The study was executed according to New Drugs and Clinical Trials Rules, Schedule Y March 2019, OECD guideline for testing of chemicals, No. 423, December 2001 and following OECD principles of GLP (as revised in 1997, issued January 1998) ENV/MC/CHEM (98) 17.

A total of 22 rats (11 male and 11 female) were procured from Global Bioresearch Solutions Pvt. Ltd. (CPCSEA Reg. No.-1899/PO/Bt/S/16/CPCSEA) for the study and were acclimatized for 6 days. During this period, observation of animals was performed daily for clinical symptoms (once), morbidity and mortality (twice). At the end of the acclimatization period, random allocation of animals was done to control and treatment groups, and animals were fasted overnight before the test. Animals from group G2 (limit dose) received a dose concentration of 2,000 mg/kg body weight. WS root extract (2 mL) 200 mg/mL stock suspension was administered orally to the animals for body weight 200 g. Control group animals were administered with vehicle alone, i.e., 2 mL of 0.1 % carboxymethylcellulose (CMC). After the dose administration, individual observation of all the animals was done for clinical signs, if any, at the first 30 min, and 1, 2, 4, and 6 h after the dose administration, and once daily thereafter for 14 days. Morbidity and mortality observations were performed for all the animals twice daily on the dosing day and for the remaining observational 14-day period. Body weights were documented before dose administration (not to exceed ±20 % of mean body weight for either sex), and weekly thereafter. Further, an evaluation of body weight gain was performed for individual animals. On the 15th day, rats were humanely euthanized by CO_2 asphyxiation and subjected to detailed gross pathological examination *viz*. external body surface, orifices, abdominal, cranial, and thoracic cavities. None of the animals from any group showed any gross pathological lesions, hence, histopathological evaluation was not performed.

Sub-chronic toxicity

The repeated dose toxicity trial (90 days) was carried out in SD rats to uncover the toxicological effect of WS root extract at the oral dose concentrations i.e., 0, 250, 500, and 1,000 mg/kg. The study was implemented in accordance with Schedule Y and OECD guideline for testing of chemicals, No. 408 entitled 'Repeated Dose 90-Day Oral Toxicity Study in Rodents', adopted on 27th June 2018 [28, 29].

A total of 104 rats (52 male and 52 female) were allotted and acclimatized for 6 days. During this period, observation of animals was performed daily for clinical signs as well as morbidity and mortality. At the end of the acclimatization period, allocation of animals was done randomly into 6 groups for different doses (Table 1). Each group, G1 to G4, contained 10 rats each male and female, except G1-R and G4-R groups which contained 5 rats. At the onset of the study, it was recommended that the weight deviation should not surpass ±20 % of the average body weight for each gender.

The formulation was prepared by taking 1,000 mg extract powder and suspended in a 10 mL vehicle i.e., 0.1 % w/v CMC to get 100 mg/mL stock solution. This stock formulation was diluted further with the vehicle to achieve concentrations for mid and low-dose groups i.e., 50 and 25 mg/mL, respectively. The exact volume of the stock solution to be administered was calculated based on the weight of an individual rat. The doses were administered by oral gavage using a 16–18-gauge needle fitted with a graduated syringe.

All the animals were examined for clinical signs along with morbidity and mortality till 91 days. Animals from the recovery group were monitored for an additional 14 days. The detailed functional observational battery comprising

clinical and neurobehavioral assessments was done weekly till terminal sacrifice that included signs like fur, skin, eye changes, mucous membranes, secretions, excretion, and autonomic activity observations (lacrimation, pupil size, unfamiliar respiratory pattern, gait, posture, locomotion, grooming). Feed consumption and body weights per cage were recorded weekly and gain was estimated for individual animals. The clinical pathology examination was done on days 45 ± 2 and 91. The samples of blood were withdrawn from retro-orbital sinuses under isoflurane anesthesia, for complete hematology, coagulation markers, and clinical chemistry analyses. Animals were sacrificed at the end of the study and various organs were observed for gross pathology examination. After recording organ weights, all the organs were subjected to histopathology study.

Data analysis

All the individual data of groups and sex was examined as mean ± standard deviation and further analyzed employing Students' t-test, and one-way ANOVA followed by Dunnett's test using Graph Pad Prism. All analyses were valued at 5 % ($p \leq 0.05$) level in contrast with control.

Results

Phytochemical analysis of WS root extract

WS root extract was analyzed for the content of total as well as individual withanosides and withanolides using HPLC-PDA. The total withanolide content in the extract was found to be 2.69 % containing the highest content of withaferin A (0.77 %) and withanolide B being the lowest (0.07 %).

Acute toxicity study

In acute toxicity, no mortality was revealed at limit tests executed at 2,000 mg/kg. Mortality and morbidity were observed in all rats twice daily on the day of dosing and 14 days thereafter. No treatment-related effects were observed clinically or pathologically throughout the observational period. Gross pathological examination of the treatment groups did not show any lesion of pathological significance compared to control groups. Hence, histopathology of organs was not conducted, and the study did not continue for lower dose levels. The body weight of male and female rats was similar to those of the control groups throughout the trial period (Table 2).

Table 2: Effect of WS root extract at 2,000 mg/kg on body weight during acute toxicity study.

Days	Body weight male, g		Body weight female, g	
	G1 0 mg/kg	G2 2,000 mg/kg	G1 0 mg/kg	G2 2,000 mg/kg
1	159.7 ± 8.87[a]	158.1 ± 11.49	150.9 ± 0.89	150.7 ± 0.84
7	186.6 ± 8.05	186.8 ± 13.04	166.1 ± 4.53	166 ± 1.41
14	203 ± 14.28	203.8 ± 10.33	181.3 ± 4.76	179.4 ± 7.37

[a]Data is shown as mean + SD, statistically no significant compared to Control (G1 Groups).

Sub-chronic toxicity

Mortality and clinical signs

Oral administration of WS root extract did not show any adverse consequences in both male and female SD rats. All animals survived throughout the trial and no morbidity was noticed. No abnormal clinical signs were perceived in comparison with control groups. Detailed weekly examinations showed no clinical abnormalities in any animal throughout the experimental period.

Effect of WS root extract on body weight and feed consumption

In male rats, a dose-dependent non-significant decrease in parameters such as body weight and body weight gain was noticed on 35th, 42nd, and 56th day, respectively (Figure 1A). In females, non-significant decline in body weight and gain was noticed on 42nd, 56th, and 90th day, respectively (Figure 1B).

The body weights through the treatment-free recovery period were comparable with those of control in both sexes; however, a significant enhancement in body weights on day 7 and 14 and a significant decrease on day 21 and 42 in male rats only was noticed.

Ophthalmic changes due to WS root extract

Ophthalmological examination was performed in the last treatment week in control (G1), high dose (G4) and in the last week of recovery period for (G1-R and G4-R) group animals. The oral administration of highest concentration of WS root extract revealed no abnormal or ophthalmic signs in both sexes during 90 days of experimental period and thereafter in the recovery duration. Hence, the animals given low and mid doses of WS root extract were not investigated.

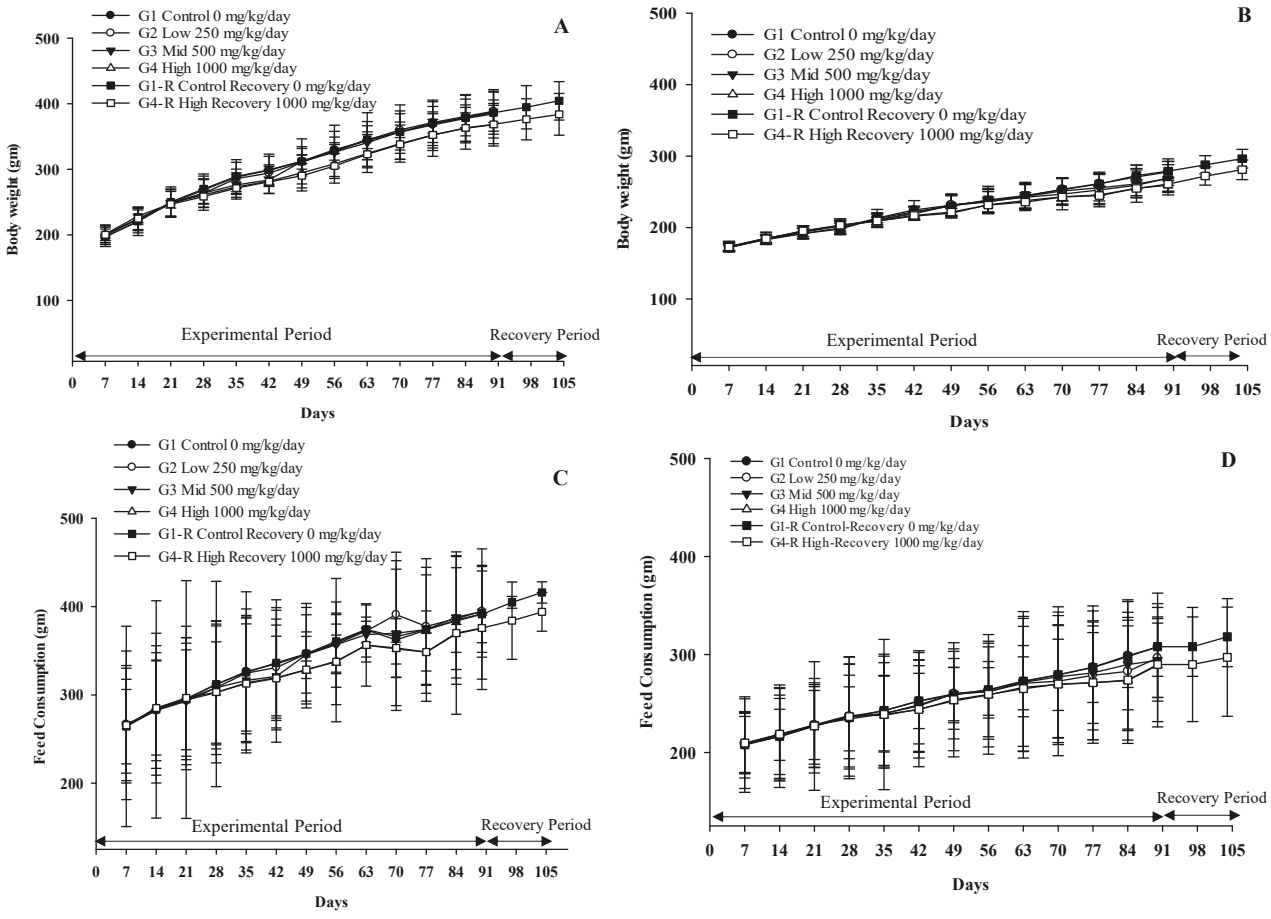

Figure 1: Variation in body weight and food intake of male (A, C) and female (B, D) SD rats administered orally with *WS* root extract at varying concentrations viz. 0, 250, 500 and 1,000 mg/kg/day for 90 days succeeded by a 14-day treatment free recovery period.

Functional observational battery

Functional observational battery examination was performed during the last week in G1, G4, and recovery G1-R, and G4-R groups. No changes were observed, hence, a further trial was not extended to lower-dose groups.

Effect of WS root extract on haematological and coagulation markers

Haematology parameters both in male and female rats were monitored on the 45th and 91st day in all groups and on 104th day in recovery groups. All hematology parameters including differential leukocyte count and reticulocytes were comparable with those of respective controls on day 91, and the treatment-free recovery period (Table 3). In the case of female SD rats, a noteworthy decline ($p<0.05$) in platelets was noticed at high dose group (1,000 mg/kg) of WS extract on day 46 compared to control group. Furthermore, in comparison to control, female rats administered with WS root extract at high dose (1,000 mg/kg) demonstrated a significant increase ($p<0.05$) in mean corpuscular hemoglobin on day 91 (Table 3). However, these changes were within the historical reference range, hence considered as clinically non-significant for control and studied animals.

In case of male rats, a decrease in platelet count has been observed in G2 low dose (250 mg/kg) groups on day 44 as compared to vehicle groups when administered the WS root extract. The differential leucocyte count and reticulocyte count were monitored only in the vehicle and highest concentration groups on 46th and 91st day and were similar with those of control groups. The coagulation markers were monitored, and the values observed were similar to the control groups.

Effect of WS root extract on clinical biochemistry markers

Clinical chemistry analysis on day 44 in male rats revealed a significant increase ($p<0.05$) in thiobarbituric acid (TBA), glucose (GLU), and sodium level in G3 mid dose (500 mg/kg)

Table 3: Haematology and coagulation analysis of SD rats during sub-chronic toxicity study.

Parameters	Sex	Dose mg/kg/day, day 91 (n=10/group)[b]				Day 105 (n=5/group)[c]	
		G1-0	G2-250	G3-500	G4-1,000	G1-0-R	G4-1000-R
WBC, 10^3/µL	Male	11.95 ± 4.29	11.07 ± 3.73	11.84 ± 3.92	12.09 ± 2.06	12.98 ± 2.28	11.72 ± 1.11
	Female	7.85 ± 2.28	7.85 ± 2.00	8.63 ± 2.18	8.35 ± 2.08	8.28 ± 1.84	7.78 ± 1.36
RBC, 10^6/µL	Male	8.20 ± 0.57	7.99 ± 0.52	8.31 ± 0.34	8.23 ± 0.33	8.40 ± 0.7	8.35 ± 0.68
	Female	7.51 ± 1.00	7.19 ± 0.89	7.62 ± 0.41	7.44 ± 0.33	7.31 ± 0.57	7.99 ± 0.60
HGB, g/dL	Male	13.23 ± 1.04	13.50 ± 1.47	13.89 ± 0.58	13.93 ± 0.24	13.84 ± 0.74	14.18 ± 0.67
	Female	13.06 ± 1.66	12.85 ± 1.61	13.50 ± 0.75	13.58 ± 0.64	12.46 ± 2.32	12.46 ± 0.27
HCT, %	Male	42.55 ± 3.05	42.61 ± 2.51	43.43 ± 1.84	42.98 ± 1.13	43.36 ± 2.55	44.82 ± 2.43
	Female	39.78 ± 5.61	38.76 ± 5.09	40.99 ± 2.29	40.18 ± 1.76	39.62 ± 3.04	43.02 ± 1.33
MCV, fL	Male	51.90 ± 1.00	53.39 ± 1.56	52.29 ± 0.96	52.26 ± 1.38	51.72 ± 1.73	53.80 ± 2.35
	Female	52.91 ± 1.25	53.84 ± 1.29	53.79 ± 1.38	54.00 ± 1.01	54.18 ± 1.44	54.18 ± 2.47
MCH, pg	Male	16.14 ± 0.55	16.87 ± 1.22	16.72 ± 0.47	16.97 ± 0.71	16.50 ± 0.98	17.04 ± 1.06
	Female	17.34 ± 0.74	17.87 ± 0.62	17.71 ± 0.63	18.25 ± 0.53[a]	16.92 ± 2.30	17.54 ± 1.21
MCHC, g/dL	Male	31.10 ± 0.86	31.53 ± 2.34	31.93 ± 0.53	32.43 ± 0.69	31.96 ± 0.83	31.66 ± 0.68
	Female	32.93 ± 1.2	33.19 ± 0.72	32.95 ± 0.66	33.81 ± 0.72	31.22 ± 3.99	32.48 ± 0.90
PLT, 10^3/µL	Male	923.50 ± 149.8	838.50 ± 155.39	985.80 ± 108.46	835.90 ± 155.46	889.00 ± 204.39	802.80 ± 149.92
	Female	908.40 ± 87.65	884.00 ± 111.84	960.50 ± 130.52	973.10 ± 73.79	964.00 ± 186.93	997.80 ± 122.49
RETICS, %	Male	0.90 ± 0.25	NP	NP	0.92 ± 0.06	NP	NP
	Female	0.96 ± 0.26	NP	NP	0.9 ± 0.25	NP	NP
Neutrophils	Male	27.90 ± 2.33	NP	NP	27.6 ± 1.71	NP	NP
	Female	29.10 ± 1.37	NP	NP	29.00 ± 1.15	NP	NP
Lymphocytes	Male	70.80 ± 2.10	NP	NP	71.0 ± 1.89	NP	NP
	Female	69.90 ± 1.06	NP	NP	69.80 ± 1.14	NP	NP
Monocytes	Male	0.90 ± 0.32	NP	NP	1.0 ± 0.36	NP	NP
	Female	0.60 ± 0.52	NP	NP	0.60 ± 0.52	NP	NP
Eosinophils	Male	0.40 ± 0.52	NP	NP	0.4 ± 0.52	NP	NP
	Female	0.40 ± 0.52	NP	NP	0.60 ± 0.70	NP	NP
Basophils	Male	0.00 ± 0.00	NP	NP	0 ±0.00	NP	NP
	Female	0.00 ± 0.00	NP	NP	0 ± 0.00	NP	NP
PT, sec	Male	30.57 ± 8.76	NP	NP	27.2 ± 0.72	NP	NP
	Female	22.44 ± 4.59	NP	NP	25.13 ± 2.22	NP	NP
APTT, sec	Male	28.00 ± 6.29	NP	NP	26.0 ± 3.56	NP	NP
	Female	23.00 ± 2.59	NP	NP	23.42 ± 2.59	NP	NP
CT, sec	Male	57.00 ± 17.03	NP	NP	60.0 ± 24.49	NP	NP
	Female	42.00 ± 20.98	NP	NP	30.00 ± 0.00	NP	NP

NP, not performed; NS, non-significant; none of the changes were significant compared to the respective control. ([b]One-way ANOVA; [c]Student's t-test for two independent means compared to control). G1-0: Group 1 (test dose: 0 mg/kg); G2-250: Group 2 (test dose: 250 mg/kg); G3-500: Group 3 (test dose: 500 mg/kg); G4-1,000: Group 4 (test dose: 1,000 mg/kg); G1-0-R: control recovery group (test dose: 0 mg/kg); G4-1000-R: high dose recovery group (test dose: 1,000 mg/kg). Values are represented as mean ± standard deviation. RBC, red blood cell count; HGB, Hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet; RETICS, reticulocyte; WBC, white blood cell count; PT, prothrombin time; APTT, activated partial thromboplastin time; CT, clotting time.

groups administered WS root extract; whereas a significant decline in blood urea level (BUL) levels and blood urea nitrogen (CBUN) ($p<0.05$) were noted in G3 mid dose groups (500 mg/kg) compared to control groups (Table 4). Moreover, in high dose G4 groups, a significant decrease ($p<0.05$) was observed in male rats on day 44 compared to control groups. However, in case of females, a considerable increase ($p<0.05$) in alkaline phosphatase (ALP) was noticed in mid dose G3 groups on day 46 comparable to vehicle groups.

Clinical chemistry analysis on day 91 in males revealed a dose-dependent and significant increase in albumin (ALB) in mid dose (G3) and high dose groups (G4), and ALB: GLO in high dose G4 groups in comparison to control groups. In case of female rats, there was a statistically significant decrease ($p<0.05$) in levels of glutamate pyruvate transaminase (GPT), TBA, albumin (ALB), total protein (PRO) in low dose G2 groups along with ALB: GLO in high dose G4 groups compared to control groups (Table 4).

Table 4: Clinical biochemical analysis of 90-day sub-chronic toxicity study on SD rats.

Biochemical markers	Sex	Dose mg/kg/day, day 91 (n=10/group)[b]				Day 105 (n=5/group)[c]	
		G1-0	G2-250	G3-500	G4-1,000	G1-0-R	G4-1000-R
GPT, U/L	Male	64.60 ± 10.2	52.10 ± 15.15	61.07 ± 10.14	63.79 ± 22.43	44.04 ± 6.79	52.96 ± 11.28
	Female	68.57 ± 15.38	49.97[a] ± 10.04	63.50 ± 14.09	57.95 ± 15.45	50.18 ± 7.14	40.84 ± 6.46
GOT, U/L	Male	113.52 ± 24.98	102.19 ± 16.52	97.08 ± 10.9	97.21 ± 12.84	85.06 ± 4.16	105.64[a] ± 18.3
	Female	109.95 ± 27.34	91.26 ± 16.72	98.10 ± 9.59	105.07 ± 16.93	120.54 ± 28.16	95.26 ± 17.61
ALP, U/L	Male	247.30 ± 76.69	183.20 ± 53.73	234.00 ± 69.25	240.80 ± 46.82	126.60 ± 27.22	169.60 ± 93.21
	Female	149.70 ± 60.07	163.00 ± 66.33	197.70 ± 96.94	173.70 ± 66.79	98.00 ± 28.84	128.80 ± 58.73
BUL, mg/dl	Male	30.58 ± 4.88	31.79 ± 2.78	29.29 ± 4.63	32.57 ± 6.09	30.30 ± 3.26	30.42 ± 3.77
	Female	38.37 ± 4.48	37.74 ± 20.05	35.61 ± 6.54	37.43 ± 2.29	32.96 ± 5.73	38.36 ± 5.01
CREAT, mg/dl	Male	0.51 ± 0.04	0.49 ± 0.05	0.53 ± 0.09	0.50 ± 0.05	0.54 ± 0.04	0.63 ± 0.11
	Female	0.64 ± 0.07	0.63 ± 0.08	0.67 ± 0.05	0.68 ± 0.05	0.61 ± 0.06	0.27 ± 0.04
GLU, mg/dl	Male	124.55 ± 7.84	120.28 ± 11.78	110.57 ± 17.16	121.68 ± 22.79	120.30 ± 16.05	116.68 ± 8.54
	Female	96.88 ± 16.41	99.70 ± 23.94	104.60 ± 16.12	114.92 ± 14.4	131.38 ± 46.3	124.10 ± 10.39
CHOLE, mg/dl	Male	53.50 ± 5.58	53.30 ± 5.33	53.20 ± 6.61	54.50 ± 6.75	54.00 ± 4	53.80 ± 6.06
	Female	73.00 ± 10.58	71.60 ± 11.83	72.30 ± 8.67	77.70 ± 9.84	54.40 ± 6.95	60.40 ± 14.77
Ca, mg/dl	Male	8.96 ± 0.47	9.07 ± 0.47	9.35 ± 0.34	9.30 ± 0.52	9.22 ± 0.19	9.32 ± 0.13
	Female	9.57 ± 0.37	9.23 ± 0.63	9.60 ± 0.32	9.59 ± 0.24	9.14 ± 0.29	9.26 ± 0.26
ALB, g/dl	Male	2.17 ± 0.11	2.24 ± 0.07	2.31[a] ± 0.14	2.34[a] ± 0.09	2.12 ± 0.18	2.36 ± 0.10
	Female	2.89 ± 0.20	2.65 ± 0.30[a]	2.86 ± 0.26	2.87 ± 0.20	2.61 ± 0.2	2.68 ± 0.17
PRO, g/dl	Male	6.60 ± 0.48	6.78 ± 0.28	6.84 ± 0.30	6.71 ± 0.26	6.55 ± 0.29	6.96 ± 0.26
	Female	7.29 ± 0.31	6.83[a] ± 0.52	7.35 ± 0.25	7.55 ± 0.27	6.83 ± 0.49	6.89 ± 0.08
TRIG, mg/dl	Male	46.39 ± 20.47	51.82 ± 37.56	41.98 ± 17.16	34.94 ± 10.3	80.04 ± 22.92	80.50 ± 19.71
	Female	48.46 ± 16.50	36.05 ± 3.95	48.47 ± 16.5	50.53 ± 23.2	49.44 ± 16.55	55.70 ± 24.28
BIT, mg/dl	Male	0.12 ± 0.05	0.12 ± 0.06	0.10 ± 0.02	0.09 ± 0.01	0.16 ± 0.09	0.16 ± 0.02
	Female	0.14 ± 0.05	0.14 ± 0.07	0.10 ± 0.02	0.12 ± 0.03	0.19 ± 0.07	0.17 ± 0.05
ALB: GLO	Male	0.49 ± 0.06	0.49 ± 0.04	0.51 ± 0.03	0.54[a] ± 0.03	0.48 ± 0.08	0.51 ± 0.04
	Female	0.66 ± 0.05	0.64 ± 0.08	0.64 ± 0.08	0.62[a] ± 0.07	0.62 ± 0.04	0.64 ± 0.07
CBUN, mg/dl	Male	14.27 ± 2.28	14.84 ± 1.3	13.67 ± 2.16	15.20 ± 2.84	14.14 ± 1.52	14.20 ± 1.76
	Female	17.91 ± 2.09	17.61 ± 9.35	16.62 ± 3.05	17.47 ± 1.07	15.38 ± 2.68	17.90 ± 2.34
GLO, g/dl	Male	4.43 ± 0.48	4.55 ± 0.31	4.53 ± 0.21	4.36 ± 0.23	4.44 ± 0.37	4.60 ± 0.26
	Female	4.41 ± 0.20	4.18 ± 0.38	4.49 ± 0.22	4.68 ± 0.28	4.22 ± 0.34	4.21 ± 0.19
Na, mmol/l	Male	143.13 ± 1.77	146.16 ± 1.07	146.77 ± 2.09	147.82 ± 2.31	141.30 ± 1.05	144.24 ± 1.08
	Female	147.54 ± 2.80	147.03 ± 2.08	149.95 ± 1.97	150.64 ± 4.08	139.62 ± 1.69	141.10 ± 2.08
K, mmol/l	Male	4.35 ± 0.56	4.41 ± 0.55	4.77 ± 0.47	4.91 ± 0.81	4.53 ± 0.42	4.50 ± 0.39
	Female	4.55 ± 0.51	4.50 ± 0. 37	4.45 ± 0.34	4.63 ± 0.72	3.87 ± 0.29	4.46 ± 0.89
Cl, mmol/l	Male	103.94 ± 1.22	104.66 ± 1.41	103.16 ± 1.00	105.16 ± 3.13	101.66 ± 2.29	102.96 ± 0.64
	Female	107.60 ± 1.96	107.37 ± 2.59	108.23 ± 1.11	107.92 ± 3.12	102.82 ± 2.15	103.68 ± 2.30
Total Bile acids, U/L	Male	72.8 ± 12.11	74.4 ± 5.36	71.6 ± 7.99	72.1 ± 11.47	NP	NP
	Female	72.9 ± 7.91	58.8 ± 21.31	68.8 ± 5.64	73.4 ± 7.39	NP	NP

NP: Not performed, [a]P<0.05 ([b]One-way ANOVA, and [c]Student's t-test for two independent means compared to control). G1-0: Group 1 (test dose: 0 mg/kg); G2-250: Group 2 (test dose: 250 mg/kg); G3-500: Group 3 (test dose: 500 mg/kg); G4-1,000: Group 4 (test dose: 1,000 mg/kg); G1-0-R: control recovery group (test dose: 0 mg/kg); G4-1000-R: high dose recovery group (test dose: 1,000 mg/kg). Values are represented as mean ± standard deviation. GPT, glutamate pyruvate transaminase; GOT, glutamate oxaloacetate transaminase; BUL, blood urea level; BUN, blood urea nitrogen; CREAT, creatinine; GLU, glucose; CHOLE, total cholesterol; PRO, total protein; ALB, albumin; BIT, total bilirubin.

The clinical chemistry observations after the treatment-free recovery period both in males and females were comparable with those of control except significant increase ($p<0.05$) in values of glutamate oxaloacetate transaminase (GOT) levels in males. The changes detected in clinical chemistry were toxicologically insignificant as no histopathological correlation was observed in any organ.

Effect of WS root extract on hormonal markers

The values of thyroid stimulating hormone, triiodothyronine, and thyroxine were measured on day 91 in low G1 and high concentration G4 groups (Supplementary Table S1). The level of thyroid markers in both sexes after treatment with the highest dose were comparable with vehicle groups and

were statistically indifferent in the animals treated with the highest dose.

Effect of WS root extract on urine markers

Qualitative urinalysis parameters of treatment groups were similar with control groups for both sexes. However, a statistically substantial rise ($p<0.05$) in the specific gravity of high dose G4 male group was noticed when compared with control group (Supplementary Table S2).

Necropsy and gross pathology

All the animals were humanely euthanized by using CO_2 asphyxiation including recovery groups G1-G4 on the 91st day and G1-R, G4-R recovery groups on the 105th day. Each animal was subjected to a detailed gross pathological examination (Figures 2 and 3). Additionally, no changes were seen in female SD rats with respect to the estrus cycle on day 91 and 104 in recovery groups. Gross pathological examination of the treatment and reference control groups showed no lesion of pathological significance in comparison to control groups (Figures 2 and 3).

Organ weight and histopathology

In comparison to controls in both sexes, weights of all the organs in relation to their terminal body weights were similar. Further, in male SD rats, a significant ($p<0.05$) enhancement in kidney weights in mid dose G3 group, a decrease ($p<0.05$) in pituitary weight in high dose G4 group as well as in relative pituitary weights in low dose G2 and high dose G4 groups was observed (Table 5).

Histopathology observations of the high-dose groups were similar to control groups and no abnormal changes were detected that could be correlated to the test item treatment. However, spontaneous and/or incidental histopathology observations of minimal severity were observed in both control and high dose groups (Supplementary Table S3).

Discussion

The use of herbal nutraceuticals has been increasing globally for their dietary and pharmaceutical purposes. The appropriate doses and length of the treatment are important to demonstrate the overall beneficial effect of the nutraceuticals. Additionally, serious adverse effects have been reported at higher doses for some of the nutraceutical products when tested for longer duration. Therefore, it is necessary to assess the long-term toxicity of any nutraceutical product prior to being utilized in the market. Thus, the current work represents the acute and sub-chronic oral toxicities of WS root extract capsules on SD rats.

Animal toxicity is crucial which provides us the information on toxic effects of specific dose of any test drug/nutraceutical. Toxicity studies performed on animals generally follow a similar pattern in humans with the use of allometric scaling and pharmacometric approaches. The acute and sub-chronic toxicity trials on SD rats were executed corresponding to standard OECD guidelines –423 and 408, respectively which include observational effects related to the endocrine system. The acute toxicity was assessed for 14 days, however sub-chronic study was performed for 90 days with repeated doses of varying concentrations of WS root extract in SD rats. In the present study, all the animals survived till the completion of the trial, and all were in active condition and looked fresh with white fur during daily observation. Moreover, weekly comprehensive clinical evaluations did not identify any clinical abnormalities in any of the animals throughout the trial period.

Body weight and general behavior are crucial parameters to determine the health status of animals and were assessed to evaluate the occurrence of toxic or non-toxic effect of the test product. The consumption of WS root extract orally at all doses did not produce any behavior changes in animals in both acute and sub-chronic toxicities. Body weight along with food intake were increased progressively in all the animals depicting insignificant effect of WS bioactives on general health throughout the toxicity studies (Figure 1). Langade et al. [19] reported an increase in body weight and food intake in Wistar rats during sub-acute toxicity of WS root extract when given a dose level of 2,000 mg/kg for 28 days. Similarly, gain in body weight and substantial increase in food intake in both sexes were observed by Balkrishna et al. [13] in sub-acute toxicity evaluation of WS whole plant extract at varying doses for 28 days. Antony et al. [11] reported enhanced weight as well as food consumption on administration of 2,000 mg/kg purified WS in female rats during acute as well as sub-chronic toxicity studies.

In the current study, mortality, abnormal clinical signs and ophthalmic aberrations were not noticed compared to respective control groups in both main and recovery rats. Further, hormonal markers showed no significant alterations comparable to control groups. At the termination of the study, there were no gross pathological alterations in any organ and organ weight in treatment groups with respect to control groups. Microscopic observation of organs at the highest dose of WS root extract revealed no alterations, although we observed statistically substantial alterations in

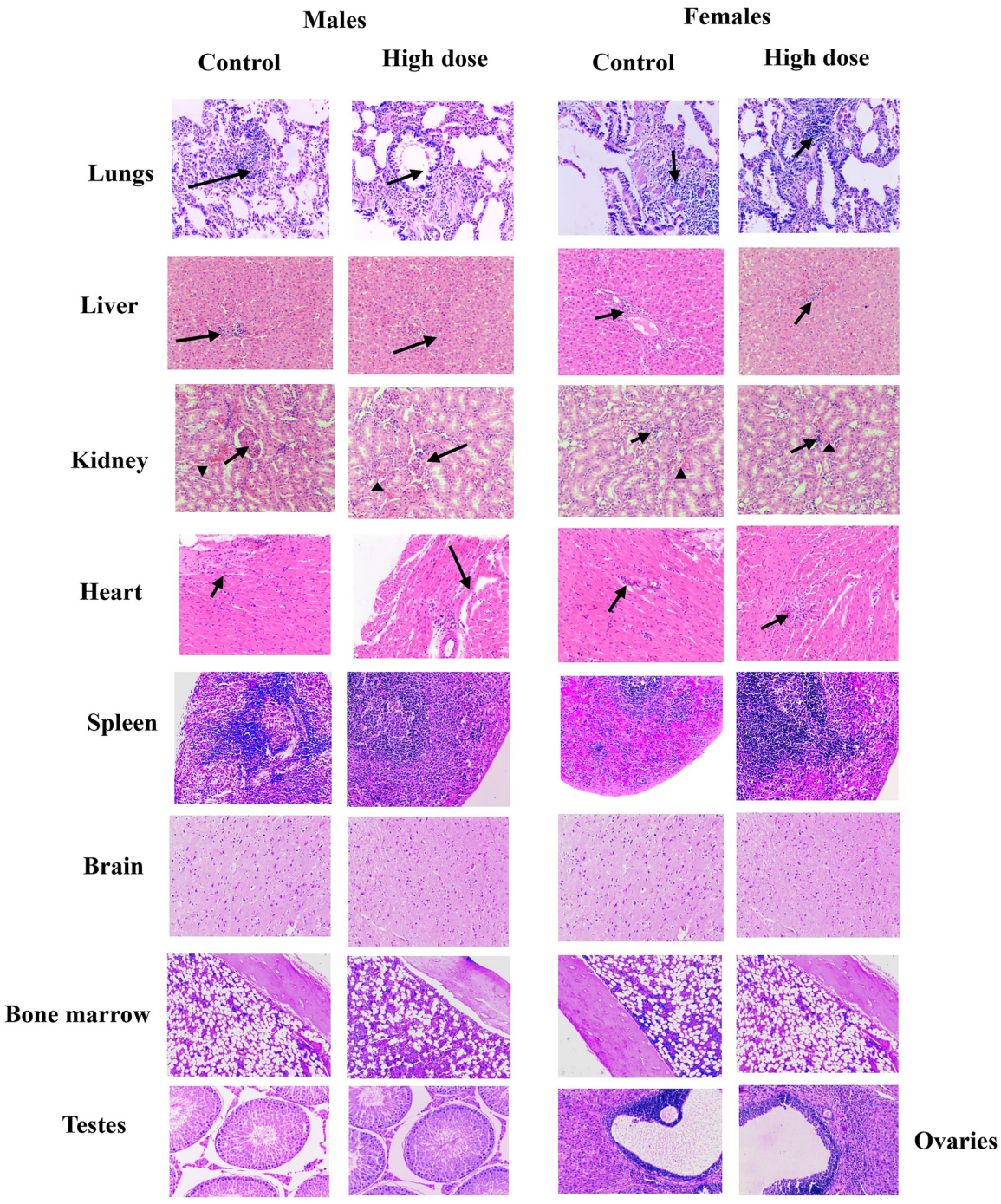

Figure 2: Histological photomicrographs of selected organs of SD rats (males and females) upon oral administration of either the control (0.1 % methylcellulose) or high dose (1,000 mg/kg/day) of *WS* root extract for a duration of 90 consecutive days. Tissue sections were stained with Haematoxylin and Eosin. Organs processed for imaging: lungs, liver, kidney, heart, brain, ovaries, testes (× 100); spleen, bone marrow (× 400).

hematology, clinical biochemistry as well as urinalysis parameters. These findings, however, were not considered to be adverse because no microscopic or macroscopic interventions were involved in the organs taken during necropsy. Moreover, these results are not likely to have clinical importance because the statistical significance found for assessed parameters falls within the reference limits that have been previously documented for SD rats. Thus, it was concluded that the NOAEL of WS root extract in SD rats could be established as 1,000 mg/kg/day.

Several *in vivo* acute toxicity studies on WS extracts depict the safer use of WS roots and plant parts at varying

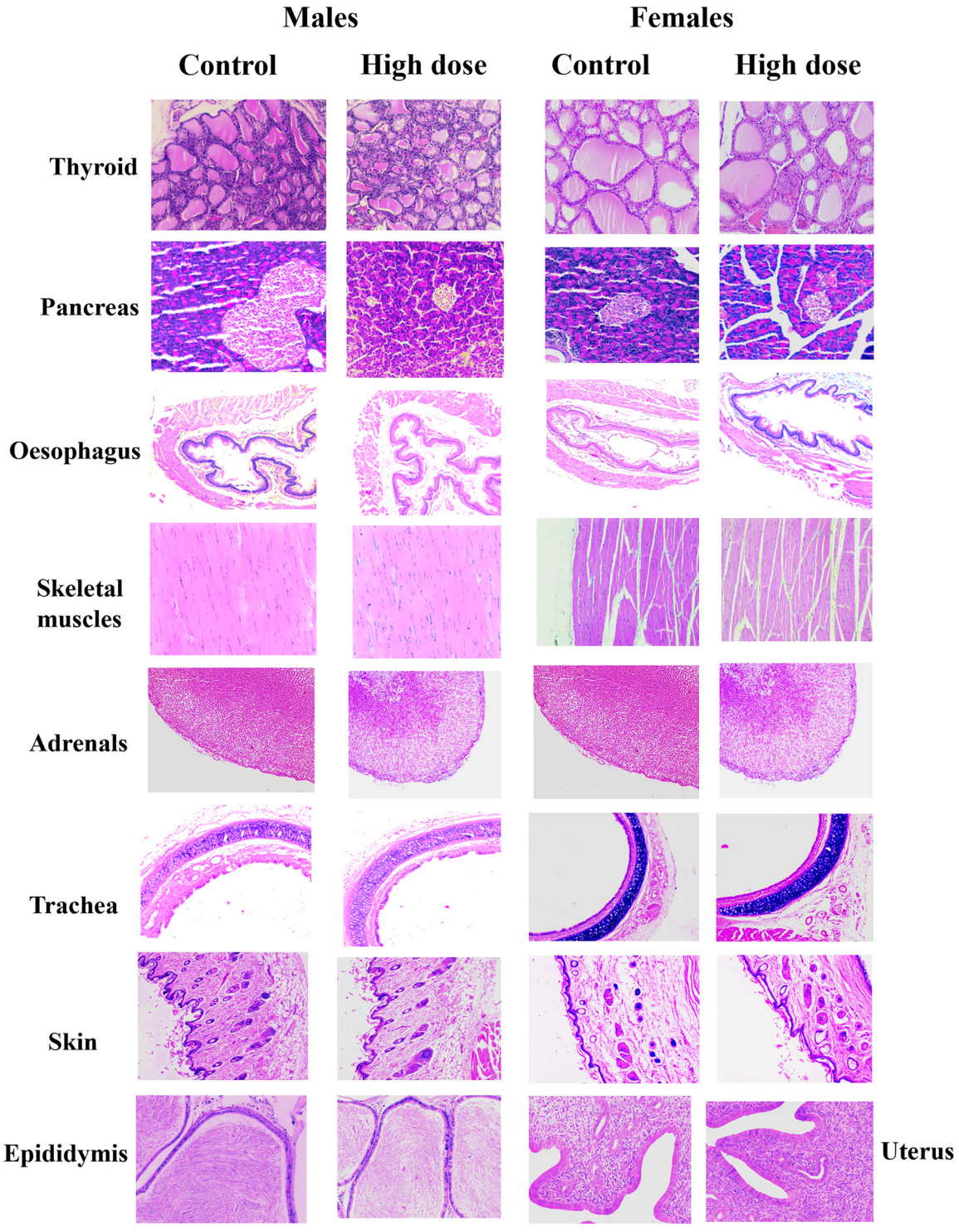

Figure 3: Histological photomicrographs of selected organs of SD rats (males and females) upon oral administration of either the control (0.1 % methylcellulose) or high dose (1,000 mg/kg/day) of WS root extract for a duration of 90 consecutive days. Tissue sections were stained with Haematoxylin and Eosin. Organs processed for imaging: thyroid, pancreas, skeletal muscle, skin, epididymis (× 100); oesophagus, adrenals, trachea (× 400).

Table 5: Effect of WS root extract on relative organ weights of male and female SD rats.

Organs	Sex	Relative organ weight, %					
		G1-0	G2-250	G3-500	G4-1,000	G1-0-R	G4-1000-R
Liver	Male	3.13 ± 0.46	3.07 ± 0.41	3.41 ± 0.48	3.00 ± 0.51	2.52 ± 0.13	3.07 ± 0.48
	Female	2.73 ± 0.38	3.06 ± 0.49	2.76 ± 0.33	2.81 ± 0.29	2.53 ± 0.29	2.39 ± 0.2
Spleen	Male	0.23 ± 0.06	0.22 ± 0.07	0.25 ± 0.08	0.21 ± 0.06	0.18 ± 0.05	0.19 ± 0.03
	Female	0.2 ± 0.04	0.24 ± 0.05	0.22 ± 0.05	0.21 ± 0.05	0.19 ± 0.01	0.18 ± 0.02
Heart	Male	0.29 ± 0.04	0.28 ± 0.02	0.31 ± 0.06	0.28 ± 0.05	0.25 ± 0.01	0.28 ± 0.02
	Female	0.28 ± 0.03	0.3 ± 0.04	0.28 ± 0.03	0.3 ± 0.03	0.26 ± 0.03	0.26 ± 0.01
Thymus	Male	0.07 ± 0.02	0.06 ± 0.02	0.08 ± 0.04	0.08 ± 0.03	0.04 ± 0.01	0.05 ± 0.01
	Female	0.07 ± 0.04	0.08 ± 0.03	0.08 ± 0.02	0.08 ± 0.02	0.04 ± 0.01	0.05 ± 0.00
Kidneys	Male	0.66 ± 0.09	0.66 ± 0.05	0.79[a] ± 0.18	0.64 ± 0.07	0.62 ± 0.03	0.72 ± 0.13
	Female	0.63 ± 0.07	0.65 ± 0.13	0.63 ± 0.07	0.62 ± 0.1	0.55 ± 0.05	0.57 ± 0.02
Adrenals	Male	0.016 ± 0.016	0.01 ± 0.004	0.011 ± 0.002	0.011 ± 0.004	0.011 ± 0.002	0.012 ± 0.003
	Female	0.021 ± 0.008	0.022 ± 0.008	0.022 ± 0.006	0.021 ± 0.007	0.018 ± 0.004	0.015 ± 0.004
Testes	Male	0.8 ± 0.09	0.82 ± 0.11	0.77 ± 0.13	0.79 ± 0.06	0.79 ± 0.03	0.75 ± 0.06
Ovaries	Female	0.023 ± 0.005	0.025 ± 0.007	0.022 ± 0.006	0.025 ± 0.01	0.017 ± 0.003	0.021 ± 0.004
Brain	Male	0.55 ± 0.09	0.51 ± 0.04	0.56 ± 0.09	0.53 ± 0.06	0.51 ± 0.05	0.55 ± 0.02
	Female	0.69 ± 0.06	0.72 ± 0.07	0.67 ± 0.08	0.7 ± 0.09	0.63 ± 0.04	0.66 ± 0.09
Pituitary	Male	0.005 ± 0.001	0.004 ± 0.00[a]	0.005 ± 0.001	0.004 ± 0.00[a]	0.005 ± 0.00	0.005 ± 0.001
	Female	0.006 ± 0.001	0.007 ± 0.001	0.005 ± 0.002	0.007 ± 0.002	0.008 ± 0.003	0.007 ± 0.001
Prostate	Male	0.1 ± 0.02	0.1 ± 0.03	0.11 ± 0.06	0.11 ± 0.05	0.06 ± 0.01	0.08 ± 0.02
Uterus	Female	0.24 ± 0.05	0.23 ± 0.04	0.25 ± 0.05	0.22 ± 0.07	0.24 ± 0.11	0.17 ± 0.03
Epididymis	Male	0.33 ± 0.06	0.31 ± 0.02	0.35 ± 0.05	0.34 ± 0.05	0.3 ± 0.04	0.33 ± 0.03
SVCG	Male	0.24 ± 0.11	0.25 ± 0.05	0.26 ± 0.07	0.27 ± 0.06	0.17 ± 0.04	0.17 ± 0.03
Thyroid/Parathyroid	Male	0.008 ± 0.001	0.008 ± 0.001	0.008 ± 0.001	0.008 ± 0.001	0.007 ± 0.001	0.046 ± 0.085
	Female	0.011 ± 0.001	0.013 ± 0.002	0.013 ± 0.003	0.013 ± 0.002	0.01 ± 0.001	0.01 ± 0.002

[a]$p<0.05$ ([b]One-Way ANOVA, and Student's *t*-test for two independent means compared to control). G1-0: Group 1 (test dose: 0 mg/kg), G2-250: Group 2 (Test Dose: 250 mg/kg), G3-500: Group 3 (test dose: 500 mg/kg), G4-1,000: Group 4 (test dose: 1,000 mg/kg), G1-0-R: control recovery froup (test dose: 0 mg/kg), G4-1000-R: high dose recovery group (test dose: 1,000 mg/kg). Values are represented as mean + standard deviation. Data is presented as mean + standard deviation.

concentrations [11, 13, 19, 21]. In acute toxicity studies of ethanolic root extract of WS when administered intraperitoneally in rats of either sex, the reported LD_{50} was 1,260 mg/kg. The repeated injections of WS root extract for 30 days at a concentration of 100 mg/kg did not result in any mortality in Wistar rats, although at the end of the study, a significant decline in spleen, thymus and adrenals was observed in male rats only and biochemical parameters were within normal range. Hematological parameters revealed an increase in hemoglobin content, but clinical chemistry was unchanged along with no histologic alterations in any organ of all rats [21].

Jain et al. [30] reported that aqueous, ethanolic and hydroalcoholic extracts of roots of WS at 2,000 mg/kg were safer in acute toxicity when administered in female Albino mice for 14 days in accordance with OECD-420. Another study evaluating the acute and sub-acute toxicities of WS hydroalcoholic root extract suggested that NOAEL was 2,000 mg/kg when given orally to Wistar rats. In sub-acute toxicity, WS root extract was administered for 28 days at different dose concentrations i.e., 500, 1,000 and 2,000 mg/kg which resulted in no toxic symptoms or mortality, no significant alterations in body, organs' weight, and haematobiochemical parameters, and no related gross/histopathological lesions at any dose concentrations [31].

The compound in WS roots, i.e., Withaferin A, has been well-recognized for its anti-angiogenic activity *in vivo* at a concentration that was 500-fold lower than that suggested for anti-cancer activity [32]. Taking this into consideration, Patel et al. [20] designed oral acute and sub-acute toxicities using a standardized extract of Withaferin A in Wistar rats. Acute toxicity was performed at 2,000 mg/kg concentration; whereas in sub-acute study conducted for 28 days, rats were fed with varying concentrations from 0–2,000 mg/kg of WS root extract. The results concluded that NOAEL for WS root extract was 2,000 mg/kg in acute toxicity; however, results of sub-acute study revealed no changes in parameters such as clinical, ophthalmic examination, and clinical pathology. Further, hematology, as well as serum chemistry parameters, were in the standard range along with no gross or histopathological findings in terminal necropsy.

Hussein et al. [16] reported an LD_{50} of 522 mg/kg body weight of alcoholic extract of WS aerial parts when injected *via* IP route at varying doses of 0–1,200 mg/kg in male albino rats in acute toxicity study. Moreover, in sub-chronic toxicity study, extracts were given IP at different doses of 10 , 20 and 40 % of the obtained LD_{50} for 60 days. Results demonstrated significant alterations in hematological, biochemical, and histopathological parameters with 15–40 % mortalities in 20 and 40 % groups by the end of the study depicting toxicity at higher doses and less than 10 % dose of WS extract was safe for further treatment studies [16]. On the other hand, oral nonclinical subacute study on hydromethanolic whole plant parts WS extract in SD rats (both sexes) at the concentrations of 100–1,000 mg/kg daily for 28 days along with a recovery period of 14 days as per OECD guideline 407 and GLP compliance concluded that oral dose level of 1,000 mg/kg daily was safer to use without detecting any toxicologically relevant findings. However, a few observations of the study included considerable alterations in hematology, clinical chemistry as well as coagulation parameters [13].

Antony et al. (2018) performed acute and sub chronic toxicities (90 days, repeated dose) of purified WS extract on female rats at a concentration of 2,000 mg/kg body weight and 100, 500, 1,000 mg/kg, respectively. The repeated oral administration of WS purified extract for 90 days did not result in any statistical changes in hematology and biochemistry profiles of treated rats comparable to control. Further, normal histopathology of major organs was observed in treated and controlled animals. The authors concluded that the NOAEL for WS purified extract was 1,000 mg/kg daily in female rats [11].

Langade et al. [19] performed sub-acute toxicity of WS root extract in Wistar rats for 28 days at different concentrations i.e., 200, 400, 800 mg/kg *via* oral administration. No mortalities, and no alteration in blood biochemistry were noticed however, variations in histological parameters were within standard prescribed range. Hence, WS root extract for 28 days did not produce any abnormalities up to 800 mg/kg; however, little increase in duration caused mortalities [19]. Kalaivani et al. [17] reported NOAEL for KSM-66 Ashwagandha root extract at a higher concentration at 2000 mg/kg for 90-day repeated toxicity study in Wistar rats. The authors reported no mortality or morbidity, and no clinical sign of toxicity in rats [17].

Due to the increased popularity of herbal medicines, there is great emphasis on augmenting our understanding of potential interactions between herbs and pharmaceuticals. Interestingly, the presence of myriad pharmacological active constituents in herbal medicines enhances the probability of herbal drug interactions (HDIs) in comparison to drug-drug interactions [33]. The herbal drug interactions could be beneficial, harmful or fatal, hence thorough and mechanistic understanding of consequences related to HDIs is necessary to achieve a successful integration of modern and complementary alternative medicines into mainstream allopathic evidence-based medicine. The prediction of HDIs is an interplay between drug metabolizing enzymes and transporters which alters directly/indirectly pharmacokinetics, pharmacodynamics, and safety profile of the drug/herb. Cytochrome P450 enzymes (Phase 1 Drug Metabolizing Enzymes) are inducible and polymorphic which play crucial roles in drug metabolism pathways and have been explored for clinically significant interactions along with risk assessment including HDIs. Various studies reported the lack of interaction of WS root extract with CYP1A, 3A4 and 2D6 *in vitro* in human and rat liver microsomes as well as CYP1A2 and 2C9 in human liver microsomes [34–36]. The methanolic extract of WS containing compounds *viz*. withaferin A, withanolide A, and withanoside IV showed no considerable effect on CYP3A activity in the liver microsomes [35]; however ethanolic extract of WS inhibited CYP3A4 activity in rat liver microsomes [36]. In another study, WS extracts reported moderate induction activity on CYP3A4 mRNA expression, whereas there was no significant effect on CYP2B6 in HepG2 cells. The methanolic extract of WS *in vivo* showed significant induction of CYP1A enzyme orally in rats, modifying phenacetin pharmacokinetics; on the contrary, pure bioactives i.e., withaferin A & withanolide A showed no interaction with CYP1A *in vitro* in rat and human liver microsomes [34]. Another *in vivo* study reported a decline in plasma concentration of ritonavir on oral administration of ritonavir with WS extract in adult Wistar rats which was attributed to P-glycoprotein (P-gp) as well as CYP3A4 induction [37]. *In vitro* study on liver microsomes revealed that the methanolic and acetate extracts of WS inhibited CYP2B6 due to presence of more withanolides and alkaloids. The moderate induction of CYP3A4 mRNA expression by WS extract in HepG2 cells was due to withaferin A, an inducer of transcription factors and receptors [38]. *In vitro* CYP450 study on human liver microsomes using combination of WS, AYUSH-64, and remdesivir for COVID-19 management suggested WS did not exhibit HDIs with CYP3A4, CYP2C8, and CYP2D6 [39]. The root extract or supplements of WS can result in clinically relevant HDIs with medications metabolized *via* CYP2B6 or 3A4. More studies are needed to assess the effect of WS extract on cytochrome P450 complex- 2A6, 2D6, 2C9, 2C8, 2C19, and 2E1 as per FDA recommendation [40].

Conclusions

The present study assessed the acute and sub-chronic oral toxicities of WS root extract at 2,000 kg/mg and escalating concentrations of 250, 500, and 1,000 mg/kg, respectively in Sprague Dawley rats for 14 and 90 days tailed by 14 days recovery time according to OECD test guidelines 423 and 408, in compliance with GLP OECD Principles. In the light of this study, it could be suggested that WS root extract studied in terms of acute and sub-chronic toxicity up to doses of 2,000 mg/kg produced no serious effects in Sprague Dawley rats. The current sub-chronic toxicity study of WS root extract helped us to determine safe starting doses in rats which can be further extrapolated to humans by allometric scaling and other pharmacometric approaches.

Acknowledgments: The authors thank the CRO (PRADO) for the conduct of the preclinical study.

Research ethics: Oral acute and sub-chronic toxicity studies were performed at the test facility of Preclinical Research and Development Organization (PRADO) Pvt. Ltd., Pune, India. The certification of test facility was by the National GLP Compliance Monitoring Authority (NGCMA), Department of Science & Technology, Government of India (GLP/C-127/2018; GLP/C-168/2021). Further, certification was by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA; Registration number: 1723/PO/RcBiBt/S/13/ CPCSEA), Ministry of Fisheries, Animal Husbandry and Dairy, Government of India.

Informed consent: Not applicable.

Author contributions: PW and PC: conducted the animal study; ES: wrote the manuscript; CG: reviewed the manuscript; AV: reviewed the manuscript; SN: conceptualized, edited, and reviewed the manuscript. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Conflict of interest: ES, CG, AV and SN are employees of Viridis Biopharma Pvt. Ltd., Mumbai, India and Phytoveda Pvt. Ltd., Mumbai, India. Other authors state no conflict of interest.

Research funding: The research is funded by Phytoveda Pvt. Ltd, Mumbai, India.

Data availability: Not applicable.

References

1. Modi SJ, Tiwari A, Ghule C, Pawar S, Saste G, Jagtap S, et al. Pharmacokinetic study of withanosides and withanolides from Withania somnifera using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Molecules 2022;27:1476.
2. Basudkar V, Gujrati G, Ajgaonkar S, Gandhi M, Mehta D, Nair S. Emerging vistas for the nutraceutical Withania somnifera in inflammaging. Pharmaceuticals 2024;17:597.
3. Kashyap VK, Peasah-Darkwah G, Dhasmana A, Jaggi M, Yallapu MM, Chauhan SC. Withania somnifera: progress towards a pharmaceutical agent for immunomodulation and cancer therapeutics. Pharmaceutics 2022;14:611.
4. Mikulska P, Malinowska M, Ignacyk M, Szustowski P, Nowak J, Pesta K, et al. Ashwagandha (Withania somnifera)-current research on the health-promoting activities: a narrative review. Pharmaceutics 2023;15: 1057.
5. Saha P, Ajgaonkar S, Maniar D, Sahare S, Mehta D, Nair S. Current insights into transcriptional role(s) for the nutraceutical Withania somnifera in inflammation and aging. Front Nutr 2024;11. https://doi.org/10.3389/fnut.2024.1370951.
6. Vazirani S, Kothari A, Fujimoto J, Gomez M. Supplements are not a synonym for safe: suspected liver injury from Ashwagandha. Fed Pract 2023;40:315–19.
7. Gómez Afonso A, Fernandez-Lazaro D, Adams DP, Monserdà-Vilaró A, Fernandez-Lazaro CI. Effects of Withania somnifera (Ashwagandha) on hematological and biochemical markers, hormonal behavior, and oxidant response in healthy adults: a systematic review. Curr Nutr Rep 2023;12:465–77.
8. Goyal M. Rasayana in perspective of the present scenario. Ayu 2018;39: 63–4.
9. Vaidya VG, Naik NN, Ganu G, Parmar V, Jagtap S, Saste G, et al. Clinical pharmacokinetic evaluation of Withania somnifera (L.) Dunal root extract in healthy human volunteers: a non-randomized, single dose study utilizing UHPLC-MS/MS analysis. J Ethnopharmacol 2024;322: 117603.
10. Verma N, Gupta SK, Tiwari S, Mishra AK. Safety of Ashwagandha root extract: a randomized, placebo-controlled, study in healthy volunteers. Compl Ther Med 2021;57:102642.
11. Antony B, Benny M, Kuruvilla B, Gupta N, Sebastian A, Jacob S. Acute and sub chronic toxicity studies of purified Withania somnifera extract in rats. Int J Pharm Pharmaceut Sci 2018;10:41.
12. Alam N, Hossain M, Khalil MI, Moniruzzaman M, Sulaiman SA, Gan SH. High catechin concentrations detected in Withania somnifera (Ashwagandha) by high performance liquid chromatography analysis. BMC Compl Alternative Med 2011;11:65.
13. Balkrishna A, Sinha S, Srivastava J, Varshney A. Withania somnifera (L.) Dunal whole-plant extract demonstrates acceptable non-clinical safety in rat 28-day subacute toxicity evaluation under GLP-compliance. Sci Rep 2022;12:11047.
14. Durg S, Bavage S, Shivaram SB. Withania somnifera (Indian ginseng) in Diabetes mellitus: a systematic review and meta-analysis of scientific evidence from experimental research to clinical application. Phytother Res 2020;34:1041–59.
15. Gopukumar K, Thanawala S, Somepalli V, Rao TSS, Thamatam VB, Chauhan S. Efficacy and safety of Ashwagandha root extract on cognitive functions in healthy, stressed adults: a randomized, double-blind, placebo-controlled study. Evid Based Complement Alternat Med 2021;2021:8254344.
16. Hussein YA, Al-Shokair SS, Ashry KM. Acute and sub-chronic toxicological potential of Withania somnifera extract on rats. Alex J Vet Sci 2017;55:10.
17. Kalaivani P, Siva R, Gayathri V, Langade D. Ninety-day repeated dose toxicity of Ashwagandha (Withania somnifera) root extract in Wistar rats. Toxicol Rep 2023;11:189–98.
18. Kelgane SB, Salve J, Sampara P, Debnath K. Efficacy and tolerability of Ashwagandha root extract in the elderly for improvement of general

well-being and sleep: a prospective, randomized, double-blind, placebo-controlled study. Cureus 2020;12:e7083.
19. Langade D, Dawane J, Dhande P. Sub-acute toxicity of Ashwagandha (Withania somnifera) root extract in Wistar rats. Toxicol Rep 2023;11: 389–95.
20. Patel SB, Rao NJ, Hingorani LL. Safety assessment of Withania somnifera extract standardized for Withaferin A: acute and sub-acute toxicity study. J Ayurveda Integr Med 2016;7:30–7.
21. Sharada AC, Solomon FE, Devi PU. Toxicity of Withania somnifera root extract in rats and mice. Int J Pharmacol 1993;31:205–12.
22. Bokan G, Glamočanin T, Mavija Z, Vidović B, Stojanović A, Björnsson ES, et al. Herb-Induced liver injury by ayurvedic ashwagandha as assessed for causality by the updated RUCAM: an emerging cause. Pharmaceuticals 2023;16:1129.
23. Lubarska M, Hałasiński P, Hryhorowicz S, Mahadea DS, Łykowska-Szuber L, Eder P, et al. Liver dangers of herbal products: a case report of Ashwagandha-induced liver injury. Int J Environ Res Publ Health 2023; 20:3921.
24. Philips CA, Valsan A, Theruvath AH, Ravindran R, Oommen TT, Rajesh S, et al. Liver Research Club India, Ashwagandha-induced liver injury-A case series from India and literature review. Hepatol Commun 2023;7: e0270.
25. Tóth M, Benedek AE, Longerich T, Seitz H. Ashwagandha induced acute liver injury: a case report. Clin Case Rep 2023;11:e7078.
26. LiverTox: Clinical and Research Information on Drug-Induced Liver Injury. National institute of diabetes and digestive and kidney diseases. Bethesda (MD). 2012. http://www.ncbi.nlm.nih.gov/books/NBK547852/ [Accessed 26 June 2024].
27. U.S. Pharmacopeia, (n.d.). https://store.usp.org/searchresults?Ntt=1043309&searchType=simple&type=search [Accessed 10 June 2024].
28. E408_1998.pdf, (n.d.). https://www.oecd.org/env/ehs/testing/E408_1998.PDF [Accessed 10 June 2024].
29. NEW DRUGS ANDctrS RULE, 2019.pdf, (n.d.). https://cdsco.gov.in/opencms/resources/UploadCDSCOWeb/2022/new_DC_rules/NEW%20DRUGS%20ANDctrS%20RULE,%202019.pdf [Accessed 10 June 2024].
30. Jain H, Parial SD, Jarald E, Daud AS, Ahmad S. Extraction of Ashwagandha by conventional extraction methods and evaluation of its anti-stress activity. Int J Green Pharm 2010;4. https://doi.org/10.22377/ijgp.v4i3.143.
31. Prabu PC, Panchapakesan S, Raj CD. Acute and sub-acute oral toxicity assessment of the hydroalcoholic extract of Withania somnifera roots in Wistar rats. Phytother Res 2013;27:1169–78.
32. Mohan R, Hammers H, Bargagna-Mohan P, Zhan XH, Herbstritt CJ, Ruiz A, et al. Withaferin A is a potent inhibitor of angiogenesis. Angiogenesis 2004;7:115–22.
33. Borse SP, Singh DP, Nivsarkar M. Understanding the relevance of herb–drug interaction studies with special focus on interplays: a prerequisite for integrative medicine. Porto Biomed J 2019;4:e15.
34. Savai J, Varghese A, Pandita N, Chintamaneni M. In vitro assessment of CYP1A2 and 2C9 inhibition potential of Withania somnifera and Centella asiatica in human liver microsomes. Drug Metab Pers Ther 2015;30: 137–41.
35. Varghese A, Pandita N, Savai J. Lack of the cytochrome P450 3A interaction of methanolic extract of Withania somnifera, Withaferin A, Withanolide A and Withanoside IV. J Pharm Negat Results 2013; 4:26.
36. Sultana R, Sultan Z. In vitro effect of Withania somnifera, Mucuna pruriens and Pausinystalia johimbe on hepatic cytochrome P450 in rat. Bangladesh Pharm J 2018;21:118.
37. Reddy ER, Bharadwaj SA, Reddy KS, Prashanth S, Sagar JVV, Goverdhan P. Possible influences of standardized herbal extract of Ashwagandha on the pharmacokinetics and toxicity of ritonavir in rats. Am J Ethnomed 2015;2:169–75.
38. Kumar S, Bouic PJ, Rosenkranz B. Investigation of CYP2B6, 3A4 and β-esterase interactions of Withania somnifera (L.) dunal in human liver microsomes and HepG2 cells. J Ethnopharmacol 2021;270: 113766.
39. Kasarla SS, Borse SP, Kumar Y, Sharma N, Dikshit M. In vitro effect of Withania somnifera, AYUSH-64, and remdesivir on the activity of CYP-450 enzymes: implications for possible herb-drug interactions in the management of COVID-19. Front Pharmacol 2022;13:973768.
40. Zanger UM, Klein K. Pharmacogenetics of cytochrome P450 2B6 (CYP2B6): advances on polymorphisms, mechanisms, and clinical relevance. Front Genet 2013;4:24.

Supplementary Material: This article contains supplementary material (https://doi.org/10.1515/dmpt-2024-0056).