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Sub-acute toxicity of Ashwagandha (*Withania somnifera)* root extract in wistar rats

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ABSTRACT

Withania somnifera (ashwagandha, WS) is widely used in traditional Indian Ayurvedic medicine. Studies indicate ashwagandha possesses antioxidant, anxiolytic, memory enhancing, antiparkinsonian, anti-venom, anti-inflammatory and antitumor properties. Present study evaluated the sub-acute toxicity of repeated dose administration of Ashwagandha root extract in wistar rats.

Material and methods: Sub-acute toxicity of Ashwagandha was done as per the OECD-407 guidelines and was carried out for 28 days where satellite group was observed for 43 days. Wistar rats, 30 male and 30 females, were included in the study with 10 [5 M, 5 F] animals per group. Laboratory procedures were performed in accordance with CPCSEA guidelines. Animals were housed in standard laboratory conditions and were administered drugs orally- vehicle to control group and Ashwagandha 200, 400, 800 mg/kg body weight/day to study group. General parameters were noted, blood collection was done for haematological and biochemical parameters. All the animals were sacrificed, dissected and observed for gross necropsy and organs of high dose groups from control and Ashwagandha groups were sent for Histopathological examination.

Result: Gradual weight gain was observed in all the animals. No signs of intoxication and no changes in blood biochemistry were observed. Histopathological changes in organs were within normal limits.

Conclusion: After repeated dose administration, Ashwagandha root powder extract did not show any major abnormality in a dose 5 times of the recommended human dose and above upto 800 mg/kg.

1. Introduction

Ashwagandha (AW), known as Withania somnifera in Ayurvedic literature, is popularly known as winter cherry and Indian ginseng which is categorized as “Rasayana” meaning rejuvenator. It can maintain health, rejuvenate the body, may enhance longevity and is used as herbal tonic and health food. This herb has been tested for various pharmacological activities like anti-inflammatory [1], analgesic [2], anxiolytic & hypnotic [3], antidepressant[4], nootropic [5], antimicrobial [6], antioxidant [7], anticonvulsant [8], cardioprotective [9], anticancer [10], etc and these activities have been attributed to the phytochemical constituents such as alkaloids, steroidal lactones, saponins, glycol-withanolides present in it [11]. As the pharmacological activities are being tested using modern evaluation methods, it is also necessary to prove its safety and tolerability in animals and humans.

Ashwagandha is used by the ayurvedic practitioners since a long time and reported benefits of it. Different parts of the plant are being used like leaves, root, stem, seeds, fruits, etc. Toxicity studies have been done on different formulations of these parts of the plant like methanolic extract [12], hydroalcoholic extract [13], decoction, seed powder, root paste, etc. [14,15] and it has been found that each part of the plant has got different concentrations of the active ingredient. [16] Balkrishna, A et al. used whole plant extract [17] while Prabu PC et al. tested the acute and sub-acute oral toxicity of hydroalcoholic extract of its roots at 10, 70 and 500 mg/kg doses [18] and Patel SB evaluated the safety of methanol extract of the roots at 500, 1000 and 2000 mg/kg doses. Authors have concluded that 2000 mg/kg is a no-observed-adverse-effect-level (NOAEL) of Withania somnifera and well tolerated dose by oral route. [19] Sharada AC et al. used the alcohol extracts from the roots of W. somnifera by intraperitoneal route and observed LD 50 of Ashwagandha at 1260 mg/kg in mice while repeated such injections of 100 mg/Kg dose for subacute toxicity in rats did not result in any mortality or changes in peripheral blood constituents [20].

All these studies have conflicting results and ambiguity about the

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toxic dose of different formulations of AW, hence we planned this study to evaluate the subacute toxicity of aqueous extract of AW roots' proprietary standardized preparation (containing more than 5% withonoids) taking into consideration the clinical dose of this medicine.

2. Material and methods

2.1. Test conditions

Study was started after obtaining the Ethics committee permission (BVDUMC/3059/2022/003/0027). Sample of the root extract was provided by the sponsor. Samples was stored away from the sunlight and moisture in a dry place. Sub-acute toxicity study was carried out as per modified OECD-407guidelines [21,22].

2.2. Experimental animals

Wistar Rats, 30 male and 30 females were obtained from Global Research Solution private limited A/P Nhavi, Tal. Bhor, Pune, Maharashtra with CPCSEA registration no. 1899/PO/Bt/S/16/CPCSEA. The animals were acclimatized to the laboratory conditions for one week and the health status was examined by a veterinary doctor before the initiation of the study. Clinical signs and symptoms were recorded a day before the study was started. 5 rats were housed per cage in polypropylene cages with autoclaved rice husk as bedding material. Humidity of 60 ± 5%, temperature 25 ± 3 °C and a 12 hr light and 12 hr dark cycle were maintained. Rodent diet of Pranav Agro was given 100 g / 5 animals / day in the morning at 10 am and was kept constant throughout the study. Aqua water was provided ad libitum. Laboratory procedures were performed in accordance to CPCSEA guidelines. Total randomization was done in all 60 animals. Five animals were randomly allocated in one cage and cages were randomly numbered.

2.3. Preparation of test solutions

Ashwagandha is a root extract of ashwagandha manufactured using an aqueous-based extraction process. Ashwagandha root extract (KSM-66®) is manufactured according to current good manufacturing practices (GMP). For the production of KSM-66® the roots of the organically cultivated ashwagandha plant are collected using Good Agricultural and Collection Practices (GACP). These roots are then quarantined and the quality roots (solid and bright, approximately 7 centimeters in length and with a diameter of 1–1.5 centimeters) are selected. The selected roots are washed with water (reverse osmosis and demineralized) to remove sand, dust, and unwanted material in a specially designed washer and then blow-dried by hot air. The dried roots are milled into a multi miller subsequently the milled roots are mixed with equal amount of hot water for 4 h in the extraction tank. The liquid mixture thus obtained is transferred into a processing reactor with the addition of more water and heated continuously for 60 min under controlled temperature and pressure. The slurry is cooled and then passed through a fine filter to remove undesired material like waste particles, and fiber. The decoction obtained is passed into a tray dryer and dried at low temperatures until a moisture content of 2–3% is obtained. The final product obtained is in powdered form, which is further subjected to a micro-pulverizer, resulting in a fine free flowing powder. This powder is then put in a vibro-sifter to obtain the final powder of the desired particle size. The powder is packed into a double polyethylene food-grade plastic. The entire manufacturing process is carried out in a clean-room facility following Good Manufacturing Practices. Inspections and testing are carried out at various points during the manufacturing of KSM-66. At the end of processing, samples are collected and in-house microbiological analysis, heavy metal analysis and phytochemical analysis is carried out. The manufacturing procedure assures a consistent and high-quality product.

The extract was dissolved in water and then the mixture was homogenized with stirrer. The solution obtained was kept in a closed plastic jar after each use. Everyday fresh solution was prepared.

2.4. Dose calculation

Dose was extrapolated from the human dose 300–600mgs daily. Low dose was double the high dose of the human dose. On the day 0, all animals were weighed and coding was done and assigned to different groups. General parameters were assessed at baseline. Dosing was done in the morning root extract low dose 200 mg/kg, medium dose 400 mg/kg and high dose 800 mg/kg were given till 28 days. Doses of each animal was calculated as per the weight of the animals. They were also observed for changes in body weight, food consumption, appearance, abnormal manifestations and mortality every day of each animal. Animals were fasted overnight, on day 28. On the next day, blood samples were collected for hematological and biochemical parameters by retro-orbital puncture under ketamine anaethesia from all the animals.

2.5. Hematological parameters

Haematocrit, haemoglobin, erythrocytes, reticulocytes, total and differential leucocyte Count, platelets and blood clotting time were measured.

2.6. Serum chemistry

Sodium, potassium, calcium, phosphorous, glucose, total cholesterol, triglycerides, bilirubin urea, creatinine, total protein, albumin, A:G ratio, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels were also assessed.

2.7. Histopathology

All the animals were sacrificed, dissected and observed for gross necropsy. All organs of normal control, high dose groups root extract (800 mg/kg) were send for the histopathological examination. Total 20 animals –i.e. 7 cassettes of each animal were prepared. Hematoxylin & Eosin (H & E) stained slides of liver, kidneys, brain, joint/bone, sex organs (testes, epididymis & ovaries, uterus), adrenal gland, spleen, heart, intestine, lungs and muscle were studied by veterinary pathologist for significant pathological changes, if any. Lesions were evaluated for severity of changes and their distribution using following criteria-Severity evaluation was graded as follows- 0 = No abnormality detected, 1 = Minimal (<1%), 2 = Mild (1–25%), 3 = Moderate (26–50%), 4 = Moderately Severe/Marked (51–75%), 5 = Severe (76–100%). Lesions were recorded as focal, multifocal and diffuse.

Animals of Satellite normal control, Satellite high dose root extract group were observed further up to 43 days. These animals were sacrificed on day 44 after taking the blood for hematological and biochemical parameters. Organs were removed and preserved for the histopathological examination.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine significant differences in groups. For Multiple comparison Dunnett's and Tukey's test was used. Analysis was performed using Graph Pad Prism 6, p values < 0.05 were taken as statistically significant. Kolmogorov-Smirnov[a] and Shapiro-Wilk tests done to check the normality of data showed p value > 0.05 which indicated that the data is normally distributed.

3. Observations and results

3.1. General parameters

No abnormality was observed in general parameters and all the animals survived till 28 days study period. No congestion was seen in mucus membrane and eyes. No change in the gait, fur etc. was observed.

One female from satellite group died on day 29. Died at night, the body was decomposed and destroyed by other animals, so could not perform the postmortem. Another female rat from the same Satellite high dose Ashwagandha group showed hunched gait with change in posture on day 30, but this problem resolved within next 6 days without any specific treatment. This animal was normal afterwards and survived till 43rd day.

Food consumption was normal in control and all the drug treated groups at different doses and so was normal weight gain seen in all animals.

3.2. Body weight

The mean body weight of rats in all test groups was similar to those of the control group at the end of 7, 14, 21, and 28 days. Initially from day 0 to day 7, the increase in body weight was not significant while from day 14 to day 28, the increase in body weight was steady.

3.3. Food consumption (Satellite group)

There was no significant difference in the values of test groups in comparison to control.

3.4. Haematology

Effect of daily oral administration of Ashawagandha Root extract for 28 days on haematological.

There was no significant difference in the haematological values of test groups in comparison to control group.

Blood Sugar, Haemoglobin, clotting time & reticulocyte count were observed in the normal range and the values were comparable to control group rats.

3.5. Biochemical parameters

Significant (**$p < 0.01$) increase in Alkaline Phosphatase levels was seen in Ashwagandha HD group compared to control & satellite Ashwagandha group. Total proteins were increased in medium (*$p < 0.05$) and high dose (*$p < 0.05$) of Ashwagandha. Significantly (*$p < 0.05$) raised levels of albumin was also seen in Ashwagandha satellite group.

3.6. Biochemical parameters

3.6.1. Biochemical parameters and haematology

Biochemical parameters -glucose, liver function tests, kidney function tests, lipid profile are summarized in Tables 1, 2 and 3. No statistical difference was seen in serum alkaline phosphatase (ALP), alanine aminotransferase (ALT) values of all treated and control group of rats, except HD satellite root extract group where the values were lower than other groups. Total proteins, albumin & globulin were also significantly high in satellite root extract group. Cholesterol, Triglycerides and HDL values of all the groups of root extract treatment were comparable to control group of animals shows normal lipid profile. No statistical difference was seen in blood urea, uric acid and serum creatinine values among all the treated groups and control group of animals. Fig. 1,2.

3.7. Histopathology observations

Fig. 3**-Liver-** Multifocal minimal hepatocellular infiltration of inflammatory cell were seen in AW high dose group (Male- G4: 1/5) and control & AW high dose group (Female- G1: 1/5, G4: 1/5). Microscopic examination of all the males treated with Ashwagandha (Gr 4) showed diffuse minimal to mild hepatocellular cytoplasmic rarefaction (glycogen deposition) when compared with control group animals (G1). However, females from G4 group did not show any effect on liver when compared with male animals in the same group.Tables 4,5.

Fig. 4**- Kidneys:** Multifocal minimal infiltration of inflammatory cell AW high dose group (Male; G4: 1/5) and multifocal minimal basophilic tubules in control and AW high dose group (Male; G1: 1/5, Female; G4: 1/5).

Fig. 5**- Lungs**.

Lungs: Multifocal minimal alveolar infiltration of inflammatory cell in control group (Male; G1: 1/5) and Microscopic examination of control G1 and Ashwagandha treated groups (G4) showed spontaneous and incidental findings. These findings were observed in both control and treated animals with minimal severity.

Table 1
Effect of Ashwagandha on Haematological parameters 28-day treatment.

SR. NO	Study groups	Blood glucose (mg/dl)	Haemoglobin (g/dl)	Clotting time	Reticulocyte count
1.	CONTROL	111.3 ± 24.44	11.29 ± 0.42	4.91 ± 0.51	0.89 ± 0.22
2.	AW LD RE (200 mg/kg)	105.9 ± 27.65	10.83 ± 1.58	4.86 ± 0.77	1.33 ± 0.22
3.	AW MD RE (400 mg/kg)	124.54 ± 17.01	10.96 ± 1.08	4.96 ± 0.47	1.73 ± 0.33
4.	AW HD RE (800 mg/kg)	121.2 ± 12.80	11.7 ± 0.97	5.2 ± 1.05	2.05 ± 0.34
5.	S-Cont	110.8 ± 24.33	10.04 ± 2.37	5.20 ± 0.91	1.96 ± 0.63
6.	S-AW RE - HD (800 mg/kg)	127.11 ± 24.03	12.23 ± 1.60	4.75 ± 0.74	1.92 ± 0.40

AW- Ashwagandha, LD- low dose, MD- medium dose, HD- high dose, RE- root extract, S Cont- satellite control, S AW RE HD- satellite Ashwagandha High dose. Results are expressed as Mean ± SD.

4. Discussion

The toxicity testing is not only done for checking the safety of the test substance but also, to characterize the possible toxic effects it can produce to develop safety guidelines for its clinical use.

In sub-acute toxicity the effect of the repeated administration of drug on all the organs is tested. The potential of the test compound to cause toxic effects of variable duration can be studies qualitatively in laboratory animals. Animal toxicity testing are essentially providing the information on the dose specific toxic effects of the test drug. Toxicity obtained in animal studies generally occurs in similar manner, incidence and severity in the human [23]. Standard guidelines are there to carry out the toxicity study. Sub-acute toxicity testing was carried out according to Modified OECD guidelines- 407 which also includes the observation related to the endocrinal system. In our study 60 animals survived till the end of study period i.e. day 28. Animals looked very active, with white fur and fresh in every day observation. No abnormal changes were observed in the general parameters during the study period like tremor, convulsions, catatonia etc. One female from satellite high dose Ashwagandha 800 mg/kg died on day 29 and one more female rat from same group showed postural abnormality but it was resolved in 6 days without any specific treatment for it. The postural change was assessed by the Veterinary doctor and reported as a nonspecific change.

General parameters including behavioural pattern was normal in all the animals. Body weight change is an important indicator of the general health status of animals [24]. Body weight loss of animals more than 20% is considered as critical according to Canadian Council on Animal

Table 2
Effect of Ashwagandha on Biochemical parameters I- in the study groups after 28-day treatment.

Study Groups	Bilirubin	SGOT (U/L)	SGPT (U/L) ALT	Alkaline Phospha ALP	Total Proteins	Albumin	Globulin	A:G Ratio
CONTROL	1.76 ± 0.90	39.82 ± 19.86	49.44 ± 18.17	323.56 ± 163.55	6.94 ± 0.55	2.99 ± 0.25	2.32 ± 0.07	1.29 ± 0.13
AW LD RE	1.6 ± 0.91	34.79 ± 15.65	41.44 ± 19.47	392.9 ± 165.65	6.77 ± 0.23	2.91 ± 0.39	2.37 ± 0.41	1.26 ± 0.28
AW MD RE	1.76 ± 0.80	47.96 ± 18.19	36.94 ± 19.99	381.8 ± 114.84	7.60 ± 0.50 *	3.03 ± 0.15	2.50 ± 0.13	1.21 ± 0.09
AW HD RE	1.45 ± 0.83	30.99 ± 17.20	39.61 ± 17.83	544.5 ± 125.59 * *	7.08 ± 0.61 *	2.93 ± 0.26	2.42 ± 0.25	1.22 ± 0.19
S-Cont	1.18 ± 0.52	18.79 ± 3.68	65.98 ± 34.60	362.36 ± 144.80	6.93 ± 0.58	3.1 ± 0.24	2.25 ± 0.29	1.40 ± 0.26
S-AW HD RE	1.13 ± 0.51	24.35 ± 6.41	61.73 ± 28.50	299.32 ± 111.99	6.67 ± 0.52	3.44 ± 0.33 *	1.95 ± 0.26	1.80 ± 0.41 * **

AW- Ashawagandha, LD- low dose, MD- medium dose, HD- high dose, RE- root extract, S Cont- satellite control, S AW RE HD- satellite Ashwagandha High dose. Results are expressed as Mean ± SD.*p < 0.05, * *p < 0.01 & * **p < 0.001 in comparison with control.

Table 3
Effect of Ashwagandha on Biochemical Parameters II in the study groups after 28-day treatment.

Study Groups	Sr.urea (mg/dl)	Sr. Creatinine (mg/dl)	Uric Acid	Sr. cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL
CONTROL	39.47 ± 8.43	0.97 ± 0.19	4.62 ± 0.39	65.5 ± 5.44	184.6 ± 73.01	37.18 ± 2.00
AW LD RE	33.84 ± 4.31	1.12 ± 0.22	5.31 ± 0.73	73.14 ± 14.16	166.9 ± 38.72	37.95 ± 2.41
AW MD RE	33.54 ± 5.11	1.04 ± 0.31	4.83 ± 0.42	59.83 ± 14.22	124.54 ± 33.77	39.38 ± 2.75
AW HD RE	32.47 ± 4.16	1.11 ± 0.26	5.17 ± 0.83	72.83 ± 16.98	178.37 ± 57.12	43.15 ± 3.22
S-Cont	39.93 ± 6.31	0.92 ± 0.13	5.18 ± 0.52	74.8 ± 12.90	161.6 ± 25.73	39.96 ± 2.31
S-AW-HD RE	29.16 ± 3.83	1.13 ± 0.28	5.35 ± 0.66	81.83 ± 20.85	133.93 ± 49.41	40.83 ± 4.22

AW- Ashawagandha, LD- low dose, MD- medium dose, HD- high dose, RE- root extract, S Cont- satellite control, S AW RE HD- satellite Ashwagandha High dose. Results are expressed as Mean ± SD.*p < 0.05,* *p < 0.01 & * **p < 0.001 in comparison with control

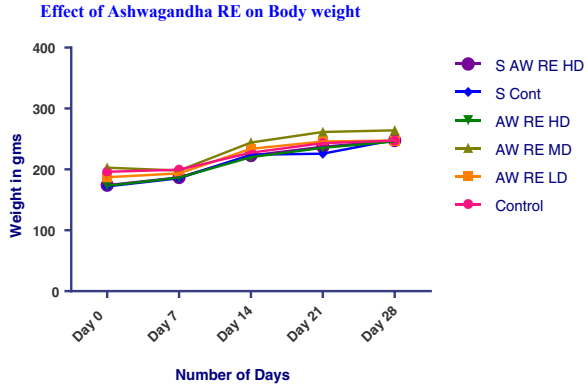

Fig. 1. : Effect of Ashwagandha on body weight of rats during the study period AW- Ashawagandha, RE- root extract, LD- low dose, MD- medium dose, HD- high dose, S Cont- satellite control, S AW RE HD- satellite Ashwagandha High dose. Results are expressed as Mean ± SD.

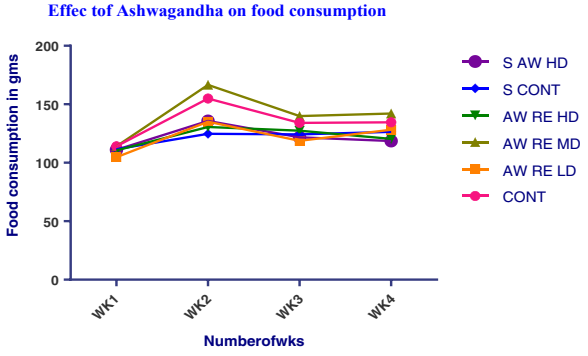

Fig. 2. : Effect of Ashwagandha on food consumption of rats during the study period AW- Ashawagandha, RE- root extract, LD- low dose, MD- medium dose, HD- high dose, S Cont- satellite control, S AW RE HD- satellite Ashwagandha High dose. Results are expressed as Mean ± SD.

Care (CACC) and OECD guidelines. Body weight and food intake was gradually increased in all the animals indicating minimal effect of the drugs on the general health of the animals during the study period (Fig. 2). But in last two weeks the weight gain was slow and minor reduction in food intake was observed.

All hematological and biochemical parameters haemogram, BSL, Liver Function tests, Renal function tests, electrolytes were found to be within normal range. (Table no.1–3). Hematological parameters are important indices of the physiological and pathological status for both animals and humans[25]. No effect of Ashwagandha root extract was observed on these parameters.

Liver status is assessed from the levels of different indicators, bilirubin, aspartate transaminase (AST) and alanine transaminase (ALT), serum alkaline phosphatase (ALP). ALP is considered for determining type of liver injury. In healthy individuals most circulating alkaline phosphatase originates from liver or bone and observed to be increased in obstructive liver diseases [26].

In the study conducted by Shruti et al., where methanolic extract was used showed safety of Ashwagandha at 2000 mg/kg, even in the sub-acute study they have used very high doses. Increase in bilirubin, AST, ALT was observed in the high dose but these high levels were within the

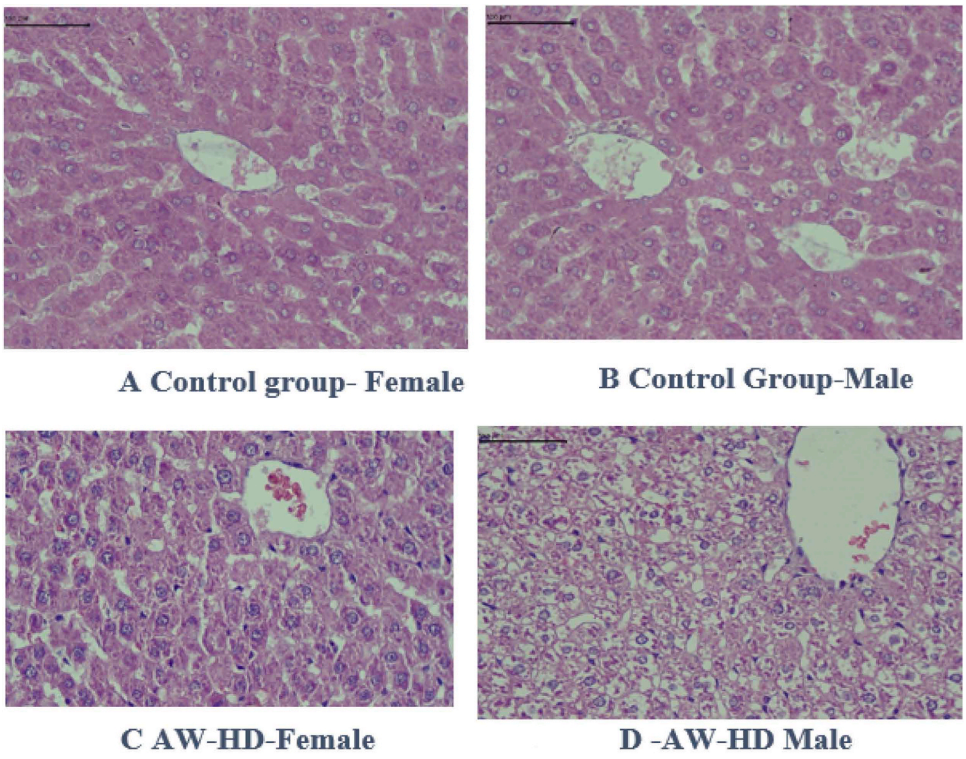

Fig. 3. : A&B-Liver (40X H-E stain): liver showing normal hepatocellular parenchyma. C&D-Showing cytoplasmic rarefaction in hepatocytes.

Table 4
Effect of Ashwagandha on serum electrolytes after 28-day treatment.

SR. NO	Study Groups	Sodium	Potassium	Calcium	Phosphorous
1.	CONTROL	138.1 ± 4.53	4.25 ± 0.45	9.43 ± 0.82	3.33 ± 0.63
2.	AW LD RE	142.6 ± 5.62	4.26 ± 0.49	9.32 ± 0.89	3.48 ± 0.81
3.	AW MD RE	141.7 ± 7.02	4.27 ± 0.47	9.92 ± 0.95	3.69 ± 0.42
4.	AW HD RE	138.9 ± 6.11	4.05 ± 0.42	9.18 ± 0.79	3.38 ± 0.61
5.	S-Cont	137.8 ± 4.91	4.18 ± 0.42	9.51 ± 1.01	3.46 ± 0.70
6.	S-AW SAT-HD RE	140.22 ± 6.99	4.18 ± 0.51	9.32 ± 0.98	3.35 ± 0.47

AW- Ashawagandha, LD- low dose, MD- medium dose, HD- high dose, RE- root extract, S Cont- satellite control, S AW RE HD- satellite Ashwagandha High dose. Results are expressed as Mean ± SD.*$p < 0.05$,* *$p < 0.01$ & * **$p < 0.001$ in comparison with control

Table 5
Effect of Ashwagandha on organ weight after 28-day treatment.

SR. NO.	Study Groups	Organ weight in gms				
		Liver	Kidney	Lung	Brain	Heart
1.	CONTROL	10.1 ± 1.52	1.79 ± 0.13	1.73 ± 0.06	1.71 ± 0.25	0.73 ± 0.07
2.	AW LD RE	11.1 ± 3.24	1.79 ± 0.11	1.65 ± 0.17	1.68 ± 0.07	0.70 ± 0.07
3.	AW MD RE	11.6 ± 2.41	1.75 ± 0.05	1.73 ± 0.09	1.75 ± 0.07	0.72 ± 0.06
4.	AW HD RE	10.6 ± 3.06	1.73 ± 0.08	1.74 ± 0.07	1.72 ± 0.05	0.69 ± 0.05
5.	S-Cont	9.6 ± 1.71	1.66 ± 0.35	1.70 ± 0.07	1.82 ± 0.06	0.72 ± 0.07
6.	S-AW SAT-HD RE	8.88 ± 0.60	1.77 ± 0.06	1.79 ± 0.09	1.77 ± 0.06	0.63 ± 0.22

AW- Ashawagandha, LD- low dose, MD- medium dose, HD- high dose, RE- root extract, S Cont- satellite control, S AW RE HD- satellite Ashwagandha High dose. Results are expressed as Mean ± SD. There was no significant difference in the values of test groups in comparison to control.

normal limits for the rodents. In our study even at the high dose of Ashwagandha 800 mg/kg increase in the ALP, Proteins, albumin was observed though it was within the normal limits for animals according to CPCSEA guidelines [27]. On 28 days administration of Ashwagandha orally in high dose (800 mg), transient increase in the Alkaline phosphatase (ALP) levels was observed which was still within the upper limit of normal values. [28] This increase in ALP normalized in the satellite group after stopping the drug and could be due to toxic metabolite of Ashwagandha which is detoxified by GSH but under limited GSH levels it can cause DNA damage and reversible liver toxicity[29]. It was also observed that in satellite AW high dose group ALP levels were returned back to the lower levels.

Gross necropsy and histopathology of all organs of high dose groups of root extract was done to observe the pathological changes if any. Multifocal minimal hepatocellular infiltration of inflammatory cell was seen in liver. (Fig. 3-C, D) This lesion suggestive of glycogen deposition in the cytoplasm of hepatocytes which could be due to effect of test item (Ashwagandha) and might have correlation and/or effect on the glucose metabolism. Multifocal minimal alveolar infiltration of inflammatory cell was seen in lungs and multifocal minimal infiltration of inflammatory cell and multifocal minimal basophilic tubules was seen in kidney (Fig. 4-C, D) but the rate of occurrence was low and severity also was less. Such lesions usually develop in the different laboratory animals to certain extent, hence were considered to be spontaneous / incidental in nature. Further, these lesions were also comparable with information available in the literature [30].

Prabhu et al., studied the toxicity of hydro alcoholic extract of Withania somnifera root in mice and reported up to 2000 mg/kg is nontoxic in acute study and in sub-acute study on observable adverse effects in hematological, biochemical and histological parameters at

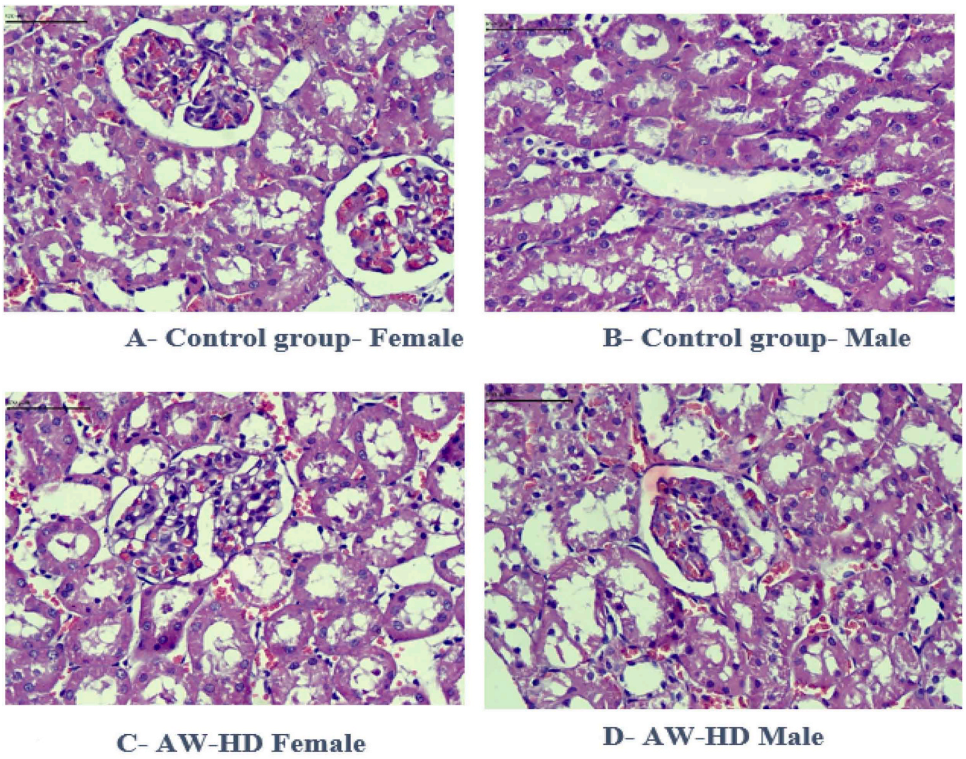

Fig. 4. A, B, C&D-Kidney (40X H-E stain): showing normal glomeruli and renal tubules.

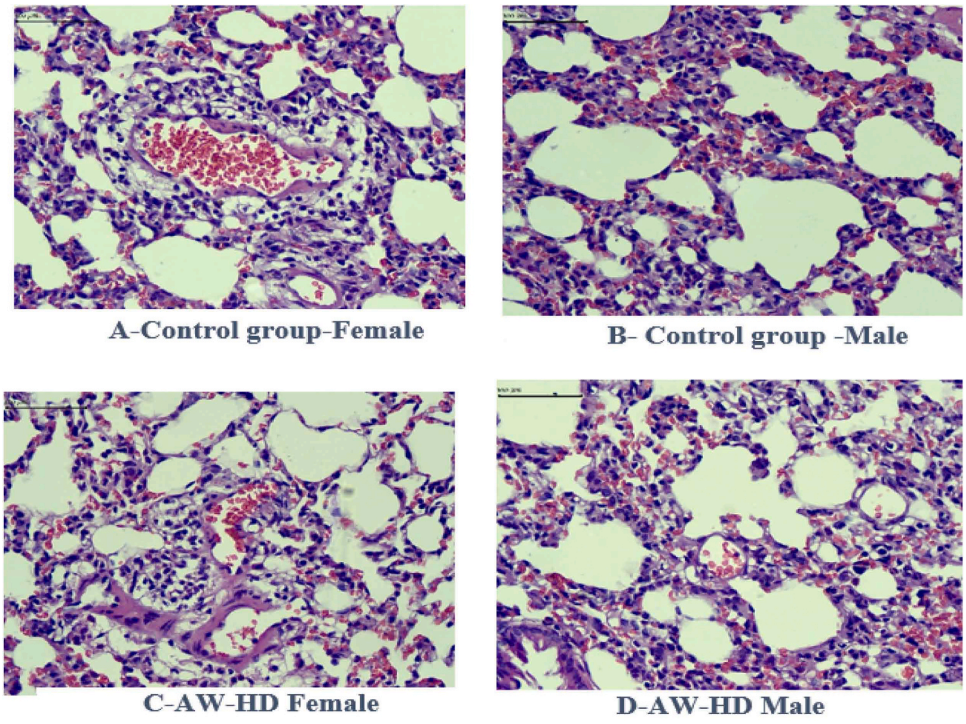

Fig. 5. A, B,C&D-Lungs (40X H-E stain): showing normal bronchial epithelium and alveloi.

doses 500, 1000 & 2000 mg/kg. Sharada et al.l have observed LD 50 of Ashwagandha at 1260 mg/kg by injectable route in mice and used 100 Mg/Kg dose for subacute toxicity. As per Prabhu et al. the difference in the LD50 values seen in the two studies may be attributable to differences in environment, method of isolation and species variation between herbs grown in different parts. In our sub-acute toxicity testing, Ashwagandha root extract (800 mg/kg) was found to be non-toxic up to 28 days period. Little increment in the duration has resulted in the mortality. Some standard guidelines need to be established to get the repeatability and generalizability of the results.

5. Conclusion

On the basis of general observations, biochemical and histopathology findings, it can be concluded that Ashwagandha is safe to be administered up to 28 days in 200 mg/kg-800 mg/kg in rats. In subacute toxicity study, root extract of Ashwagandha did not cause any pathological effects on any organ and was found safe over 28 days treatment in the said dose.

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Author statement

Authors are thankful to the reviewer's for their comments to improve the manuscript. We have addressed each and every comment raised by them. Sending herewith the Revised manuscript entitled " **Subacute toxicity of Ashwagandha (Withania somnifera) root extract in wistar rats"** as per the reviewers comment with the intension to publish it in your esteemed journal. Authors declare -No conflict of interest. On behalf of all the contributors I will act and guarantor and will correspond with the journal.

CRediT authorship contribution statement

Dr. Deepak Langade: Conceptualization, Supervision, Project administration. **Dr. Jayshree Dawane:** Formal analysis, Writing - original draft, Writing - review & editing. Data curation. Project administration, Methodology implementation. **Dr. Priti Dhande:** Investigation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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