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Acute and Sub-Chronic Toxicological Potential of *Withania Somnifera* Extract on Rats

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ABSTRACT

Withania somnifera (WS) has a wide range of therapeutic applications for several disorders. Toxicity studies on WS are very limited. The purpose of the present work was to study the acute and sub-chronic toxicity of WS extracts in rats and to determine the LD_{50} value, which in turn helps in determining the dose range for the plant extract to be used with no harmful or lethal effects on the animal. Rats in acute study were IP injected with the alcoholic extract of WS (the aerial parts) at doses of (0), 150, 300, 600 and 1200 mg/kg bwt. In sub-chronic toxicity study WS extract was administered IP at a dose of 10%, 20% and 40% of the obtained LD_{50} (52 mg/kg bwt, 104 mg/kg bwt and 208 mg/kg bwt respectively) for 60 days. Acute toxicity revealed an IP LD_{50} of 522 mg/kg wt. Significant changes in body weight, hematological, biochemical and histopathological changes were recorded in 20% and 40% LD_{50} groups with 15 – 40 % mortalities respectively, by the end of the experiments. Based on this study, WS showed toxicity at doses higher than 10% LD_{50} when given IP and it was suggested to use ≤ 10% LD_{50} (IP) doses of WS extracts, than the tested here to start with in setup protection or treatment studies.

1. INTRODUCTION

Withania somnifera (WS) is a small woody shrub belongs to the family Solanaceae and commonly known in different part of the world as Winter cherry, Indian ginseng, Somm El-Ferakh and Ashwagandha which means "odor of the horse", probably originating from the odor of its root, which resembles that of a sweaty horse. While the specie name "*somnifera*" in Latin means "sleep-inducer" which probably refers to its extensive use as a stress-busting remedy (Ven Murthy et al., 2010 & Seenivasagam et al., 2011). *Withania somnifera* is widely distributed in tropical and subtropical zones in Asia, Africa, Middle East and Southern Europe. In Saudi Arabia it grows in Western (North and South Hijaz), Southern and Eastern Regions (Al-Yahya et al., 1990; Hepper 1991; Al-Hindawi et al., 1992). Phytochemical analysis of WS have shown the presence of more than 12 alkaloids, 40 withanolides and several sitoindosides in different part of the plant (Mirjalili et al., 2009). The alkaloids includes somniferine A, Withanine, withasomnine, somninine, nicotine, pseudowithanine (Mossa et al. 1987 and Mishra et al., 2000). WS recorded as an official drug in Indian Pharmacopoeia-1985 (Singh et al., 2011 & Uddin et al., 2012). It has a wide range of therapeutic applications as arthritis, lumbago, carbuncle, spermatorrhoea, asthma, leukoderma, general debility, sexual debility, anxiety, neurosis, scabies, ulcers, and leucorrhoea (Ali et al., 1997 and Tiwari et al., 2014). The leaves of the plant are bitter and have some medicinal uses in fever and painful swelling. The flowers are astringent, depurative, diuretic, and aphrodisiac. The seeds are anthelminthic, remove white spots from the cornea, increase sperm count, as well as testicular growth. The fruits traditionally used as a topical treatment for tumors and tubercular glands, and skin ulcers (Chopra et al., 2004, Kaur et al

2004, Singh et al., 2011). No significant changes recorded in the body weight, organ weight, hematological and biochemical parameters, when the alcoholic root extract of WS administered once orally to Wister rats at 2000 mg/kg and observed for 14 days for acute toxicity and daily at 500, 1000 and 2000 mg/kg and observed for 28 days for sub-acute toxicity (Prabu et al. 2013). Moreover, teratology profile of WS, monitored on the developing fetus of pregnant rats including mortality, structural abnormalities, and changes in growth revealed no evident changes found in the mother or in the fetus. In addition, no changes noticed in the body weight of prenatal females, number of corpora lutea, implantations, viable fetuses, and skeletal and visceral formations (Prabu and Panchapakesan 2015). Similarly, Swiss albino mice injected intraperitoneally with a single dose of 1100 mg/kg WS root extract, did not produce any deaths within 24 h, but small increases have led to mortality with an LD_{50} of 1260 mg/kg of body weight. Moreover, repeated IP injections of 100 mg/kg (1/12 LD_{50}) in Wister rats for 30 days did not result in any mortality or changes in blood constituents. However, significant weight reductions recorded in the spleen, thymus, and adrenal weights with significant increase in the activity of serum acid phosphatase (Tiwari et al., 2014 and Sharada et al. 1993). Back in 1965, Malhotra et al found that, the oral LD_{50} of a 2 % suspension of ashwagandholine (total alkaloids from the roots of WS) prepared in 10 % propylene glycol using two percent gum acacia as suspending agent was 465 mg/kg (332-651 mg/kg) in rats and 432 mg/kg (299-626 mg/kg) in mice. Moreover, the acute oral LD_{50} of alcohol extract from defatted WS seeds dissolved in normal saline was 1750 ± 41 mg/kg in albino mice (Singh, et al. 1982). In another study, the acute IP LD_{50} of aqueous-methanol extracts of WS roots from one-year-old cultivated WS injected in mice was 1076 ±78 mg/kg (Grandhi, et al 1994). Despite the wide use of WS plant as food and medicine in traditional societies of Africa and Asia, including Saudi Arabia, reports of toxicity studies, either on WS root or whole plant or different extracts of the plant, are still limited. The present study aimed to determine the IP LD_{50} of alcoholic extract of the areal parts of WS. In addition, the toxicological effects of fractions of the obtained IP LD_{50} studied in the sub-chronic toxicity study.

2. MATERIAL AND METHODS

2.1 Animals: Adult male albino rats apparently healthy and weighed 120-130 g obtained from animal house, College of Veterinary Medicine, King Faisal University (KFU). Rats were housed in hygienic fiberglass cages (five /cage) and ad libitum access to water and commercial pellets (obtained from the Grain Silos and Flour-Mills Organization, Riyadh). Animals were maintained at a controlled temperature (22 ± 3^0C) and humidity (55± 5 %), with a 12 h dark/light cycle. All experiments carried out according to the rule and ethics followed by KFU animal Care Committee.

2.2 Plant extraction: The aerial parts of WS, (leaves, stem and fruits), freshly collected from various farms in Al-Ahsa, KSA. Approximately 2kg of dried plant material were ground and extracted with 80% ethanol by shaking and percolation for 24 hours at room temperature. The extract then centrifuged at 1000 rpm for 10 minutes and the supernatant evaporated completely under vacuums in a rotary evaporator at 40 ^{o}C. Prior administration, the residue was dissolved in sterile distilled water.

2.3 Experimental design:

2.3.1 Determination of the IP LD50: According to the method of Weil (1952) for determination of the dose of LD_{50}, an exploratory trial were performed in five groups each of five rats. WS alcoholic extract was administered IP in doses of 25, 50, 75, 100 and 125 mg/kg b.wt. in the five groups to find the smallest toxic dose to start with. The dose 75 mg/kg b.wt. was then the least dose to cause signs of toxicity, it was multiplied by a constant factor (2) for each succeeding group of rats. Therefore, five groups of rats were used, (10 each). The 1st, 2nd, 3rd, 4th and 5th were injected 0, 150, 300, 600 and 1200 mg/kg b.wt. of WS alcoholic extract, respectively. Mortality rate was recorded after 24 hours.

2.3.2 Sub-chronic toxicity studies: Adult male rats were allocated into four equal groups (20 rats each). The 1st, 2nd and 3rd groups were daily injected IP (4 times /week) with 2/5, 1/5, and 1/10 of the obtained LD_{50}, corresponding to 208.9 (high dose), 104.5 (medium dose) and 52.2 (low dose) mg/kg b.wt. aqueous solution of WS alcoholic extract, respectively. The fourth group injected by sterile distilled water and serve as control. All rats kept under observation throughout the experiment. Six rats, from each group killed after 30 and 60 days. All

animals clinically observed daily for signs of toxicity. Body weight monitored weekly.

2.3.2.1 Hematological and biochemical assessments:

Blood samples taken from the median eye canthus of each rats at 30 and 60 days of treatment. Samples obtained in clean dry test tube containing EDTA (Analar, BDH) as an anticoagulant used for determination of red blood cell count (RBCs), the packed cell volume (PCV), hemoglobin concentration (Hb) and white blood cell count (WBCs) (Kelly, 1974). While other blood samples lift to clotting and then serum obtained after centrifugation at 3000 rpm/15 min and kept at –20ºC until used for liver and kidney functions using commercial assay kits (Bayer Corporation), which includes; alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), glucose, creatinine and urea. Measurements performed using spectrophotometer chemistry analyzer (Miles Inc., Germany).

2.3.2.2 Histopathological Studies

All animals, either dead spontaneously or killed at the end of the experimental period, were dissected and thoroughly examined for detection of any abnormalities. Tissue specimens taken from the liver and kidneys then fixed in 10 % neutral buffered formalin, later processed in paraffin and sectioned at (5-6 μm) in thickness. Finally, all sections stained with hematoxylin and eosin (H & E) according to Junqueira and Carneiro (2003).

2.4. Statistical analysis

All values presented as mean (± S.D). Data analyzed statistically by one-way ANOVA followed by Tukey's multiple comparison tests using SPSS software. Calculation done using the SAS System. The minimum level of significance was set at ($P < 0.05$).

3. RESULTS

3.1. Determination of LD50:

The obtained IP LD_{50} of alcoholic extracts of WS in rats was 522.27 mg/kg bwt. as calculated according to Weil (1952) and shown in table(1).

Table (1): IP LD50 determination of WS extract in rats

Groups	Rats /group	Dose mg/kg bwt	Dose ratio	No of deaths
1	10	0 (control)		0
2	10	150	-	0
3	10	300	2	1
4	10	600	2	7
5	10	1200	2	10

- Log m = Log Da + d. (f+1) Where
  - Log m = the log of LD_{50}
  - Log Da = the log of the lowest of the four dosage level used.
  - d = the logarithm of the constant ratio between dosage levels
  - f = a constant factor obtained from Weil's tables.
- log m = log 150 + log 2 (0.8 + 1.0)
  = 2.17609 + 0.30103 (1.80)
  = 2.17609 + 0.541854
  = 2.7179
- Antilog m = 522. 27
  LD_{50} = 522. 27 mg/kg b.wt.

3.2. Clinical observation and body weight:

Symptoms of acute toxicity (LD_{50}) in rats injected (IP) with alcoholic extracts of WS appeared within 1/2 - 1 hours after injection of the toxic dose. A small number of rats died within 10 hours of injection, while the others died within 24 hours. In those animals died earlier, the most prominent signs were increased heart and respiratory rates, unconsciousness, closed eyes, stupor and paralysis of the hind legs, after which the animals became drowsy and finally died. While in those died later, various signs appeared such as shaking of the head, licking of the legs, petechial hemorrhages from the eye-canthus, lying on the sternum, creeping on the abdomen, diarrhea, irregular gasping fits ending by coma and death. Symptoms of toxicity due to successive administration, observed first after one week of treatment at the high dose level (208.9 mg/kg B.wt; 40% of LD_{50}) and includes, piloerection, frequent urination, increased heart and respiratory rate, loss of appetite, decreased body weight, closed eyes, tendency to deep sleep, diarrhea. Mortalities start at the 4^{th} week and reached 40 % by

the end of the experiment. While at the medium and low dose levels (104.45 and 52.275 mg/kg B.wt; 20% & 10% of LD_{50} respectively), the same preceding signs appeared, but after a latent period of 15 days and in moderate to mild condition. However, mortalities in medium dose group reached 10 % by the end of experiment. Rats in the control group showed no clinical signs of illness.

Body weight was significantly decrease, starting at 4th week and 3rd week in low and medium dose group, respectively, reaching maximum at 6th week where the percent of decrease in both group were 21.77 % and 34.68%, respectively, when compared to control group. However, the decrease in the body weight in the high dose group start earlier at the second week and become marked at the 6th week, where the percent of loss in weight reached 51.62 % when compared to the control group (table 2).

3.3. Hematological and biochemical findings:

Results showed in table (3) indicated that RBCs count, Hb concentration and PCV significantly increased in both high and medium dose groups. However, the high dose group affected more and start earlier than the medium dose group at 30 and 60 days of treatment. While in the medium dose group, these effects noticed only at 60 days. No significant changes recorded in WBCs count in all WS treated groups. Liver transaminases (ALT-AST) and kidney parameters (creatinine –urea) were significantly increased in the high dose group at both 30 and 60 days of WS treatment (table 4). While in the medium dose group, the significant increase in liver and kidney parameters delayed at 60 days of WS treatment and in milder form. Alkaline phosphatase, (ALP) significantly increased only in high dose group and at 60 days of WS treatment. Glucose level significantly decreased in both medium (60 day) and high (30 and 60 day) dose groups. In addition, low dose group showed non-significant changes in hematological and biochemical parameters tested

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Table (2): Effect of IP injections of WS extract on the body weight (g) of rats in sub-chronic toxicity study.

Week	*Control*	Low dose *1/10 LD50*	Medium dose *1/5 LD50*	High dose *2/5 LD50*
0	124 ±3.65	123 ±2.12	127 ±2.41	125 ±1.92
1	136 ±5.59	134 ±2.30	139 ±2.59	136 ±2.70
2	152 ±5.94 [a]	149 ±3.96 [a]	150 ±2.70 [a]	135 ±3.11 [b]
3	163 ±4.66 [a]	160 ±3.70 [a]	151 ±2.77 [b]	139 ±2.30 [c]
4	178 ±3.65 [a]	158 ±1.92 [b]	148 ±2.24 [c]	134 ±2.30 [d]
5	189 ±3.42 [a]	172 ±3.42 [b]	156 ±2.30 [c]	130 ±1.92 [d]
6	200 ±2.68 [a]	187 ±3.42 [b]	157 ±3.21 [c]	135 ±1.14 [d]
7	217 ±3.44 [a]	190 ±3.42 [b]	162 ±3.08 [c]	127 ±3.21 [d]
8	228 ±2.86 [a]	195 ±3.42 [b]	161 ±2.92 [c]	126 ±3.96 [d]
9	248 ±5.63 [a]	194 ±1.53 [b]	162 ±2.79 [c]	120 ±3.08 [d]

- Values within columns represent the Mean (±SD).
- Means with different letters indicate significant differences ($p<0.05$) between groups within the same period of exposure.

Table (3): Effects of repeated intraperitoneal injections of WS extract on some hematological parameters in male rats along 60 days.

Days	*Groups*	RBCs x10^6	Hb *gm/dl*	PCV *%*	WBCs x10^3
0	*Control*	8.04 ±0.50	13.42 ±0.35	40.26 ±1.10	9.40 ±0.91
	Low dose (1/10 LD50)	8.08 ±0.41	13.58 ±0.48	40.74 ±1.30	8.90 ±0.37
	Medium dose (1/5 LD50)	8.05 ±0.92	13.45 ±0.39	40.35 ±1.14	8.95 ±0.61
	High dose (2/5 LD50)	8.08 ±0.44	13.61 ±0.44	40.83 ±1.30	8.76 ±0.53
30	*Control*	8.07 ±0.53	13.57 ±0.85	40.71 ±1.50	8.54 ±0.78
	Low dose (1/10 LD50)	8.10 ±0.51	13.95 ±0.28	41.85 ±1.14	8.67 ±0.58
	Medium dose (1/5 LD50)	8.12 ±0.21	14.21±0.27	42.60 ±1.10	8.80 ±0.47
	High dose (2/5 LD50)	8.95 ±0.12*	14.85 ±0.12*	44.55 ±1.00*	9.06 ±0.14
60	*Control*	8.40 ±0.25	14.10 ±0.25	42.30 ±1.10	8.65 ±0.51
	Low dose (1/10 LD50)	8.45 ±0.19	14.50 ±0.12	43.50 ±1.00	8.71 ±0.42
	Medium dose (1/5 LD50)	8.95 ±0.12*	14.90 ±0.12*	44.70 ±1.15*	8.79 ±0.46
	High dose (2/5 LD50)	9.50 ±0.31*	15.50 ±0.13*	46.50 ±1.10*	9.28 ±0.33

- Values within columns represent the Mean (±SD).
- Significance from control: *p<0.05; ** p< 0.01.

Table (4): Effects of repeated intraperitoneal injections of WS extract on some serum biochemical parameters in male rats along 60 days.

days	*Groups*	ALT *U/l*	AST *U/l*	ALP *U/l*	Creatinine *mg/dl*	Urea *mg/dl*	Glucose *mg/dl*
0	*Control*	40.00 ±3.81	88.00 ±5.70	145 ±7.91	0.92 ±0.42	22.00 ±4.69	135.20 ±7.60
	Low dose (1/10 LD50)	39.80 ±3.35	91.60 ±5.13	144.40 ±8.47	0.72 ±0.28	21.40 ±4.66	133.60 ±8.62
	Medium dose (1/5 LD50)	40.00 ±3.61	89.80 ±3.96	145.00 ±7.91	0.80 ±0.48	20.80 ±5.12	136.40 ±5.86
	High dose (2/5 LD50)	40.40 ±3.85	87.40 ±2.59	144.60 ±8.23	0.97 ±0.45	21.80 ±4.66	135.20 ±7.60
30	*Control*	42.80 ±2.59	87.00 ±5.70	143.20 ±8.58	0.96 ±0.45	25.80 ±3.96	136.00 ±7.97
	Low dose (1/10 LD50)	40.20 ±3.70	88.20 ±5.63	145.20 ±8.11	0.80 ±0.48	21.40 ±3.05	137.20 ±5.93
	Medium dose (1/5 LD50)	42.40 ±2.79	90.55 ±6.11	143.00 ±5.70	0.83 ±0.49	23.40 ±3.65	133.20 ±6.46
	High dose (2/5 LD50)	50.40 ±3.85*	114.40* ±7.30	151.20 ±2.59	2.00 ±0.29*	32.80 ±4.32	120.80 ±4.66*
60	*Control*	40.20 ±4.32	87.80 ±5.93	147.00 ±7.91	0.82 ±0.45	24.80 ±5.81	136.80 ±7.40
	Low dose (1/10 LD50)	41.20 ±3.70	88.60 ±5.18	143.60 ±8.99	0.70 ±0.31	23.00 ±5.66	136.80 ±6.30
	Medium dose (1/5 LD50)	50.80 ±3.27*	103.00 ±12.04*	151.60 ±7.02	1.96 ±0.16*	31.40 ±3.05*	126.00 ±2.92*
	High dose (2/5 LD50)	115.00 ±7.91**	131.40 ±6.11**	173.60 ±6.11**	2.43 ±0.26**	64.80 ±7.46**	98.00 ±9.62**

- Values within columns represent the Mean (±SD).
- Significance from control: *p<0.05; ** p< 0.01.

3.4 . Histopathological changes

In the control and low dose groups, gross examination revealed no significant lesions. Microscopically, liver showed single and/or few foci of hepatic cell necrosis associated with mononuclear cell infiltration, lymphocytes and plasma cell aggregations in preportal areas. Mild Kupffer cell proliferation also observed (Fig. 1-a). Kidneys showed few foci of interstitial round cell infiltration at cortico-medullary junction (Fig. 1-b). Results of the other groups revealed that, the more severe lesions observed in the high dose group while the moderate lesions noticed in the medium dose group. Grossly the liver of rats in high dose group appeared swollen and congested.

Microscopically, the hepatocytes appeared diffusely swollen with coarse cytoplasmic vacuoles. Thickening of the cellular membrane was so obvious. Multifocal areas of hepatic necrosis with mononuclear inflammatory cell aggregation noticed in different parenchymal areas. Kupffer cells were enlarged and prominent (Fig. 1-c). Kidneys grossly appeared enlarged with rough irregular surface besides whitish-yellowish discolored sub-capsular patches extending to include cortex and medulla. Microscopically, proximal convoluted tubules showed vascular degeneration associated with presence of intracytoplasmic brownish granular deposits of variable size (Fig. 1-d). Hyaline and granular casts observed in many tubules. Foci of perivascular interstitial mononuclear cell aggregation seen in cortex. In addition, chronic pyelonephritis characterized by fibrosis and chronic inflammatory cell aggregations in multiple areas including medulla and cortex associated with tubular atrophy or dilatation (Fig. 1-e). In the medium dose group, liver showed moderate infiltration of inflammatory cells in portal tracts besides Kupffer cells proliferation. Kidneys showed foci of interstitial round cell infiltration at cortico-medullary junction. The proximal convoluted tubules showed vacuolation of the lining epithelium with intracytoplasmic accumulation of brownish bodies (Fig. 1-f).

4. DISCUSSION

The numerical value of LD_{50} usually used to classify and to compare toxicity potential of different chemicals. Our results showed that, the single IP LD_{50} of WS alcoholic extract (areal parts), 24 hours after injection, was 522.27 mg/kg bwt. This result is contradictory with those of Grandhi, et al (1994) where their recorded IP LD_{50} was 1076 ±78 mg/kg bwt. in mice and 1260 mg/kg bwt. in Wister rats (Tiwari et al., 2014). This difference between the results may be due to the stage of plant growth, the climatic and environmental conditions under which the plants grows, the species difference, part and method of extraction. Studies on sub-chronic toxicity of IP treatment of WS extract are very scarce. Results of sub-chronic study revealed that, body weight was significantly decreased, in a dose dependent manner, 51.62% in high dose group (40% LD_{50}), 34.68% in medium dose group (20% LD_{50}) and 21.77% in low dose group (10% LD_{50}). Such result together with the observed diarrhea and depressed appetite in both medium and high dose groups, culminating the 10% and 40% mortalities recorded in them. This could explained by, when 20-40% of the obtained LD_{50} (104-208 mg/kg bwt) injected IP and repeatedly along 2 months, may accumulate toxic doses of WS constituents and causing toxicity.

Moreover, WS extract, as several species of solanaeceae, had a narcotic effect on rats due to their parasympatholytic alkaloids such as hyosyamine, hyoscine, solanine and other tropane derivatives (Sahni et al., 1995). Similarly, hematological and biochemical changes observed here showed that, RBCs count, Hb concentration and PCV significantly increased in the high and medium dose groups, where the high dose group affected earlier and more than the medium dose group. This rises in blood parameters are usually associating the dehydration caused by diarrhea (Billett, 1990). These results are inconsistent with those obtained by Prabu et al., (2013) and Patel, et al., (2016) who found no changes in both hematological and biochemical parameters in rats dosed orally with 2000 mg/kg bwt.

WS root extract for 42 days. This may be due to difference in the route of administration, where the absorption rate in IP is more than the oral route. Biochemical findings of liver and kidney functions revealed a significant increase of (ALT, AST, Urea and creatinine) in both high and medium dose groups. These changes were more severe in high dose group and begin earlier than medium dose group. In addition, ALP increased significantly at 60 day in high dose group. This may attributed to the accumulating toxic constituents of WS extract, which may cause hepatic and renal cell damage. The histopathological findings noticed in liver and kidneys of high and medium dose groups explain more such

obtained results. The low dose group (10% LD_{50}= 52 mg/kg bwt) showed non-significant changes in hematological and biochemical parameters tested. Hence, it seems to be safer than the medium or high doses.

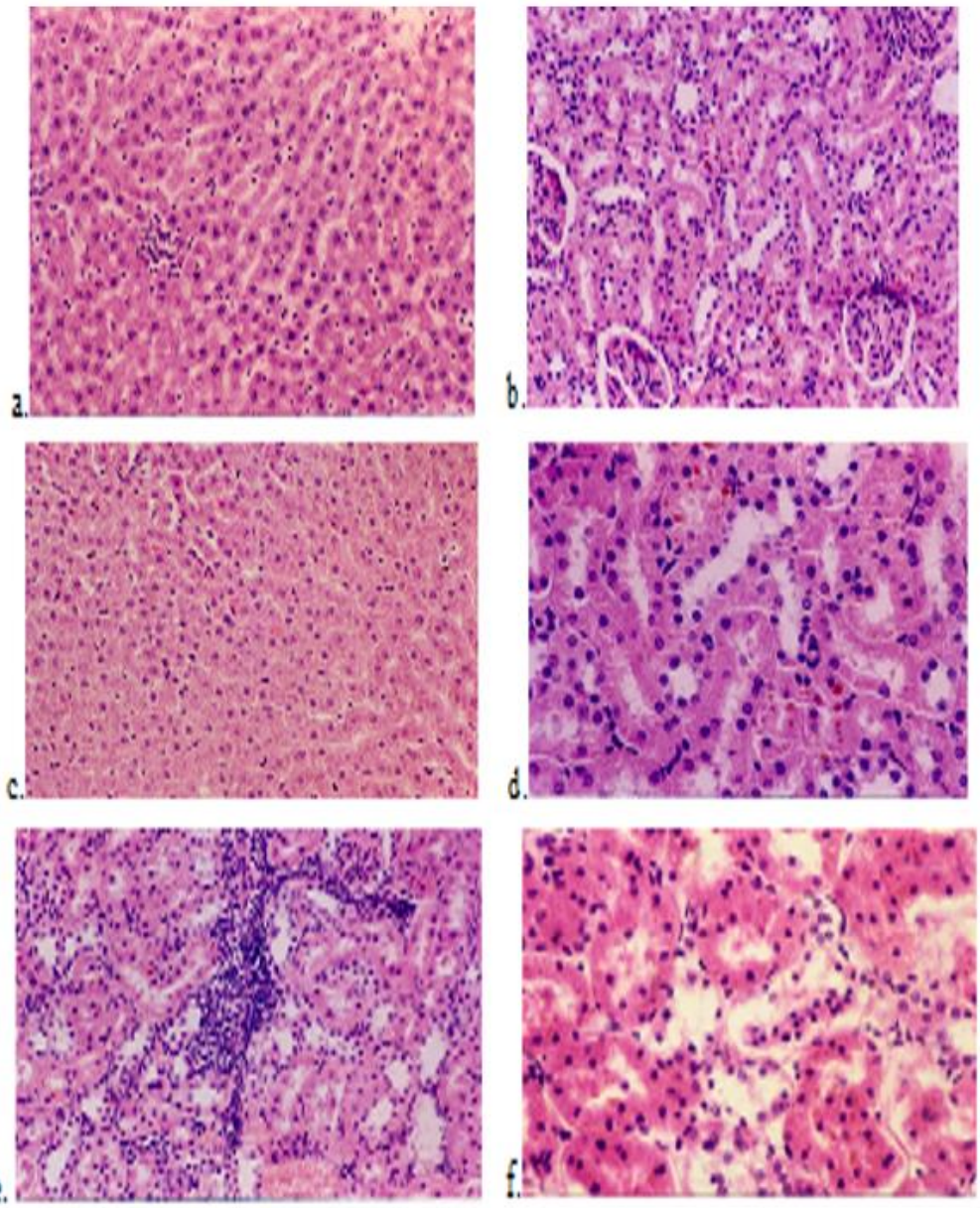

Fig. (1): Histopathology of rats treated IP with alcoholic extract of WS. In normal and low dose groups (a) Liver of normal and low dose group showing single and/or few foci of hepatic cell necrosis associated with mononuclear cell infiltration in periportal areas and mild Kupffer cell proliferation (X200 H&E). (b) Kidneys showed few foci of interstitial round cell infiltration at cortico-medullary junction (X100 H&E). In high dose group (c) Liver showed multifocal areas of hepatic necrosis with mononuclear inflammatory cell aggregation, hepatocytes swollen with coarse cytoplasmic vacuoles and thickening of the cellular membrane (X200 H&E). (d) Kidney showed vascular degeneration of proximal convoluted tubules with presence of intracytoplasmic brownish granular deposits (X200 H&E). (e) Kidney showed multiple foci of perivascular interstitial mononuclear cell aggregation in medulla and cortex with tubular atrophy or dilatation (X200 H&E). In medium dose group (f) Kidneys showed foci of interstitial round cell infiltration at cortico-medullary junction and vacuolation of the lining epithelium (X100 H&E).

5. CONCLUSIONS

As a conclusion, sub-chronic toxicity studies with WS extracts help to calculate safe starting doses in humans & animals to explore more about the beneficial role of WS extracts in treating different body disorders. Therefore, from this study it is recommended to use lower doses (IP) than the tested here to start with in setup protection or treatment studies.

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