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Two Weeks Dose Range Finding Study of KSM-66 Ashwagandha Root Extract by Oral Route in Wistar Rats – A Toxicity Study

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ABSTRACT

Ashwagandha (Withania Somnifera) is widely used as an adaptogenic medicinal herbin Ayurveda, the Indian system of Medicine. This study assessedpotential toxic effects associated with repeated oral administration of Ashwagandha root extract (ARE) for two weeks in Wistar rats. Twenty male and female Wistar rats were assigned to four groups viz., control, ARE (500/1000/2000 mg/kg body weight per day). Animals were observed for mortality/morbidity, clinical signs of toxicity (daily cage side observation), clinical examination (gait, mobility, arousal level, respiration, clonic/tonic movement, stereotype, bizarre behaviour, defecation, urine pools, vocalization, and rearing) prior to dosing and before necropsy day, body weight (weekly), and feed consumption. After 14 days, the animals were euthanized (CO_2) and subjected to detailed gross necropsy (examination of external orifices, cranial, thoracic, and abdominal cavities, and their contents). Organs (liver, kidney, heart, spleen, brain, adrenal, thymus, ovaries, uterus with cervix, testes, and epididymis) were removed and weighed. No clinical signs of toxicity were observed with Ashwagandhatreated groups, and it had nosignificant ($p>0.05$) effect on body weight and feed consumption by animals compared to control. No differences ($p>0.05$) were observed between ARE treated animals and control with respect to the absolute and relative organ weightand gross pathological findings. Ashwagandha root extract was found to be well tolerated in doses up to 2000 mg/kg body weight administered orally for 14 days in Wistar rats.

Key Words: *Ashwagandha, Withania somnifera, KSM-66, Medicinal herbs, Toxicity study.*

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INTRODUCTION

Ashwagandha (Withania somnifera), is a revered plant in Ayurveda, an Indian holistic medical system. The roots of ashwagandha are used as an astringent, thermogenic, adaptogenic, tonic, anti-inflammatory, aphrodisiac, and anthelmintic [1]. The ashwagandha plant also contains other components that have antibacterial and antioxidant properties [2]. The root extract of ashwagandha includes more than 200 primary and secondary metabolites, including sterols, glyco-withanolides, alkaloids, and flavanol glycosides. Ashwagandha extracts include substantial concentrations of Withanone (win), one of the withanolides [3]. It has been found that the amount of withanone (win) in leaves is considerable (19 mg/gram of extract) and negligible in roots. Its relevance in Ayurvedic practises is shown by the fact that it has been used traditionally to promote health, reduce anxiety, and increase the body's adaptability to stress. Ashwagandha extract, is notable for its remarkable efficacy and all-encompassing holistic wellness benefits. Ashwagandha, is also recognized as a *Rasayana* in Ayurvedic tradition [4].

Because of their unique effects and relatively low side effects, herbal medicines like Ashwagandha have been gaining popularity all over the world. Quality control is a challenge to ensure the safety, efficacy, and batch-to-batch consistency of herbal products due to the complexity of phytochemical constituents. Generally, it is believed that the risk

associated with herbal drugs is much less, but several reports on serious adverse reactions are indicating the need for the development of safety profiles; effective regulatory guidelines; and quality control systems for authentication, isolation, and standardization of herbal medicine [5, 6].

Toxicology testing is one of the preclinical procedures used to verify a formulation's safety. The oral acute toxicity tests are performed before subchronic toxicity, mutagenesis, teratogenic and carcinogenic testing [6, 7]. The OECD Guidelines for testing of chemicals is the most relevant internationally agreed testing method used by government, industry, and independent laboratories to identify and characterise potential hazards of chemicals [8, 9]. Establishing data on the toxicity of any medicinal extract is an integral prerequisite prior to the development of drugs. In a recent study, the no observed adverse effect level (NOAEL) of Ashwagandha Root Extract (ARE) was 2000 mg/kg body weight/day in rats after repeated oral administration for 90-days [10]. In another study conducted by Langade D. et al. [11] reported that with 28- day repeated dose administration, Ashwagandha root extract does not show any major abnormality in rats with doses up to 5 times of the recommended human dose [11]. In recentin-vitro study, acute oral toxicity was conducted in Wistar rats (n = 25) with doses of 500/1000/2000 mg/kg body weight in male Swiss albino mice for morbidity and mortality for 3 days. No mortality, morbidity, or any clinical signs were observed up to 3 days following ARE administration. Ashwagandha root extract failed to show any mortality in doses up to 2000 mg/kg oral dosage and did not show any mutagenic (genotoxic) effects in high concentrations [10]. These findings substantiate the safety of ashwagandha root extract.

The present study was conducted to provide information on the possible health hazards likely to arise from repeated oral administration to the test item "KSM-66 Ashwagandha Root Extract" for a period of two weeks (14 days) in Wistar rats using the OECD Guidelines. In addition, the dose levels for subsequent 90 Day Repeated Dose Toxicity Study of KSM- 66 Ashwagandha Root Extract by Oral Route in Wistar Rats was determined [10]. Mortality, clinical signs of toxicity, food intake, body and organ weight changes and gross pathological examinations were undertaken to assess the safety profile of KSM-66 Ashwagandha Root Extract.

MATERIAL AND METHODS

Ashwagandha Root Extract

KSM-66 formulation of Ashwagandha is a root extract of ashwagandha manufactured using an aqueous based extraction process. It is slightly hygroscopic and yellowish brown in color. It is standardized to >5% of total withanolide content, and also consists of <0.1% of withaferin A [12, 13]. KSM-66 formulation of Ashwagandha was developed based on principles of both ancient wisdom and scientific methods [14]. KSM-66 has been recognized as a potent and concentrated form of the holy herb Ashwagandha.

Experimental Animals

Adult male or female (Nulliparous and non-pregnant), Wistar rats, 8-9 weeks old, were used in the study. The study protocol confirmed the Indian National Science Academy Guidelines for the Use and Care of Experimental Animals in Research. Animals were housed in the Animal Facility under standard laboratory conditions. Temperature and relative humidity were maintained, monitored, and recorded twice a day and found in the range of 19.7°C to 22.6°C and 48.6 to 67.1% respectively. 12-15 air changes/h were maintained in animal confinements. The animals were maintained in 12h light artificial photoperiod and 12h dark. Animals were housed in groups in polypropylene cages covered with stainless steel grid tops. Dedusted and autoclaved paddy husk was used as bedding material. A copy of the periodic bedding material analysis report was retained in the study file. Cages were changed on alternative days and the cage grill once a week. Animals were fed with laboratory rodent pelleted feed procured from M/s. VRK Nutritional Solutions, Pune, ad libi-tum except on the day of fasting. The study was approved by the Institutional Animal Ethics Committee (IAEC/61/SRIHER/691/2020).

Acclimatation and Randomization

Rats were acclimatized for five days under laboratory conditions before initiating the study. Randomization was performed at the end day of acclimatization. Young healthy rats were selected and grouped based on the stratified body weight. The weight variation of animals was minimal and did not exceed ± 20% of the mean weight of each sex. For identification, animals were marked with permanent markers on the base of the tail during acclimatization, randomization, and experimental phase.

Suspendability, Stability, and Homogeneity

KSM-66 Ashwagandha Root Extract (test item) was suspendable in 0.1 % carboxymethycellulose (CMC) sodium (vehicle). Suspendability of the test item in the vehicle was ensured a day prior to dosing. Homogeneity of the test item was maintained by constant stirring up using a glass rod during dosing.

Justification for Route of Administration

The proposed route of administration of test item is oral in human. Hence, oral route was chosen.

Justification for selection of doses

Based on existing information and the safety profile of the Ashwagandha Root Extract is likely to be non-toxic and the No-Observed-Adverse-Effect-Level for 28 days in rats is 2000 mg/kg body weight [15], the highest level tested. Hence, additional doses such as 500 mg/kg b.wt. (Low dose), 1000 mg/kg b.wt. (Mid dose) and 2000 mg/kg b.wt. (High dose) were selected for the study.

Study Grouping

The study was conducted using a total of forty Wistar rats (twenty male and female each). These Wistar rats were randomly assigned to four groups viz., Vehicle Control (G1), Low Dose (G2), Mid Dose (G3), and High Dose (G4) Test compound groups consisting of five animals /sex/group.

- **Vehicle control (G1):** animals received 0.1% CMC sodium solution as the vehicle for period of 14 days
- **Low Dose (G2):** G2 animals received 500 mg/kg b.wt of KSM-66 Ashwagandha Root Extract via oral route once daily for period of 14 days
- **Mid Dose (G3):**G3 animals received 1000 mg/kg b.wt of KSM-66 Ashwagandha Root Extract via oral route once daily for period of 14 days
- **High Dose (G4):**G4 animals received 2000 mg/kg b.wt of KSM-66 Ashwagandha Root Extract via oral route once daily for period of 14 days

Study Design

Three graduated doses of test items (low, mid, and high doses) were used for the study. Dosing of animals (5 animals/sex/group) was performed as mentioned below.

Group No.	Treatment	KSM-66 Ashwagandha Root Extract Dose (mg/kg b.wt.)	Male		Female	
			No. of Animals	Animal No.	No. of Animals	Animal No.
G1	Control (Vehicle)	0	5	1-5	5	21-25
G2	Low dose	500	5	6-10	5	26-30
G3	Mid dose	1000	5	11-15	5	31-35
G4	High dose	2000	5	16-20	5	36-40

Evaluation Parameters

Mortality and Morbidity

Animals from all groups were observed for mortality and morbidity twice daily from acclimatization, till the day of necropsy.

Clinical Signs of Toxicity

General clinical observations of animals of all groups were performed once daily (after the dosing performed for the day) till the day of the necropsy. Detailed clinical examination was performed prior to first dosing and thereafter before a day of necropsy.

Body Weight

Individual body weight of the animals was recorded on the first day of treatment before dosing (day 1), middle of treatment (day 8), and on the last day of treatment (day14). The terminal body weight of the animals was taken on the day of scheduled necropsy (day 15). The body weight changes for all the animals were calculated.

Feed Consumption

Cage-wise feed consumption of animals was recorded daily from the day of dosing (day 1), till the day of necropsy. The average feed consumption was calculated per week.

Pathology

Necropsy - Gross Pathology and Tissue Collection

All animals were fasted overnight on day 14 and necropsied on day 15, using CO_2 euthanasia, and were subjected to detailed gross necropsy which included gross examination of external orifices including body cavities viz. thoracic, abdominal, cervical, and cranial cavity were opened and observed including its content for gross pathological changes. Most of the Organs {Skin, lymph node, mesenteric, eyes, brain (cerebrum, cerebellum, and mid-brain medulla/pons),

pituitary, trachea, oesophagus, thyroid, parathyroid, thymus, heart, lungs, stomach, small and large intestines, spleen, pancreas, liver, adrenals, kidneys, urinary bladder, gonads (epididymides, testes; ovaries, uterus with cervix, vagina), skeletal muscle, bone, and spinal cord} were observed for gross lesions in all animals. As there was no test item-related gross lesion observed in any of animals, tissues were not collected for a histopathology examination.

Organ Weights

The absolute wet weight of the organs was recorded during necropsy after trimming off the excess fascia. Individual terminal body weight of all animals was recorded on the day of necropsy and used to calculate relative organ weight.
Relative organ weight was calculated as mentioned below,

$$\text{Relative organ weight (\%)} = \frac{\text{Weight of organ (g)}}{\text{Terminal body weight of the animal (g)}} \times 100$$

Statistical Analysis

Statistical analysis was performed to analyse the mean differences between test item and control. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc using Sigma Plot 12.3 software. Each group mean were presented along with Standard Deviation and the number of ani-mals/observations (N). The "p" value ≤0.05 was fixed as significance criterion. Data, including weight along with body weight changes, feed consumption (cage wise) and organ weight (absolute and relative) were subjected to statistical analysis.

RESULTS

Mortality and Morbidity

No mortality or morbidity was observed in any of the KSM-66 Ashwagandha root extract treated and control group animals during the experimental period (Table 1)

Table 1: Summary of Mortality and Morbidity

Groups	KSM-66 Ashwa-gandha Dose (mg/kg) of body weight	Animal gender	Number of animals	Mortality	Morbidity	Duration in days
G1	0	Male	5	0/5	0/5	1-15
		Female	5	0/5	0/5	1-15
G2	500	Male	5	0/5	0/5	1-15
		Female	5	0/5	0/5	1-15
G3	1000	Male	5	0/5	0/5	1-15
		Female	5	0/5	0/5	1-15
G4	2000	Male	5	0/5	0/5	1-15
		Female	5	0/5	0/5	1-15

Clinical Signs of Toxicity

All animals were found to be normal and exhibited no abnormal signs of toxicity from first day of dosing till necropsy. Summary of cage side clinical signs observation is depicted in Table 2 and summary of detailed clinical examination is presented in Table 3.

Table 2: Summary of Detailed Clinical Signs Observation

Parameters	Male rats (n=5)								Female rats (n=5)							
	Aday priortodos-ing				Aday priortone-cropsy				Aday priortodos-ing				Aday priortone-cropsy			
	G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3	G4
Bodyposture																
Curledupoftenasleep	0	0	0	0	1	3	3	1	0	0	0	0	2	1	2	3
Flattened,limbsmaybespread out	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0
Lyingonside	0	0	0	0	3	2	1	1	0	2	0	1	2	3	2	2
Rearing	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SittingNormally,feettuckedin	1	2	1	1	0	0	0	0	2	1	3	1	0	0	0	0
Sittingorstandinga-lert,watching	3	2	4	4	1	0	1	3	2	2	1	2	1	1	1	0
Convulsions																

ClonicTonicmovement-Absent	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
Easeofremoving																	
Aggressive, attemptstobite	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	
Animalrears	3	3	3	2	0	3	2	1	1	1	3	1	0	3	3	2	
Animalsitsquietly	0	1	0	1	3	0	2	1	0	4	1	0	2	1	1	2	
Runsaroundcage	2	0	0	1	1	1	1	0	1	0	1	3	1	1	0	0	
Vocalizationswithoutresis-tance	0	1	2	1	1	1	0	3	2	0	0	1	1	0	1	1	
Handlingreactivity																	
Alert,limbsputagainstthebody	2	2	2	1	1	3	5	1	2	3	2	3	1	2	2	3	
Animaltotallyinactive	0	0	2	2	3	0	0	1	1	1	2	1	1	0	0	0	
Rigidinhand	2	2	0	1	0	0	0	1	1	0	1	0	2	0	1	1	
Squirms twists tendstobite	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	
Vocalizationswithoutresis-tance	0	1	1	1	0	2	0	1	1	1	0	1	1	3	2	1	
Palpebralclosure																	
Eyelidswideopen	5	5	5	4	5	5	5	5	5	5	5	5	5	5	5	5	
Slightlyclosed	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	
Lacrimation																	
Noexternallacrimation	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
EyeExamination																	
Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
Piloerection																	
Absent	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
Salivation																	
Noexternalsalivation	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	

Table 3: Summary of Detailed Clinical Examination

Parameters (Open field test)		**Male rats (n=5)**								**Female rats (n=5)**							
		Aday priortodosing				**Aday priortonecropsy**				**Aday priortodosing**				**Aday priortonecropsy**			
		G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3	G4
Gait	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Mobility score	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Arousal Level	Low	4	4	2	1	1	1	2	1	1	3	1	3	1	1	1	1
	High	0	1	3	3	4	3	3	3	3	2	3	1	4	4	3	4
	Veryhigh	1	0	0	1	0	1	0	1	1	0	1	1	0	0	1	0
Respiration	Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Clonicmove-ment	Absent	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Tonicmovement	Absent	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Stereotypy	Absent	5	5	5	5	5	5	5	4	5	5	5	5	5	5	5	5
	Excess Groom-ing	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Bizarrebehavior	Absent	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Number ofdefe-cations	0-2	4	5	5	4	4	2	3	5	4	3	4	4	4	4	3	3
	4-7	1	0	0	1	1	3	2	0	1	2	1	1	1	1	2	2
Numberofurine pools	0-2	4	5	4	5	5	4	5	5	3	4	5	3	5	5	3	5
	3-4	1	0	1	0	0	1	0	0	2	1	0	2	0	0	2	0
Number ofVoca-lization	0	4	5	3	3	4	5	4	5	3	5	4	4	4	5	2	4
	1	1	0	2	2	1	0	1	0	2	0	1	1	1	0	3	1
Number of Rearing	10-20	4	3	3	4	4	3	2	4	3	2	4	1	3	1	2	4
	21-30	1	2	2	0	1	2	3	1	2	3	1	4	2	3	3	1
	31-43	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0

Body Weight and Body Weight Changes

No statistically significant changes were noted in any of the KSM-66 Ashwagandha root extract-treated groups of boththe sex when compared with the respective control groups throughout the experimental periodand thecomputed p-values are not ≤ 0.05 (Table 4).

Table 4: Summary of Body Weight and Body Weight Change

Animal gender	Groups	KSM-66 Ashwagandha Dose (mg/kg) of body weight		BodyWeight (ingrams)			BodyWeightChange (ingrams)	
				Day1	Day 8	Day 14	Day1- 8	Day1-14
Male	G1	0	Mean	187.17	193.73	205.77	6.55	18.60
			SD	18.29	19.80	13.52	16.61	5.93
			N	5	5	5	5	5
	G2	500	Mean	196.48	210.83	225.44	14.35	28.96
			SD	23.67	22.23	23.42	7.05	12.27
			N	5	5	5	5	5
	G3	1000	Mean	183.15	205.34	212.08	22.19	28.93
			SD	18.27	28.10	14.02	12.36	5.36
			N	5	5	5	5	5
	G4	2000	Mean	186.50	203.83	215.09	17.32	28.59
			SD	19.21	19.90	17.24	10.12	6.04
			N	5	5	5	5	5
Female	G1	0	Mean	147.50	158.35	164.16	10.86	16.67
			SD	10.75	14.69	20.19	4.65	9.82
			N	5	5	5	5	5
	G2	500	Mean	145.97	158.19	167.25	12.22	21.28
			SD	7.29	9.14	12.51	6.21	8.26
			N	5	5	5	5	5
	G3	1000	Mean	143.56	155.07	160.23	11.51	16.66
			SD	4.86	7.12	12.57	3.29	8.18
			N	5	5	5	5	5
	G4	2000	Mean	145.67	154.84	163.09	9.17	17.42
			SD	4.25	9.17	6.87	5.38	3.22
			N	5	5	5	5	5

SD-standard deviation, N -Number, G-Group

Feed Consumption

In males, a statistically significant increase in mean feed consumption was observed in all test ite KSM-66 Ashwagandha root extract treated groups, i.e., G2-Low dose (500 mg/kg, b.wt.), G3-Mid dose (1000 mg/kg, b.wt.) and G4-High dose (2000 mg/kg, b.wt.) when compared with G1- Control (0 mg/kg b.wt.) group during week-1. However, no statistically significant changes were noted in any of the test item treated groups when compared with the control group during week-2. In females, no statistically significant changes were noted in any of the KSM-66 Ashwagandha root extract treated groups when compared with the control group throughout the experimental period and the computed p-values are not ≤ 0.05. (Table 5).

Table 5: Summary of Average Feed Consumption

Animal gender	Groups	KSM-66 Ashwagandha Dose (mg/kg) of body weight		Feed Consumption (in grams/animal/day)	
				Week 1	Week 2
Male	G1	0	Mean	13.22	16.01
			SD	0.94	0.63
			N	5	5
	G2	500	Mean	15.45*	16.12
			SD	1.56	1.22
			N	5	5
	G3	1000	Mean	15.16*	14.65
			SD	1.56	1.36
			N	5	5

	G4	2000	Mean	15.20*	14.87
			SD	1.51	1.24
			N	5	5
Female	G1	0	Mean	11.81	11.69
			SD	0.74	2.97
			N	5	5
	G2	500	Mean	12.43	13.02
			SD	1.48	1.63
			N	5	5
	G3	1000	Mean	11.43	11.51
			SD	0.87	1.12
			N	5	5
	G4	2000	Mean	11.38	13.37
			SD	1.10	1.30
			N	5	5

** denotes significantlyhigherthanG1(pvalue ≤0.05), SD-standard deviation, N -Number, G-Group*

Gross Pathology

External: No external gross pathological findings were observed in any of the animals at all treated dose levels including control group animals.

Internal: No KSM-66 Ashwagandha root extract treatment-related internal gross pathological findings were observed in any animals of the treated and control group (Table 6).

Table 6: Summary of Gross Pathology

Groups	**KSM-66 Ashwagandha Dose (mg/kg of body weight)**	**Animal gender**	**Gross pathology Abnormalities**			
			External Lesions		**Internal Lesions**	
			No Abnormality Detected	Abnormality Detected	No Abnormality Detected	Abnormality Detected
G1	0	Male (n=5)	5	0	5	0
		Female (n=5)	5	0	5	0
G2	500	Male (n=5)	5	0	5	0
		Female(n=5)	5	0	5	0
G3	1000	Male (n=5)	5	0	5	0
		Female(n=5)	5	0	5	0
G4	2000	Male (n=5)	5	0	5	0
		Female(n=5)	5	0	5	0

N -Number, G-Group

Organ Weight

Absolute Organ Weight

Mean absolute organ weight, terminal body weight, and the percentage value of relative organ weight of male and female animals from respective control groups (G1-0 mg/kg b.wt.) were calculated and the p-value of the difference between the treated groups and the corresponding G1- Control (0 mg/kg b.wt.) group was determined. Nostatistically significant changes were noted in any of the organs in any of the treated groups when compared with the Control group (Table 7).

Table 7: Summary of Absolute Organ Weight (Gross Pathology)

Male	**Groups**	**KSM-66 Dose (mg/kg of body weig**	**Mean Weight(ingrams)**									
			TerminalBodyWeight (Day15)	Liver	Kidneys	Heart	Spleen	Brain	Adrenals	Thymus	Testes	Epididymides

		ht										
	G1	0	185.146 ±	8.110	1.466	0.810	1.165	1.666	0.050	0.301	2.587	0.835
			10.98	1.09	0.13	0.10	0.25	0.15	0.01	0.07	0.22	0.11
			5	5	5	5	5	5	5	5	5	5
	G2	500	216.834*	9.803	1.701	0.845	1.592	1.707	0.038	0.304	2.750	0.840
			21.96	1.10	0.12	0.12	0.25	0.04	0.01	0.07	0.221	0.03
			5	5	5	5	5	5	5	5	5	5
	G3	1000	205.814	8.634	1.581	0.788	1.159	1.785	0.038	0.325	2.703	0.85
			15.54	1.12	0.19	0.13	0.21	0.11	0.01	0.06	0.29	0.126
			5	5	5	5	5	5	5	5	5	5
	G4	2000	200.360	9.538	1.635	0.854	1.324	1.772	0.042	0.323	2.799	0.968
			17.11	0.88	0.28	0.09	0.35	0.11	0.01	0.09	0.17	0.13
			5	5	5	5	5	5	5	5	5	5
Female	**Groups**	**KSM-66 Dose (mg/kg of body weight**	**Mean Weight(ingrams)**									
			TerminalBodyWeight (Day15)	Liver	Kidneys	Heart	Spleen	Brain	Adrenals	Thymus	Ovaries	Uteruswith-cervix
	G1	0	154.252	6.900	1.375	0.664	1.183	1.598	0.055	0.396	0.069	0.504
			19.36	1.39	0.21	0.09	0.31	0.19	0.01	0.16	0.01	0.27
			5	5	5	5	5	5	5	5	5	5
	G2	500	156.474	6.802	1.227	0.660	0.942	1.528	0.046	0.337	0.080	0.559
			11.32	1.23	0.10	0.04	0.27	0.20	0.01	0.04	0.02	0.30
			5	5	5	5	5	5	5	5	5	5
	G3	1000	151.410	6.342	1.232	0.744	0.802	1.768	0.054	0.370	0.069	0.362
			13.32	1.24	0.12	0.13	0.08	0.105	0.02	0.05	0.02	0.14
			5	5	5	5	5	5	5	5	5	5
	G4	2000	152.992	6.816	1.275	0.653	0.943	1.652	0.054	0.300	0.071	0.446
			10.23	0.34	0.04	0.08	0.22	0.21	0.018	0.07	0.01	0.17
			5	5	5	5	5	5	5	5	5	5

** denotes significantly higher than G1 in male rat groups (p value ≤ 0.05), SD-standard deviation, N -Number, G-Group*

Terminal Body Weight

In males, a statistically significant increase in terminal body weight was observed in the G2-Low dose (500 mg/kg, b.wt.) group when compared with G1- Control (0 mg/kg b.wt.) group. All other treated groups were comparable to Control group animals. In females, no statistically significant changes were noted in terminal body weight in any of the treated groups when compared with the Control group.

Relative Organ Weight

No statistically significant changes were observed in any of the percentage values of relative organs in any of the animals of both the sexes of G3-mid dose (1000 mg/kg, b.wt.) and G4-high dose (2000 mg/kg, b.wt.) when compared with respective G1- Control group (0 mg/kg b.wt.) animals. However, in the G2-Low dose (500 mg/kg, b.wt.) group, a statistically significant decrease in the adrenals of females and kidneys of male were noted when compared with the respective control (G1) group animals.

All other organs of both the sex of G2-low dose (500 mg/kg, b.wt.) were comparable to respective G1-Control group animals. The statistically significant changes observed in the G2-Low dose (500 mg/kg, b.wt.) group (Increase in terminal body weight of male, decrease in adrenals of female and kidneys of male when compared to respective control groups) were considered toxicologically insignificant as there was no dose correlation and gross changes were not evident. Hence, these changes were not considered as KSM-66 Ashwagandha root extract related.

DISCUSSION

Ashwagandha (*Withania somnifera*) is a well-established and a reputed herb in Ayurvedic medicine. It has been used as a "Rasayana" (rejuvenator), nootropic, and as a powerful natural adaptogen. The herb extract is extensively used for general wellbeing and in specific ailments. The root of the Ashwagandha plant (also known as Withania somnifera), has a long history of use as an adaptogen in the ayurvedic system of complementary medicine, and is used to counteract the negative effects of stress [1, 16].

Modern research has identified a number of active ingredients in Ashwagandha that may have therapeutic applications. The plant contains a range of bioactive constituents, including withanolides, glycowithanolides, sitoindosides, withaferin A besides other therapeutically active phytochemicals responsible for its anticancer, antidepressant, anxiolytic, cardioprotective, antioxidant, immunomodulating, neuroprotective and anti-inflammatory properties. KSM-66 formulation of Ashwagandha has been standardized to >5% of total withanolide content, - and <0.1% of withaferin A [17].

Generally, plant-based products used in Ayurvedic medicine are considered as safe. Such products are used after rigorous evaluation following the Ayurvedic practices and are time-tested, have a long history of application in various health-related aspects [8, 9]. Ashwagandha the medical herb has been consumed since time immemorial in Ayurveda. Several studies have investigated the toxicity of the oral administration of Ashwagandha root extracts in animal models and in humans. A study conducted in India on a group of 80 fully healthy individuals confirmed the lack of toxicity of this raw material. The participants were each administered 300 mg of Ashwagandha root extract orally, twice daily for 8 weeks. This was assessed by monitoring parameters such as body weight, systolic and diastolic blood pressure, hemoglobin, alkaline phosphatase, alanine transaminase, aspartate transaminase and plasma neutrophil and platelet counts [18].

The present study was undertaken to assess the oral toxicity of KSM-66 Ashwagandha extract in rats as per OECD Guidelines [19, 20]. These studies included a 14-day range finding study in male and female Wistar rats to select the dose levels for subsequent 90 Day repeated dose toxicity study. Three doses of KSM-66 Ashwagandha (500 mg/kg, 1000 mg/kg and 2000 mg/kg were studied. All animals were observed for mortality/morbidity (twice daily); clinical signs of toxicity (daily cage side observation), detailed clinical examination (once prior to dosing and before necropsy day), weekly body weight and feed consumption. At the end of treatment period, the animals were sacrificed and subjected to detailed gross necropsy which includes gross examination of external orifices, the cranial, thoracic, and abdominal cavities and their contents. On completion of the gross pathology examination, the selected organs were weighed from all animals.

Detailed clinical examination showed no significant effect of KSM-66 on body posture (curled up often asleep position, flattened, limbs often spread out, lying on side, rearing, sitting position, feet tucked in sitting or standing alert) [21]. Clonic Tonic movements was found to be absent. Ease of removing from cage (Aggressive nature, attempts to bite, animal rears, sits quietly, runs around the cage, vocalizations without resistance) was unaffected. Handling reactivity (Alert, limbs put against the body, animal totally inactive, Rigid in hand, squirms, twists, tends to bite and vocalizations without resistance) was found to be absent. Palpebral closure was absent, and eyelids were wide open. Eye on examination was found to be normal, piloerection and salivation were absent among the experimental rats.In majority of the animals, gait was found to be normal, arousal level low, respiration normal, clonic, and tonic movements as well as stereotypywas found to be absent. Defecation and urination behavior was normal among the treated groups. In addition, no significant difference in number of vocalizations and rearing behavior was seen in the treated groups as compared to normal control. Thus, objective end points for clinical examination provided preliminary evidence regarding the safety of KSM-66.

All three of the KSM-66 Ashwagandha formulations that were tested—500 mg/kg, 1000 mg/kg, and 2000 mg/kg—

were found to be safe. No Mortality or morbidity was observed in any of the animals of KSM-66 Ashwagandha treated group during the study. Results of the study concur with previously reported investigations where Ashwagandha extract was found to be safe in experimental animals [15, 22 & 23]. Acute Ashwagandha toxicity testing was done in 2016 by Patel et al. at a dose of 2000 mg/kg. In the sub-acute toxicity study, Ashwagandha extract was gavaged to Wistar rats at doses of 500, 1000, and 2000 mg/kg body weight/day for 28 days. Ashwagandha administration did not result in any toxicologically significant treatment-related changes in clinical observations, ocular examination, body weight increase, feed consumption, clinical pathology assessment, or organ weight when compared to the control group. Serum chemistry and haematological parameters were within the expected ranges. The highest dosage of Ashwagandha reported to have no deleterious effects was found to be 2000 mg/kg body weight [15].

The 2012 study by Prabu et al. also assessed the acute and subacute oral toxicity of *Withania somnifera* root (WSR) extract in Wistar rats. In the 14-day observation period following oral administration of 2000 mg/kg of WSR extract, acute toxicity was studied. No toxic signs or deaths were observed. In the subacute study, rats received oral doses of 500, 1000, and 2000 mg/kg of WSR extract once daily for 28 days. The body weights, organ weights, and haemato-biochemical parameters did not change significantly for any of the dosing levels. No gross or histological lesions caused by the WSR were observed. The no observed adverse effect level was found to be 2000 mg/kg body weight per day which is consistent with the present findings [22].

Another study examined the acute (24 h) and subacute (30 d) toxicity of alcohol extracts from the roots of W. somnifera in typical Swiss albino mice and Wistar rats. In mice, an intraperitoneal injection of the extract at a dose of 1100 mg/kg did not result in any deaths within 24 hours, but modest increments did cause mortality. The determined ID50 value was 1260 mg/kg body weight. In subacute toxicity trials, Wistar rats of either sex received repeated injections of ashwagandha extract at a dose of 100 mg/kg body weight for 30 days without experiencing any mortality or changes in the components of their peripheral blood. While other biochemical parameters identified in the study were within the normal range, the peripheral blood acid phosphatase level in both sexes significantly increased from the control [23].

The changes in the body weights in the KSM-66 Ashwagandha treated animals did not reveal any treatment-related adverse effects when compared to the vehicle control throughout the experimental period [6]. In the present study, although KSM-66 Ashwagandha treatment significantly increased the food intake of the treated group at one-weekpost-treatment, it did not translate into weight gain. Nonetheless by the end of the second week, the food intake pattern stabilized and no significantimpact on food consumption was observedwith KSM-66 Ashwagandha treatment. However contrary to our results, Choudhary et al., [12], reported that Ashwagandha reduced body weight and body mass index which further supports the hypothesis that Ashwagandha root extract exerts antistress activity, resulting in reduced food cravings and better eating behaviors [6].

Pathological assessment of injury is the prerequisite for the safety assessment of any test compound. In the present study in the treatment group, no significant effect on the absolute and relative organ weight of the liver, kidneys, heart, spleen, brain, adrenals, thymus, testes, epididymides, male and female sex gonadswas observed. However, study by Sharada et al., [23] reported that the male Wistar rats' spleen, thymus, and adrenal weights were significantly decreased when they received repeated injections of ashwagandha extract at a dose of 100 mg/kg body weight for 30 days. However, in the present study,only the dose of 500 mg/kg of KSM-66 Ashwagandha significantly reduced the weight of adrenals in the male rats [23].

Additionally, no test drug-related adverse gross pathological findings were observed in any organ or tissue in KSM-66 Ashwagandha treatment groups of either sex. Under the conditions of this study and based on the toxicological endpoints evaluated, the KSM-66 formulation of Ashwagandha root extract was found to be well tolerated up to 2000 mg/kg b.wt. when administered orally for a period of 14 days in Wistar rats. However long-term safety studies are needed to confirm the safety of Ashwagandha.

CONCLUSION

Based on the toxicological endpoints evaluated, the KSM-66 formulation of Ashwagandha root extract was found to be well tolerated at 500, 1000, and2000 mg/kg b.wt. when administered orally for a period of two weeks (14 days) to Wistar rats.

Authors Contributions

Authors PK, RS, and VG contributed towards the conduct of the study, other study activities, data collection and analysis.

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Conflict of Interest

Authors PK, RS, and VG are employed with Centre for Toxicology and Developmental Research (CEFTE), Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India. CEFTE received grant from Shri Kartikeya Pharma, Telangana, India.

Ethical Approval

The study was approved by the Institutional Animal Ethics Committee (IAEC/61/SRIHER/691/2020) registered under CCSEA, India.

Data Availability

The data that support the findings of this study is available from the corresponding author on reasonable request. The data is not publicly available due to privacy or ethical restrictions.

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