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90-days Repeated Oral Dose Toxicity Study on Jawarish Bisbasa (A Polyherbal Unani Formulation)

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Abstract

Jawarish Bisbasa (JBS) is a polyherbal Unani formulation clinically indicated for the treatment of Zof-e-Meda (Stomach Weakness), Zof-e-Hazm (Dyspepsia), Bawaseer Amya (Blind piles), Nafkh-e-Shikam (Flatulence) and Ghasiyan (Nausea). The present study was designed to investigate toxicity of JBS following 90 days repeated oral administration in rats. JBS was orally administered at the dose levels of 1000 and 2000 mg/kg bw/day. The results obtained showed that oral administration of JBS at doses 1000 and 2000 mg/kg bw did not show any significant adverse effects on survival, body weight gain, hematology, clinical biochemistry, gross necropsy and histopathology. Therefore, No-Observed-Adverse-Effect-Level (NOAEL) is found to be greater than 2000 mg/kg body weight.

Keywords: Jawarish Bisbasa, Polyherbal, Toxicity, Unani

Abbreviations

Jawarish-e-Bisbasa (JBS)
No-Observed-Adverse-Effect-Level (NOAEL)
body weight (bw)
Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA);
Standard Error of Mean (SEM)

1. Introduction

Jawarish-e-Bisbasa (JBS) is a compound Unani pharmacopoeial formulation mentioned in the National Formulary of Unani Medicine, Part-I and other classical text of Unani system of medicine[1]. It is recommended for the treatment of Zof-e-Meda (Weakness of the stomach), Zof-e-Hazm (Dyspepsia), Bawaseer Amya (Blind piles), Nafkh-e-Shikam (Flatulence) and Ghasiyan (Nausea)[1]. JBS is a classical Unani formulation; however safety data of JBS is not available in literature. Therefore, present study was undertaken to evaluate the toxic effect of JBS following repeated oral administration in rats. For repeated dose oral toxicity, animals were treated with different doses of JBS as aqueous suspension via oral gavage for 90 days. The ingredients of the JBS are follows:

Unani Name	Botanical Name
Heel-e-Khurd	*Elettaria cardamomum* Maton.
Jaiphal	*Myristica fragrans* Houtt.
Saleekha	*Cinnamomum cassia* Blume.
Heel-e-kalan	*Ammomum subulatum* Roxb.
Zanjabeel	*Zingiber officinale* Rosc.
Daar Chini	*Cinnamomum zeylanicum* BL.
Asaroon	*Valeriana hardwickii* Wall.
Filfil Siyah	*Piper nigrum* Linn.
Qaranfal	*Syzygium aromaticum* (L.) Merrill and Perry.
Asal/Qand Safaid	Honey/sugar.

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2. Materials and Methods

2.1 Experimental Animals

SD rats (100 ± 20 g, 6 weeks old) were procured from ICMR-National Institute of Nutrition, Hyderabad, India. Female animals used were nulliparous and non-pregnant. Rats were group housed in polycarbonate cages in the air conditioned room maintained at the temperature of 22°C ± 3°C and relative humidity of 30–70%, with a 12:12 h light/dark illumination cycle. CPCSEA (Committee for the purpose of control and supervision of experiments on animals) guidelines of laboratory animal care was followed throughout the experiment[2] Protocol of the study was approved by the Institutional Animals Ethics Committee vide protocol No. CRIUM/IAEC/2016/02/P01.

Animals were maintained on standard pellet diet (National Institute of Nutrition, Hyderabad) and free access to water, unless mentioned in the experiment. Animals were housed for one week for acclimatization to the laboratory conditions before initiation of experiment.

2.2 Drug/Formulation

JBS was prepared in GMP certified pharmacy of Central Research Institute of Unani Medicine as per formulae mentioned in National Formulary of Unani Medicine[1]. An aqueous suspension in 0.3% CMC (<2 mL/100 gm body weight (bw)) was freshly prepared every day. The drug was orally administered as an aqueous suspension at the maximum volume of 2 mL/100 gm bw. The control animals were administered with vehicle only. Test drug was administered at dose levels of 1000 mg/kg bw and 2000 mg/kg bw once daily for 90 consecutive days at same time each day to minimize variations.

2.3 Vehicle

0.3% CMC aqueous suspension was used as vehicle for oral administration of formulations.

2.4 Dose Selection

Recommended therapeutic dose of JBS is 5–10 g[1]. As a conservative approach, 10 g human dose was used for dose translation (back calculation) for rats as per body surface area conversion[3]. For 90-day repeated dose oral toxicity on JBS, two dose groups were included viz. 1,000 mg/kg bw per day (i.e., Therapeutic Equivalent Dose) and 2,000 mg/kg bw per day (i.e., approximately two times of therapeutically equivalent dose). Third higher dose group is not feasible because two times of TED is already equivalent to limit dose as per ICH guidelines[4].

2.5 Drug Administration

JBS was administered in the form of 0.3% CMC aqueous suspension prepared using mortar and pestle. Drug or vehicle was orally administered via stainless steel gavage, by calculating the dose based on the body weight of rats, for a period of 90 days. Duration of toxicity study for JBS was decided as 3 months (i.e. 90-days) based on its duration of clinical use in Unani system.

2.6 Experimental Design

The 90-day repeated dose oral toxicity study was performed according to the OECD test guideline-408[5]. Male and female Sprague Dawley rats were divided into 3 groups with 20 animals (10 males + 10 females) in each group as follows:

- Vehicle control (0.3% Aq. CMC suspension).
- JBS Low Dose (X = 1,000 mg/kg bw).
- JBS High Dose (2X = 2,000 mg/kg bw).

Experimental rats were observed for mortality and morbidity twice a day, throughout the study period. Detailed clinical examination (i.e., functional observation parameters) was conducted periodically to detect any sign of toxicity, at a fixed schedule (1 h after vehicle or drug administration). Body weight of the animals was recorded once in a week. Average feed consumption was recorded for male and female animals at weekly interval by weighing the amounts of feed given to a cage group and leftovers on the next day. At the end of the treatment period, rats were deprived of feed overnight but water was provided ad *libitum*. Rats were anaesthetized with isoflurane vapours using EZ anaesthesia chamber. Blood samples were collected under anesthesia by retro-orbital puncture in the EDTA vacutainers for hematological investigations and in plain vacutainers for biochemical analysis as reported earlier[6].

Haematology parameters such as Hemoglobin (Hb), Red Blood Cell count (RBC), White Blood Cell count (WBC), Hematocrit (HCT) and Platelet (PLT) were analyzed using fully automated haematology cell counter (Swelab Autocounter-920EO+). Serum biochemical parameters such as fasting glucose, Alanine

Transaminase (ALT), Aspartate Transaminase (AST), Alkaline Phosphatase (ALP), total bilirubin, creatinine, Blood Urea Nitrogen (BUN), Total Cholesterol (TC), Triglycerides (TG), Total Protein (TP) and albumin were analyzed using fully automatic biochemistry analyzer (Erba-EM200) using commercially available kits (Make: Erba). At the end of study duration, all animals were euthanized using carbon dioxide and gross necropsy was performed by trained Veterinarian. Organs, tissues and internal cavities were examined macroscopically and internal organs/tissues were isolated, trimmed and weighed. Organs/tissues were stored in the 10% neutral buffer formalin and embedded in paraffin for histopathological analysis. The tissues were routinely processed and stained with haematoxylin and eosin and examined under microscope. Any change in the normal tissue architecture was observed and recorded.

2.7 Statistical Analyses

Data is expressed as mean ± Standard Error of Mean (SEM). The mean difference between the control and treatment groups was analyzed by one-way analysis of variance using Graph Pad prism (version 5) Graph Pad Software, Inc., CA, USA. p value ≤ 0.05 was considered statistically significant.

3. Results

3.1 Survival and Clinical Examination

No post dose adverse effect was reported on survival of both male and female rats after oral administration of JBS for 90 consecutive days. No incidences of mortality were reported in JBS treated male and female rats at all tested dose levels. There were no report for any abnormal clinical signs based on clinical examination conducted at different time points in JBS treated animals at 1000 mg/kg bw and 2000 mg/kg bw as compared to animals of control group.

3.2 Body Weight

There were no significant differences in body weight of JBS treated animals as compared to control group. The expected pattern of weight gain was observed in both male and female rats of drug treated group as compared to control group (Figures 1 and 2).

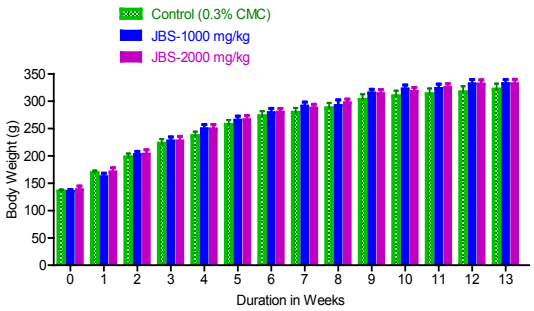


Figure 1. Average body weight of control and JBS treated male rats.

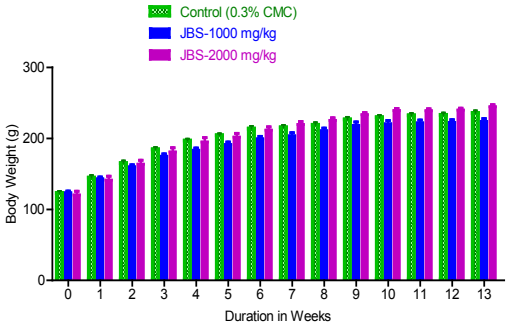


Figure 2. Average body weight of control and JBS treated female rats.

3.3 Feed Consumption

The feed consumption was measured weekly throughout study duration. Male rats treated with JBS at all tested dose levels did not show significant difference in food consumption as compared to control group. No consistent difference observed in food consumption in female group receiving test drug as compared to control group (Figures 3 and 4).

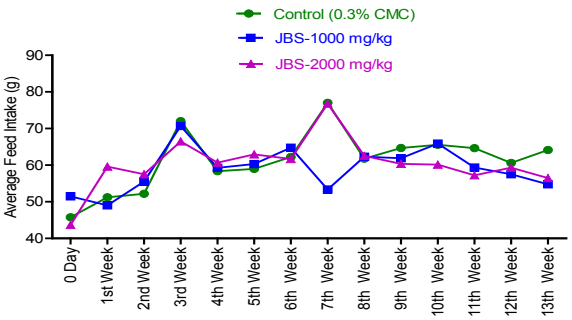


Figure 3. Average feed intake of control and JBS treated male rats.

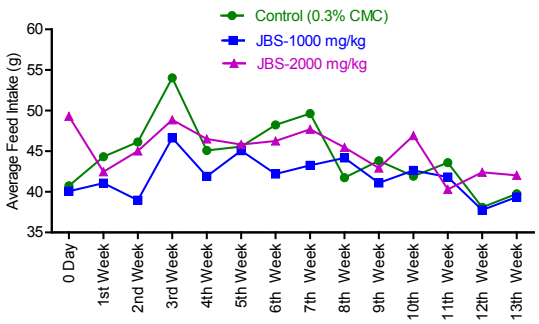


Figure 4. Average feed intake of control and JBS treated female rats.

3.4 Hematology

JBS did not produce any significant effect on hematological parameters after 90 days administration except in respect of platelets count in female group at low dose level (1000 mg/kg bw) which showed significant increase ($p<0.05$) as compared to control female group (Table 1). Oral administration of JBS consecutively for 90 days did not produce any significant alterations in other hematological parameters like Hb, RBC, WBC, HCT, neutrophils, lymphocyte and eosinophils.

3.5 Clinical Chemistry

The oral administration of JBS consecutively for 90 days did not produce any significant alteration in biochemical profile except significant decrease ($p<0.01$) in serum glucose level in drug treated female group at high dose level (2000 mg/kg bw) as compared to control group (Table 2). There was a significant increase ($p<0.05$) in total protein in drug treated female group at high dose level (2000 mg/kg bw) as compared to control animals. There was also significant decrease in albumin level ($p<0.01$) in female group at high dose level (2000 mg/kg bw) as compared to control group. It was also observed that there was significant increase in globulin level ($p<0.01$) in drug treated female group at high dose level (2000 mg/kg bw) as compared to control animals. There was a significant decrease in triglyceride level ($p<0.01$) in drug treated female group at low dose level (1000 mg/kg bw) as compared to control animals.

3.6 Organ Weights

The oral administration of JBS at dose levels 1000 and 2000 mg/kg bw did not induce any alterations in actual organ weight and relative organ weight in the organ/tissues such as Brain, Thymus, Heart, Lungs, Liver, Adrenals, Kidney, Testis, Epididymis, Uterus and Ovaries. However, there was a significant decrease ($p<0.01$) in relative organ weight of spleen in male group at both dose levels as compared to control animals. Additionally, there was a significant decrease in relative organ weight of left kidney in male group at low dose level (1000 mg/kg bw) as compared to control group (Figures 5 and 6).

Table 1. Effect of JBS on haematological parameters in rats

Parameter	Male			Female		
	Control	JBS		Control	JBS	
		1000 mg/kg bw	2000 mg/kg bw		1000 mg/kg bw	2000 mg/kg bw
Hb (gm%)	14.5 ± 0.44	15.3 ± 0.46	15.3 ± 0.33	15.6 ± 0.21	15.6 ± 0.17	15.5 ± 0.15
RBC (Million/mm^3)	7.5 ± 0.24	8.0 ± 0.29	7.8 ± 0.19	7.5 ± 0.10	7.8 ± 0.11	7.7 ± 0.10
WBC (/mm^3)	4581.8 ± 307.8	5254.5 ± 359	4854.5 ± 284	3400.0 ± 312	4018.2 ± 343	3481.8 ± 347
Platelet (lakhs/mm^3)	4.7 ± 0.32	4.7 ± 0.21	4.3 ± 0.24	3.4 ± 0.15	4.1 ± 0.25*	3.5 ± 0.12
HCT (%)	38.3 ± 1.07	41.4 ± 1.07	40.8 ± 0.85	41.4 ± 0.61	40.5 ± 1.20	41.0 ± 0.67
Neutrophil (%)	7.6 ± 1.03	7.2 ± 0.76	6.0 ± 0.68	7.8 ± 1.41	6.5 ± 0.88	6.6 ± 0.52
Lymphocyte (%)	79.4 ± 1.32	78.8 ± 1.41	80.3 ± 1.27	80.0 ± 2.11	79.8 ± 1.77	80.0 ± 0.91
Eosinophil (%)	5.5 ± 0.22	5.8 ± 0.28	6.0 ± 0.35	5.0 ± 0.47	5.6 ± 0.45	5.5 ± 0.22
Monocyte (%)	7.5 ± 0.36	7.3 ± 0.72	8.0 ± 0.51	7.2 ± 0.70	7.8 ± 0.65	7.9 ± 0.48

(Values presented as Mean ± SEM; n = 10/ sex; ANOVA; * = $p<0.05$)

Table 2. Effect of JBS on blood biochemical parameters in rats

Parameter	Male			Female		
	Control	JBS		Control	JBS	
		1000 mg/kg bw	2000 mg/kg bw		1000 mg/kg bw	2000 mg/kg bw
Glucose (mg/dL)	85.2 ± 4.10	77.0 ± 3.42	88.1 ± 6.62	97.5 ± 3.19	89.1 ± 7.89	74.7 ± 2.56**
ALT (IU/L)	76.1 ± 3.91	79.2 ± 6.93	81.9 ± 5.26	63.5 ± 4.44	64.4 ± 3.10	62.2 ± 4.73
AST (IU/L)	183.1 ± 4.32	176.5 ± 5.55	180.5 ± 9.41	146.0 ± 5.55	168.4 ± 12.47	152.9 ± 7.11
Bilirubin (mg/dL)	0.5 ± 0.13	0.3 ± 0.11	0.3 ± 0.11	0.2 ± 0.01	0.2 ± 0.07	0.2 ± 0.01
ALP (IU/L)	117.0 ± 6.07	125.6 ± 10.79	119.5 ± 11.36	111.4 ± 9.54	112.5 ± 7.61	96.6 ± 7.87
Total Protein (g/dL)	6.4 ± 0.37	7.2 ± 0.36	6.5 ± 0.29	6.2 ± 0.31	7.0 ± 0.32	7.1 ± 0.41*
Albumin (g/dL)	3.5 ± 0.06	3.3 ± 0.05	3.8 ± 0.15	4.2 ± 0.13	3.8 ± 0.14	3.7 ± 0.08**
Globulin (g/dL)	2.9 ± 0.42	3.9 ± 0.40	2.7 ± 0.32	2.0 ± 0.29	3.2 ± 0.33	3.4 ± 0.48**
BUN (mg/dL)	15.2 ± 2.01	11.0 ± 1.21	9.8 ± 0.32	14.3 ± 2.15	10.5 ± 0.37	10.3 ± 0.34
Creatinine (mg/dL)	0.79 ± 0.01	0.87 ± 0.03	0.78 ± 0.04	0.79 ± 0.03	0.82 ± 0.03	0.85 ± 0.02
Cholesterol mg/dL)	62.8 ± 2.25	64.1 ± 3.63	64.5 ± 3.61	107.3 ± 6.39	95.7 ± 7.52	97.9 ± 6.53
Triglycerides(mg/dL)	69.5 ± 4.26	54.9 ± 3.95	58.6 ± 2.05	86.2 ± 7.33	55.5 ± 3.05**	72.1 ± 5.83
HDL (mg/dL)	34.8 ± 1.55	33.7 ± 1.39	33.4 ± 1.83	31.3 ± 1.42	32.5 ± 1.82	33.2 ± 1.81
LDL (mg/dL)	14.0 ± 2.73	19.3 ± 3.26	19.4 ± 4.48	60.0 ± 5.09	51.9 ± 7.38	50.5 ± 5.87

(Values presented as Mean ± SEM; n = 10/ sex; ANOVA; * = p<0.05, ** = p<0.01)

3.7 Histopathological Analysis

Histopathological analysis of organ portion (Figured 7A and B) after treatment with JBS 2000 mg/kg bw revealed histological changes in liver of animals. Liver examination showed focal periportal and chronic parenchymal inflammation in both sexes of control and drug treated animals. The hepatocytes also showed microvacuolation and vascular congestion. The abnormalities were observed both in control and drug treated animals which may not be attributed to drug treatment. Chronic interstitial pneumonitis of different grades was observed in both control and JBS treated animals hence was not considered significant. Rest of the organs were found normal histologically.

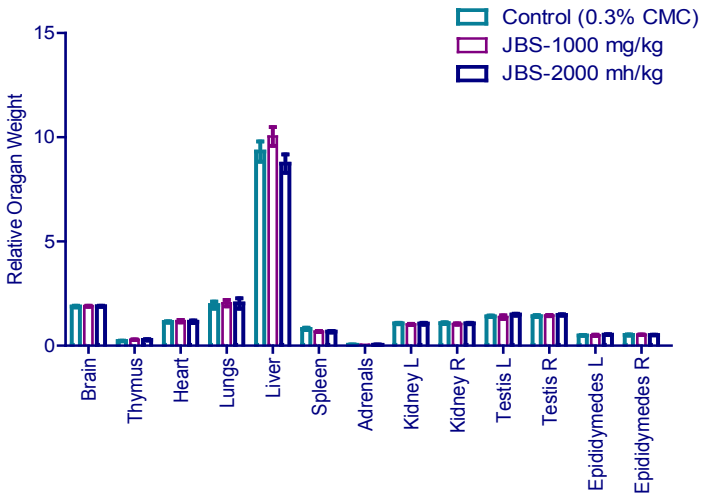


Figure 5. Relative organ weight of control and JBS treated male.

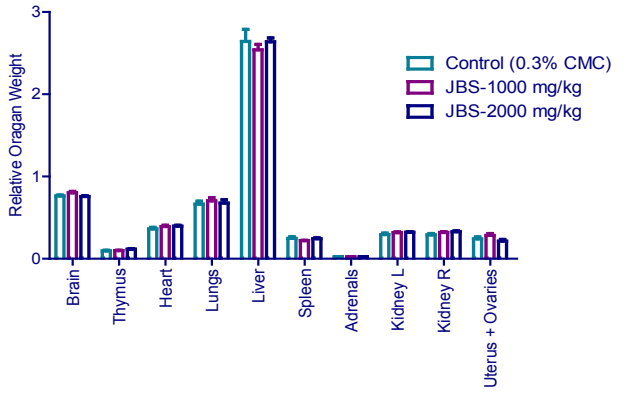


Figure 6. Relative organ weight of control and JBS treated female.

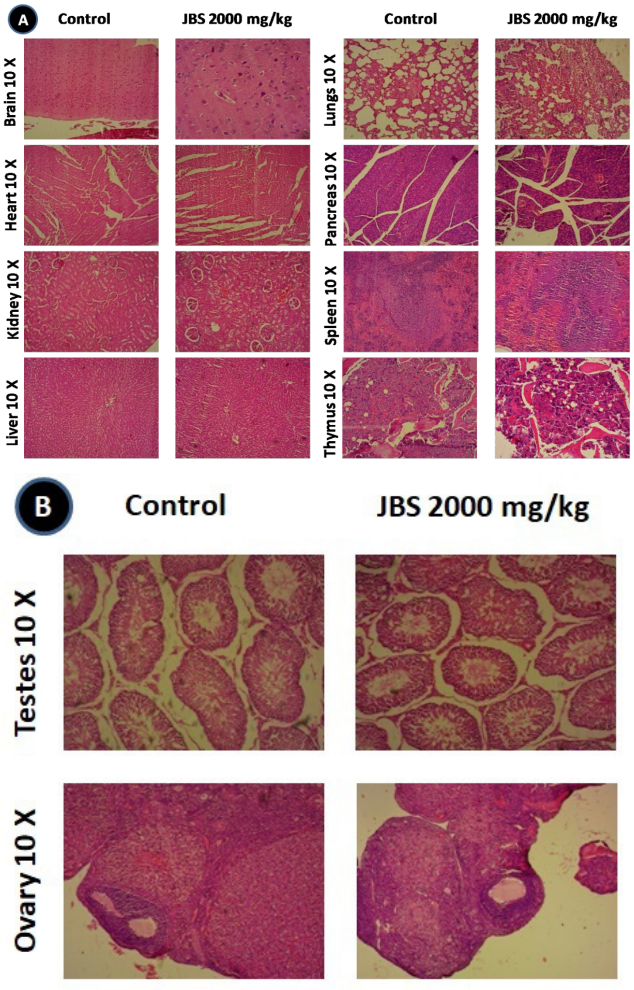


Figure 7 **(A) (B).** Histological images of different organs of control and JBS (2000 mg/kg bw) treated animals at 10X magnification.

4. Discussion

Although JBS is being used for decades for various indications by Unani physicians but scientific data regarding its safety on long-term usage is not available. Greatest challenge for scientific personnel involved in research on herbal medicine is to identify the toxic/adverse effect of herbal based pharmaceutical products. Inadequate standardization and contamination are the possible reason for herbal drug toxicity[7]. It is estimated that only about 10% of herbal products in the world market are truly standardized otherwise, the majority of these products are in clinical use with little knowledge about their active and /or toxic constituents[8]. Hence, the present study was conducted to evaluate the safety of JBS, a polyherbal Unani formulation, by performing 90 days repeated dose oral toxicity study in Sprague Dawley rats. No incidence of mortality and no clinical signs indicative systemic toxicity were observed during the study duration. The pattern of body weight gain measured weekly throughout 90 days was comparable to normal control group at all dose levels and showed consistent normal growth pattern. The feed consumption was measured weekly throughout study duration. Male rats treated with JBS at all tested dose levels showed no significant alteration in feed intake as compared to control group. Inconsistent difference was observed in feed consumption in female group receiving test drug compared to control group.

Haematopoietic system, especially the bone marrow where the production of red blood cell takes place is at major risk following administration of toxic substances[9,10]. Based on haematological assay, most of the values in JBS treated group were found normal in comparison to control except an increase in platelets count in female group at low dose level (1000 mg/kg bw) as compared to control group. The significant elevation of platelets count is a predisposing factor for hyper coagulating state and risk of spontaneous intravascular clotting and thromboembolism[11]. In present study significant increase in platelets count may not be considered toxicologically significant as the values were well within normal physiological range and similar changes were neither observed in high dose females nor in males at any dose.

The biochemical parameters are important tool to assess the integrity and functionality of organs. It is also

important to assess risk, pathological condition and general health status of body[12]. Based on biochemical results observed in present study, no significant alterations were observed with respect to different biochemical parameters except certain isolated/incidental changes. There was a significant decrease in blood glucose level only in female group at high dose (2000 mg/kg bw), however the values remained well within the normal physiological range and considered toxicologically insignificant. Albumin is the most abundant plasma proteins possessing physiological role in maintaining osmotic pressure and transportation of endogenous and exogenous substance. Reduction in albumin level is an indicator of liver dysfunction likely apparent in hepatitis and liver cirrhosis. In present study there was significant reduction in albumin level in drug treated female group at high dose level (2000 mg/kg bw) as compared to control animals. However, the reduction in albumin will be considered toxicologically insignificant as the observed values remains with normal range and similar change was not observed in any other JBS treated group. Additionally, reduction observed is dose independent and liver functions found normal in drug treated animals as compared to control. It was also observed that there was a significant increase in total protein and globulin level in high dose (2000 mg/kg bw) JBS treated female group as compared to control group. One of the possible causes for increase plasma total proteins is dehydration however; the assay of total proteins could not reveal clarity in reference to metabolic state of an individual[13]. Moreover, the rise in proteins levels in present study was found within normal physiological range. The effects observed were not dose dependent and could not be considered toxicologically significant. There was significant reduction in triglyceride level in drug treated female group at low dose (1000 mg/kg bw) as compared to control animals. However, alterations may not be considered toxicologically significant as the values were found to be within the normal physiological range and no other JBS treated group reflect such changes.

Evaluation of organ/body weight ratios is recommended by The Society of Toxicologic Pathology in toxicity studies lasting from 7 days to one year[14,15]. Relative organ weight data of JBS treated male and female rats at the end of 90 days dosing period were comparable with respective control groups except significant reduction in relative organ weight of spleen in male at both tested dose levels and left kidney in male group at low dose levels. Since no noteworthy findings were observed during gross necropsy in any organ/tissue in JBS treated or control animals. Moreover, in the absence of associated changes in the clinical pathology parameters such as RBC count (spleen), BUN and creatinine (renal function biomarkers)[16] and absence of histopathological changes, the alteration in relative organ weight may not be considered toxicologically significant.

Histopathological examination revealed abnormality in liver and lungs. Vascular congestion observed in hepatocytes which could possibly be due to inflammation, blockage and vasoconstrictor effect of JBS/Vehicle on the walls of blood vessel of hepatocytes. The pneumonitis in lungs may be attributed due to aspiration of drug /vehicle in lungs for repeated oral administration for 90 days. The presence of micro vacuole in hepatocytes may be due to abnormal infiltration of extracellular substance into the hepatocytes.

5. Conclusion

Based on the findings of present study following 90 days oral administration of JBS at doses 1000 and 2000 mg/kg bw/day, it can be concluded that JBS did not show any significant adverse effects on haematology, clinical biochemistry, body weight, feed intake, gross necropsy and histopathology. Therefore, No-observed-adverse-effect-level (NOAEL) may be considered greater than 2000 mg/kg body weight.

6. Acknowledgement

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7. Conflicts of Interests

The authors declare that they have no conflicts of interest.

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