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Original Research Article

Acute and Sub-Acute Oral Toxicity Studies of Hydroalcoholic Extract of *Terminalia arjuna* (Roxb.) Bark in Rodents

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ABSTRACT

Terminalia arjuna (T.arjuna) bark is extensively used by Indian herbal medicine practitioners for treating various cardiovascular ailments. Though multiple clinical trials and non-clinical studies have been reported on *T.arjuna bark*, the toxicology data was found to be inadequate. Hence, studies were conducted to establish the toxicology profile of *T.arjuna bark*. In the acute toxicity study, animals were administered with a single dose of 2000 mg/kg of *T. arjuna* bark hydro-alcoholic extract, whereas in repeated dose toxicity study animals were administered with the extract at doses of 0, 100, 500, and 1000 mg/kg, once daily, for 28 consecutive days, and on day 29, all the animals were humanely sacrificed after the collection of required blood samples. There were no mortality or clinical signs of toxicity observed after administration of a single dose of 2000 mg/kg of *T. arjuna* bark hydro-alcoholic extract. In the repeated dose toxicity study, there were no treatment-related adverse effects in any of the dose groups and control group animals in respect to clinical signs, body weight, feed consumption, haematology, clinical chemistry, urinalysis, and histopathology. Based on the results, LD_{50} of *T. arjuna* bark hydro-alcoholic extract is concluded as >2000 mg/kg. No-Observed-Adverse-effect Level of *T. arjuna* bark hydro-alcoholic extract from 28-day repeated dose toxicity study is established at 1000 mg/kg/day.

Keywords: *Terminalia arjuna* bark extract, LD_{50}, No-Observed-Adverse-effect Level, Rat.

Introduction

Many decades ago, herbal medicines were the only system that is used for treating human ailments. Approximately 25% of modern medicines are from plant sources. Currently, about 80% of people worldwide rely on herbal medicines. It is being advocated that herbal medicines are devoid of any adverse effects. However, studies reported that they are not completely free from toxicity or adverse effects. Herbal medical system lacks classification based on its toxicity, in the absence of sufficient basic research. It is to be noted that there is no adequate data about target organs, safe dose range, safety window of effective dose, and minimum toxic dose in respect to herbal medicine. Thus, specifying the toxic and adverse effects of each herbal medicine/medicinal herbs is a vital base to ensure the safe use of herbal medicine. [1]

Terminalia arjuna (Roxb.) Wight & Arn. (*T. arjuna*) is one of the most accepted and beneficial medicinal plants in the indigenous system of medicine for the treatment of various critical diseases. It belongs to the *Combretaceae* family comprising nearly 200 species distributed around the world. *T. arjuna* is an evergreen and deciduous tree that grows up to 25–30 m in height, distributed in India, Burma, Mauritius, and Sri Lanka. It is known by its various vernacular names, the most commonly used ones are Arjuna (Common Name), Arjun (Hindi), Marudhu (Tamil and Malayalam), Tella Maddi (Telugu), Arjhan (Bengali), Sadaru (Marathi), Sadado (Gujarati), Neer matti (Kannada). [2, 3]

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T. arjuna bark contains various triterpenoids, glycosides, flavonoids, phenolics, tannin, minerals, and trace elements. Triterpenoids include arjunin, arjunic acid, arjunenin, terminic acid, terminoltin, and arjunolic acid. Glycosides includes, arjunetin, arjunoside I, II, arjunolone, arjunolitin arjunaphthanoloside, arjunglucoside IV and V, arjunasides a-e, olean-3b, 22b-diol-12-en-28 b-d-glucopyranosie-oic acid, terminarjunoside I and II, terminoside a and termionic acid. Flavonoids and phenolics includes, arjunone, luteolin, baicalein, ethyl gallate, gallic acid, kempferol, oligomeric, proanthocyanidins, pelargonidin, quercetin, (þ)-catechin, (þ)-gallocatechin and (-)-epigallocatechin, ellagic acid and its derivatives such as 3-O-methyl-ellagic acid 4-O-b-dxylopyranoside, 3-O-methyl ellagic acid 3-O-rhamnoside, 3-O-methyl ellagic acid 40-O-a-L-rhamnophranoside, (-)-epicatechin. Tannins include, pyrocatechols, punicallin, castalagin, casuariin, casuarinin, punicalagin, terchebulin, and terflavin C. Minerals and trace elements includes, calcium, magnesium, aluminum, zinc, copper, silica.[4]

It has been reported that *T.arjuna* possesses multiple medicinal properties, such as antioxidant, antidiabetic, antimicrobial, cardioprotective, antiarthritic, antidiarrheal, anti-dysenteric, CVS and CNS stimulator, diuretic in cirrhosis of liver and strangury, abortifacient, and analgesic activity. It also has been reported to decrease the level of serum triglycerides and cholesterol, recover the level of high-density lipoproteins, act as an anti-ischemic agent, relieve myocardial necrosis, modulate platelet aggregation and act as an effective antioxidant. Ayurveda pharmacopeia of India recommends the powder of the stem bark in emaciation, chest diseases, lipid imbalances and polyuria, hypercholesterolemia, as well as in fungal and microbial infections, or as anti-fertility and antidote against poisons. *Terminalia arjuna*, traditionally has been used as a cardiotonic and cardioprotective, and has been designated for instability of three humours *viz*., *vata*, *pitta* and *kapha as per* Ayurveda. [5-9]

T.arjuna bark is used widely by herbal medicine practitioners for treating cardiovascular diseases. Few clinical trials and preclinical studies have already been reported, however, there are no kinds of literature available on repeated dose toxicology studies conducted in rats for the bark of *T.arjuna.* Hence, this study was designed to find out the greatest concentration, that causes no detectable adverse alteration

of morphology, functional capacity, growth, development, or life span of the target organism under defined conditions of exposure of *T. arjuna* bark hydro-alcoholic extract *i.e.,* No-Observed-Adverse-effect Level (NOAEL)and target organs of toxicity, if any.

Materials and Methods

T. arjuna bark was freshly collected from the campus of Oushadhi, Thrissur, Kerala, India in April 2017. The plant material was authenticated (Voucher specimen no. BSI/SRC/5/2023-24/Tech-482) by Dr. M. U. Sharief, Scientist F and Head of Office, Southern Regional Centre, Botanical Survey of India, T.N.A.U. Campus, Coimbatore, Tamil Nadu. The bark was cut into small pieces and sun-dried for approximately one month and minced before pulverizing, coarsely powdered bark was passed through a sieve of no.10 to obtain uniform particle size. The coarsely powdered bark was extracted using soxhlet apparatus with water and ethanol mixture in the proportion of 1:1 as a solvent. The extractive value of its hydro-alcoholic extract was found to be 22.52%.

Animals

Sprague-Dawley rats (6-8 weeks old, male and female) were obtained from the breeding facility of *in vivo* Biosciences (Bangalore, Karnataka). Experiments were performed following the committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines for laboratory animal facility, the gazette of India, 2018[10] Experimental procedures and protocol were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of Invivo Biosciences, (CPCSEA reg.no. 1165/PO/RcBit/S/08/ CPCSEA; Protocol proposal number 27/2017 (Acute toxicity study), protocol number 36/2017(28-day repeated dose toxicity study).

Animals were housed under standard laboratory conditions, air-conditioned with adequate fresh air supply (air changes 12-15 per hour), room temperature of 22 ± 3°C and relative humidity of 30-70 %, with 12 hours' light and 12 hours' dark cycle was maintained in the experimental room. A group of three animals was housed in standard polycarbonate cages with stainless steel mesh tops having facilities for holding pelleted feed and drinking water. Clean sterilized corn cob was provided as bedding material. Animals were fed with laboratory animal feed (*ad libitum*) throughout the experimental period. Animals were provided with *ad libitum* filtered drinking water passed through a water filter system (Aquaguard™) in autoclaved polypropylene bottles. Water bottles were changed daily.

Experimental design

Single-dose acute oral toxicity study

The main objective of conducting an acute toxicity study was to determine the LD_{50} of the *T.arjuna* extract *via* oral route. Acute toxicity of *T. arjuna* bark extract was evaluated following OECD (organisation for economic co-operation and development) test guideline 423 and Schedule Y guidelines.[11, 12] Based on OECD 423 guidelines, the substance is tested for its acute toxicity, using a stepwise procedure, each step using three animals of single sex. The absence or presence of compound-related mortality of the animals dosed at one step will determine the next step. As per the guideline, the starting dose level should be that which is most likely to produce mortality in some of the dosed animals, however, considering its widespread use, 2000 mg/kg is selected as the initial dose following the OECD test guideline 423 Annex 2d.[11]

After the acclimatization period of five days, three female rats were administered orally with 2000 mg/kg of *T. arjuna* bark extract suspended in 0.5% carboxymethylcellulose in distilled water as a single dose using oral gavage. Animals were observed for mortality, morbidity, and clinical signs of toxicity for 14 days, results of the first step of the test were confirmed using another set of 3 female rats.

28-day repeated dose toxicity study

The purpose of this study was to find NOAEL *T. arjuna* bark hydro-alcoholic extract and target organs, if any. A repeated dose toxicity study of twenty-eight days was performed following OECD Guideline (Test No. 407) and Schedule 'Y' of Drugs & Cosmetics Appendix III, Repeated-dose Systemic Toxicity Studies.[13,14] Forty-eight animals (24 Males and 24 Females) were acclimatized for five days and were randomly allocated to four groups of six animals, based on the body weight. Test animals received *T.arjuna* bark extract formulations orally once daily for 28 consecutive days.

Selection of dose levels and justification

In the case of repeated-dose toxicity studies, dose selection is paramount, therefore an extensive literature search was performed for finding an appropriate maximum daily human dose. We found many published literature on clinical studies using *T.arjuna* bark powder, with doses ranging from 500 mg to 5 grams.[15-22] Based on the literature available, 5 grams/day of bark powder is considered as the maximum daily human dose of *T. arjuna* bark extract for deriving human equivalent animal dose. In the study, low dose is the human equivalent rat dose (100 mg/kg), mid-dose is 5 times of low dose (500 mg/kg), and the high dose is 10 times of low dose (1000 mg/kg), human equivalent rat dose is calculated as below:

MDD (Maximum Daily Dose) of *T.arjuna* bark powder = 5000 mg per day

MDD of *T. arjuna* extract based on extractive value = 5000 mg × 22.52% (extractive value)

= 1125mg per day;

i.e., $= \frac{1125 \text{ mg/ day}}{60\text{kg (adult human bodyweight)}} = 18.75$ mg/kg/day

Human equivalent rat dose = 18.75 mg/kg/day × 6.17 (Rat conversion factor[23] = 115.69 mg/kg/day

Dose formulation preparation and administration

Three different dose formulations at a concentration of 10, 50, and 100 mg/ml were prepared using 0.5% carboxymethylcellulose in distilled water, daily before dosing. The required quantity of *T. arjuna* bark extract was weighed separately in a dry mortar and then the vehicle was added drop-wise and triturated with the help of a pestle to make a homogenous suspension. The resultant suspensions were then transferred into a beaker and the final volume was made up with a vehicle to get the final concentration. The resultant suspension was stirred using the magnetic stirrer during administration. The maximum volume of administration used was 10 mL/ kg animal body weight.

Parameters

Clinical signs and mortality

All the animals were observed twice daily for mortality and morbidity. Routine observations for checking general clinical signs were performed twice a day (pre-dose and post-dose) during treatment days.

Detailed clinical examination

Detailed clinical examination was done before the administration of *T.arjuna* extract on Day 1 and at weekly intervals thereafter, on Days 7, 14, 21, 28 of the treatment period. During detailed clinical examination, all rats were observed for changes in the skin, fur, eyes, mucous membranes, the occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern), changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypes (e.g., excessive grooming, repetitive circling and bizarre behavior (e.g. self-mutilation, walking backward). On days of detailed clinical examination, these observations replaced the daily clinical sign (post-dose) observations except for Day 1.

Table 1: Details of grouping

Group	Dosage
Group 1	Vehicle control (0 mg/kg p.o., *T.arjuna extract)* for 28 days
Group 2	Low dose (100 mg/kg p.o., *T.arjuna extract*) for 28 days
Group 3	Mid dose (500 mg/kg p.o., *T.arjuna extract*) for 28 days
Group 4	High dose (1000 mg/kg p.o., *T.arjuna extract*) for 28 days

Neurological examination
The following neurological examination was conducted during week 4 of the treatment period for all the animals.

Home cage observations
Each animal was observed in the home cage for posture and presence of abnormal vocalizations, tremors, and convulsions.

Open field observation
The rat was placed (one at a time) in an open arena, on a flat surface placed with a clean absorbent paper and observed for 2 minutes. The absorbent paper was replaced for each group. During this observation period, the rat was evaluated as it moves about freely/unperturbed and the observations (eg, gait, posture, tremors, mobility score, arousal level, clonic or tonic movements, stereotypic behaviour, bizarre behaviour, abnormal vocalizations) were made and recorded..

Sensory reactivity measurements
After the 2 minutes of the observation period, while the rat is in the open field arena, the rat could move freely in the open field box for these tests. During sensory reactivity measurements, rats were observed for the approach response, touch response, click response, tail-pinch response, pupil response, aerial righting reflex, and all the observations were recorded using scores/ranks.

Bodyweight and feed consumption
Body weights were recorded individually for all animals on acclimatization day 1, on randomization day, the start of dosing, and thereafter, at weekly intervals and at the time of necropsy. The measured weekly body weights were used to optimize and adjust the individual doses each week. Feed consumption was calculated weekly from the first day of treatment until sacrifice. Feed input and leftovers were measured once weekly for each group.

Clinical biochemistry and haematology
Blood samples were collected from all animals on 29th day of the experiment. Animals were fasted overnight before blood sample collection. Blood samples were collected by puncturing the retro-orbital plexus with the help of a fine capillary tube. A sample of 0.5 mL of blood was collected into tubes containing 10 µL of 10% EDTA solution for haematology tests includes Complete Blood Count (CBC) analysis, using the automated blood cell count analyzer.[24] Approximately 2 mL of blood was collected in tubes containing heparin sodium (20 IU/mL) for clinical chemistry analysis. The plasma was separated by centrifugation at 4000 rpm for 8 minutes at 4 ºC, stored at -20 ºC (± 2 ºC) and used for clinical chemistry analysis. Urine samples were collected under fasting conditions (with *ad libitum* water) using metabolic cages to evaluate appearance, color, volume, specific gravity, and pH.

Pathology
All animals were euthanized by CO_2 asphyxiation and subjected to necropsy followed by gross pathology on 29th day of the experiment. Histopathological observations were performed in control and high dose groups. Viscera was collected from the high-dose test and the control group of animals. The wet weight of the collected organs was noticed after trimming the adherent tissues and paired organs were weighted together.
For histopathological evaluation, fixed tissues were dehydrated in ascending ethanol, cleared in xylene, and embedded in paraffin. Serial sections were trimmed to a thickness of 10-20µ and later to 3-6µ to obtain thin tissue sections using a rotary microtome. The sections were mounted on 3-aminopropyl triethoxysilane coated slides and dried for 24 h at 37^{0}C. The sections on the slides were deparaffinised with xylene and hydrated in a descending series of alcohol. They were thereafter stained with Mayer's haematoxylin and eosin dyes, dried and mounted on a light microscope for histopathological examination. [25]

Statistical analysis
The results of the 28-day repeated dose toxicity study *viz.*, body weight, feed consumption, organ weight, clinical chemistry, haematology and urinalysis parameters were analyzed using one-way ANOVA followed by Dunnett's t-test using GraphPad Prism version 6.0. Each group mean was presented with the Standard Deviation (SD) and the number of animals/observations (N) used to calculate the mean. The "p" value $\leq$ 0.05 was considered statistically significant. Males and females were considered separately for analysis. G1 group (vehicle control) was compared with G2 (100mg/kg), G3 (500mg/kg), and G4 (1000mg/kg) groups.

Results and Discussion

Terminalia arjuna is an ayurvedic remedy for heart diseases, it has been mentioned since the vedic period in many ancient Indian medicinal texts including *charaka saṃhitā, sushruta saṃhitā* and *ashtānga hridayam*.[26] Many studies have been conducted in *T.arjuna*, however, no sufficient safety data is reported for repeated dose toxicological tests in animal models as per standard regulatory guidelines. We, therefore, evaluated the safety of hydroalcoholic extract of *T.arjuna* bark in acute oral toxicity study followed by 28 -repeated dose toxicity study in rats.

Acute oral toxicity study in rats
In the first step of the acute oral toxicity study, a set of three female animals were administered with a single dose of 2000 mg/kg of *T.arjuna* extract. There were no mortality or clinical signs of toxicity observed during the study duration of 14 days. And the result was confirmed by administering the same dose (2000 mg/kg) to another set of 3 female rats. Based on the result, Oral LD_{50} cutoff value of the *T. arjuna* hydroalcoholic extract was concluded as >2000 mg/kg in rats.

Repeated dose toxicity study in rats
Major target organs of toxicity are usually found to be the liver, kidney, and blood, as they are involved in the metabolism or excretion of xenobiotics. The parameters such as AST, ALT, ALP, hematological parameters, urinalysis parameters, histopathology, bodyweight, feed consumption, and clinical signs will help to assess the severity of the toxicity of the test formulation to the biological systems. The high dose selected for the repeated dose toxicity study in rats has covered 10 times the human maximum daily dose (MDD). Hence, we believe that the results of this study can help to establish a sufficient margin of safety for the extract.

Clinical signs, morbidity and mortality
There were no clinical signs of toxicity, morbidity and mortality observed during the home cage observation in any of the dose levels tested.

Detailed clinical examination
There were no clinical signs of toxicity observed during the detailed clinical examination in any of the dose levels tested (Table 2 and 3).

Table 2: Summary of clinical signs (daily observation)

	Male				**Female**			
From Day 1 to Day 29	**G1**	**G2**	**G3**	**G4**	**G1**	**G2**	**G3**	**G4**
	0 mg/kg	**100 mg/kg**	**500 mg/kg**	**1000 mg/kg**	**0 mg/kg**	**100 mg/kg**	**500 mg/kg**	**1000 mg/kg**
Normal	6	6	6	6	6	6	6	6

The number of rats in each group was 12 (6 male and 6 female). G1-vehicle control; G2-Low dose, G3-Mid dose; G4-High dose.

Table 3: Summary of neurological examinations

Parameter	Group	G1	G2	G3	G4
	Dose mg/kg	0	100	500	1000
	No of rats	12*	12*	12*	12*
Home Cage Observations					
Posture					
Sitting normally, feet tucked, Sitting or Standing alert, watching		12	12	12	12
Abnormal Vocalizations					
Absent		12	12	12	12
Tremors					
Absent		12	12	12	12
Convulsions - Clonic Movement					
Absent		12	12	12	12
Convulsions - Tonic Movement					
Absent		12	12	12	12
Handling Observations Ease of Removing from Cage					
Easy (minimally avoids capture but is not aggressive)		12	12	12	12
Handling Reactivity					
Moderately low (slight resistance)		12	12	12	12
Palpebral Closure					
Eyelids wide open i.e., Normal		12	12	12	12
Piloerection					
Absent		12	12	12	12
Lacrimation					
Absent		12	12	12	12
Salivation					
Absent		12	12	12	12
Stereotypic Behavior					
Absent		12	12	12	12
Bizarre Behavior					
Absent		12	12	12	12
Abnormal Vocalization					
Absent		12	12	12	12
Sensory Reactivity Measurements					
Approach Response					
Normal response (approaches		12	12	12	12
Touch response					
Normal Response (spontaneously lifts tail when touched)		12	12	12	12
Click Response					
Normal Response (clear movements of the head, neck and ears)		12	12	12	12
Tail-Pinch Response					
Normal Response (Flinches, moves rapidly and/or vocalizes)		12	12	12	12
Pupil Response					
Normal		12	12	12	12
Aerial Righting Reflex					
Normal		12	12	12	12

*6 male + 6 female. Values represents the number of animals affected

Table 4: Summary of weekly average bodyweight (g)

Male					
Day→	1[#]	**8**	**15**	**22**	**29[##]**
G1	142.98 ± 11.17	184.14 ± 14.56	229.36 ± 13.29	256.69 ± 14.67	276.61 ± 14.25
G2	136.18 ± 11.3	183.65 ± 13.85	227.57 ± 14.28	257.42 ± 16.6	279.25 ± 13.83
G3	138.4 ± 9.04	187.35 ± 15.54	229.97 ± 17.53	261.08 ± 13.04	279.7 ± 15.83
G4	139.25 ± 5.17	186.56 ± 14.36	229.45 ± 15.98	263.89 ± 13.69	283.3 ± 14.91
Female					
Day→	**1[#]**	**8**	**15**	**22**	**29[##]**
G1	121.38 ± 6.82	138.87 ± 7.09	157.07 ± 5.45	172.81 ± 5.48	183.08 ± 8.37
G2	122.64 ± 7.49	143.31 ± 7.37	160.05 ± 7.01	173.5 ± 5.45	182.61 ± 7.69
G3	119.7 ± 6.8	141.04 ± 7.17	158.12 ± 8.19	172.7 ± 7.43	183.41 ± 5.97
G4	123.24 ± 7.56	142.63 ± 6.66	158.96 ± 5.16	172.42 ± 6.03	184.16 ± 6.17

The number of rats in each group was 12 (6 male and 6 female). The results were mean ± SD. The "p" value ≤ 0.05 was considered as significant, statistical test using one-way ANOVA followed by Dunnett's t-test. [#]first day of dosing,[##]day of necropsy. SD - Standard deviation.

Effect on Body weight, body weight changes and feed consumption

Measures of animal growth are routinely evaluated in toxicology studies and are key to the interpretation of compound-related effects. The major parameter for evaluating the growth includes body weight gain from initial body weight, daily feed consumption, etc. As feed consumption may be affected by the animal's body weight, comparing daily feed consumption among treatment groups without accounting for the bodyweight differential may be misleading. Therefore, compound-related effects on feed consumption are evaluated by calculating relative daily feed consumption, which is defined as daily feed consumption divided by average body weight.[27, 28] In this study, to decrease the bias, randomization by bodyweight stratification at the beginning of the study was performed before allocation to the experimental groups. Body weight changes in both sexes were not significant ($p > 0.05$) at any of the dose levels (0, 100, 500, and 1000 mg/kg) tested compared to control group animals. Mean body weight and body weight changes of *T. arjuna* hydroalcoholic extract treated animals were comparable with vehicle control group (Table 4).

Feed consumption in rats was found to be normal for both sexes for the entire duration of the study with no significant changes ($p > 0.05$) compared to control groups (Table 5).

However, a marginal increase in feed consumption and body weight was noticed in dose groups compared to vehicle control groups in both sexes, yet this increase in body weight was not related to the toxicity of *Terminalia arjuna* hydroalcoholic extract.

Effect on haematology parameters

Assessment of haematological indices can be diagnostic of adverse effects of foreign compounds on the blood constituents since such haematological alterations have higher predictive value for human toxicity when the data are interpreted from animal studies. [29] In this study, there was a marginal increase in haemoglobin and platelet count in male animals across the dose groups compared to the vehicle control group. And a marginal increase in RBC count and platelet in female animals was observed across the dose groups compared to vehicle control groups. These changes did not show statistical significance ($p > 0.05$), besides, there was no histopathological correlation. Hence, these changes were considered incidental, and not related to the toxicity of the extract. The haematological and coagulation parameters did not reveal treatment-related effect in any of the dose levels tested (Table 6).

Effect on clinical chemistry parameters

Changes in clinical pathology parameters are then assessed for changes in the concurrent data sets such as clinical signs and anatomic pathology to determine the underlying pathophysiology. These parameters generally indicate a response to a change in a particular tissue or metabolic pathway, they often correlate with structural changes that are detected by light microscopy and are less likely to correlate with in-life changes.[30] In the current study, the male animals showed a marginal decrease in ALT and AST levels, a marginal increase in urea level, and blood urea nitrogen across groups. Female animals showed a marginal decrease in ALP levels in all the dose groups, a slight increase in total protein, creatinine, and bilirubin level. AST and ALT are enzymes found mainly in the liver, but also found in red blood cells, heart cells, muscle tissue, and other organs, such as the pancreas and kidneys. AST or ALT levels are a valuable aid primarily in the diagnosis of liver damage. When body tissue or an organ such as the liver or heart is diseased or damaged, additional AST and ALT are released into the bloodstream, causing levels of the enzyme to rise. Therefore, the amount of AST and ALT in the blood is directly related to the extent of the tissue damage. After severe damage, AST levels rise 10 to 20 times and greater than normal, whereas ALT can reach higher levels (up to 50 times greater than normal).[30, 31] However, in this study, there were no increase in the levels of these parameters, and histopathological changes in both sexes. Hence, these changes are not related to the toxicity of the extract.

Bilirubin is an important metabolite of heme (ferroprotoporphyrin IX), a coordination complex that serves to coordinate iron in various proteins. It is a potentially toxic substance. However, the body has developed mechanisms for its safe detoxification and disposition.[32] An increase in bilirubin level is a sign of hepatic damage. The total protein test measures the total amount of albumin and globulin, a decrease in the levels is caused in the case of liver or kidney disease. Nonetheless, the change in bilirubin and protein levels in this study were marginal and considered incidental, which are not related to the toxicity of the extract.

Urea is the final metabolite of protein nitrogen balance, the measurement of which allows the evaluation of the generic metabolism of proteins and amino acids through the urea cycle, which is exclusively hepatic. Once in the blood, urea is excreted primarily by the kidneys. After this glomerular filtration, between 40% and 60% is reabsorbed at the tubular level, constituting a marker of renal function. Creatinine is an excretion product of muscle activity, which circulates in the blood. Its elimination is exclusively renal, so there is a correlation between creatinine levels and renal function. Most creatinine that is eliminated by the kidneys is freely filtered in renal glomeruli, and a small fraction is filtered by the tubular component, which is a good indicator of renal-glomerular function. BUN is the amount of nitrogen that circulates in the form of urea through the bloodstream. In healthy animals, urea is filtered from plasma by the renal glomerulus. It returns to the blood through renal tubules, but most of it is excreted through urine. If the kidney is not functioning properly, then sufficient urea cannot be removed from plasma, leading to higher BUN levels.[33] However, the marginal increase in the levels of urea, creatinine, and BUN are considered incidental, which is not related to the toxicity of the extract. Hence it can be concluded that the clinical chemistry parameters did not reveal treatment-related effect in any of the dose levels tested (Table 7). Further, no treatment-related effect was observed in urine analysis parameters like appearance, colour, volume, specific gravity, and pH either in the group mean values or in the incidence of semi-quantitative observations made across different dose groups ($p > 0.05$) (Table 8).

Effect on Organ weights

The group mean values of the absolute weights of organs such as brain, heart, liver, kidneys, adrenals, testis/ovaries of all groups did not reveal any treatment-related effect in any of the dose levels tested ($p > 0.05$) (Table 9).

Table 5: Summary of average daily feed consumption (g/rat/cage/day)

Male				
Week→	**Week 1 (Day 8)**	**Week 2 (Day 15)**	**Week 3 (Day 22)**	**Week 4 (Day 29)**
G1	16.8 ± 0.74	17.29 ± 0.45	18.2 ± 0.23	18.57 ± 0.4
G2	17.33 ± 0.62	17.45 ± 0.29	18.4 ± 0.66	18.4 ± 0.66
G3	18.14 ± 0.31	17.28 ± 0.91	18.48 ± 0.54	17.77 ± 0.72
G4	16.68 ± 0.28	16.98 ± 0.81	19.35 ± 0.94	19.02 ± 0.53
Female				
G1	10.88 ± 0.51	12.68 ± 0.61	12.62 ± 0.47	12.88 ± 0.94
G2	11.06 ± 0.92	13.23 ± 0.43	12.47 ± 0.48	13.62 ± 0.56

G3	11.29 ± 0.64	13.62 ± 0.41	12.68 ± 0.22	13.61 ± 0.41
G4	11.28 ± 0.62	13.28 ± 0.64	13.5 ± 0.51	14.00 ± 0.97

The number of rats in each group was 12 (6 male and 6 female). The results were mean ± SD. The "p" value ≤ 0.05 was considered as significant, statistical test using one-way ANOVA followed by Dunnett's t-test. SD - Standard deviation.

Table 6: Summary of haematology parameters

Male				
Group	**RBC (10^6/ µl)**	**WBC (10^3/ µl)**	**Hb (g/dl)**	**Pt (10^3/ µl)**
G1	9.52 ± 0.34	10.33 ± 0.49	17.28 ± 0.24	1047.67 ± 114.01
G2	8.74 ± 0.28	11.28 ± 3.5	18.38 ± 0.2	1063.25 ± 103.14
G3	9.12 ± 0.41	9.28 ± 1.19	18.61 ± 0.42	1135.1 ± 127.16
G4	9.77 ± 0.14	10.72 ± 0.95	18.30 ± 0.18	1050.75 ± 97.28
Female				
Group	**RBC (106/ µl)**	**WBC (103/ µl)**	**Hb (g/dl)**	**Pt (103/ µl)**
G1	7.73 ± 0.81	7.35 ± 1.14	17.12 ± 0.29	1034.07 ± 140.87
G2	8.92 ± 0.43	7.69 ± 0.77	17.17 ± 0.36	1172.92 ± 113.82
G3	8.92 ± 0.20	7.47 ± 1.09	16.93 ± 0.58	1004.69 ± 120.06
G4	8.78 ± 0.21	7.27 ± 1.57	17.06 ± 0.49	1101.25 ± 176.46

The number of rats in each group was 12 (6 male and 6 female). The results were mean ± SD. The "p" value ≤ 0.05 was considered as significant, statistical test using one-way ANOVA followed by Dunnett's t-test. SD - Standard deviation. RBC- red blood cell count, WBC-white blood cell count, Hb-Hemoglobin, Pt-Platelet.

Gross pathology and histopathology findings

There were no treatment-related gross or histopathological lesions observed in the organs examined such as brain, heart, kidney, liver, spleen, stomach, intestine, ovary, uterus, epididymis, and testis across 100mg/kg and 1000mg/kg groups. (Figure 1)

In summary, there were no test item-related adverse effects noted in feed consumption and body weight. The changes AST, ALT, ALP were found to be not significant when compared to control group animals. Also, there were no significant changes in parameters like urea, blood urea nitrogen, and creatinine, and urine analysis parameters, which reveals that treatment with *T. arjuna* bark extract is not altering hepatic and kidney functions concerning tested parameters. Haematology parameters also did not reveal any kind of adverse conditions and changes were not significant compared to control group animals. Histopathology of various organs *viz.*, brain, heart, liver, kidney, spleen, stomach, intestine, ovary, uterus, epididymis, and testis did not reveal any treatment-related lesions. As there were no adverse effects in any of the tested parameters detected in any of the dose levels, NOAEL of *T. arjuna* bark extract in rats is considered as the highest dose level tested in the study i.e., 1000 mg/kg/day.

Conclusion

From the acute toxicity results, it is concluded that oral LD_{50} of hydroalcoholic extract of *T. arjuna* bark is >2000 mg/kg in rats. Repeated dose toxicity study of *T. arjuna* bark extract did not exhibit any mortality/morbidity or untoward clinical signs at any of the dose levels. The clinical chemistry, haematology, urinalysis, and histopathology did not reveal any toxicity in any of the dose levels tested. Hence, based on the results of this study NOAEL of *T. arjuna* bark extract in rats is 1000 mg/kg/day.

However, considering the long-term use of *T. arjuna* bark in a preventive or curative aspect, a chronic toxicity study would be required to further establish its safety for prolonged use.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 7: Summary of clinical chemistry parameters

Male					
Group	**Albumin (g/dl)**	**ALT (IU/l)**	**AST (IU/l)**	**ALP (IU/l)**	**Bilirubin (mg/dl)**
G1	4.03 ± 0.29	62.09 ± 14.34	103.98 ± 17.06	125.12 ± 21.81	0.13 ± 0.02
G2	4.17 ± 0.12	59.72 ± 13.6	99.32 ± 15.44	121.77 ± 21.74	0.12 ± 0.02
G3	4.13 ± 0.17	57.17 ± 9.72	102.79 ± 13.75	127.32 ± 15.52	0.12 ± 0.01
G4	4.08 ± 0.17	58.08 ± 8.74	96.96 ± 10.63	118.3 ± 15.86	0.13 ± 0.02
Group	**TP (g/dl)**	**Urea (mg/dl)**	**BUN (mg/dl)**	**Creatinine (mg/dl)**	**Glucose (mg/dl)**
G1	7.16 ± 0.30	31.48 ± 2.44	14.4 ± 1.03	0.53 ± 0.02	129.77 ± 14.30
G2	7.02 ± 0.24	31.91 ± 2.35	14.15 ± 0.95	0.57 ± 0.02	142.45 ± 7.36

G3	7.1 ± 0.39	33.59 ± 2.8	14.2 ± 1.74	0.54 ± 0.03	138.22 ± 9.66
G4	7.09 ± 0.37	32.32 ± 2.62	15.25 ± 0.55	0.56 ± 0.03	140.26 ± 7.92
Female					
Group	**Albumin (g/dl)**	**ALT (IU/l)**	**AST (IU/l)**	**ALP (IU/l)**	**Bilirubin (mg/dl)**
G1	4.37 ± 0.12	38.89 ± 9.83	81.02 ± 9.73	89.58 ± 6.04	0.12 ± 0.03
G2	4.19 ± 0.05	37.33 ± 6.45	83.5 ± 11.06	75.43 ± 12.31	0.11 ± 0.02
G3	4.36 ± 0.12	40.74 ± 6.12	85.9 ± 8.80	81.35 ± 9.41	0.13 ± 0.01
G4	4.29 ± 0.12	37.04 ± 4.67	82.59 ± 11.37	84.59 ± 8.61	0.13 ± 0.02
Group	**TP(g/dl)**	**Urea(mg/dl)**	**BUN(mg/dl)**	**Creatinine (mg/dl)**	**Glucose (mg/dl)**
G1	6.49 ± 0.11	31.8 ± 3.22	14.28 ± 1.83	0.49 ± 0.03	118.42 ± 4.62
G2	6.75 ± 0.19	29.35 ± 2.82	12.66 ± 0.75	0.50 ± 0.03	121.12 ± 5.67
G3	6.56 ± 0.19	30.5 ± 3.11	13.69 ± 1.24	0.51 ± 0.02	118.64 ± 7.38
G4	6.66 ± 0.12	29.03 ± 3.64	14.06 ± 0.64	0.50 ± 0.03	124.46 ± 8.21

The number of rats in each group was 12 (6 male and 6 female). The results were mean ± SD. The "p" value ≤ 0.05 was considered as significant, statistical test using one-way ANOVA followed by Dunnett's t-test. SD - Standard deviation. ALT- Alanine aminotransferase; AST- Aspartate aminotransferase; ALP- Alkaline phosphatase; TP-total protein; BUN-blood urea nitrogen.

Table 8: Summary of urine analysis parameters

Male					
	Appearance	**Colour**	**Volume (ml)**	**Specific Gravity**	**pH**
G1	Clear	Pale Yellow	8.63 ± 1.68	1.02 ± 0.01	7.11 ± 0.37
G2	Clear	Pale Yellow	8.31 ± 1.87	1.013 ± 0.01	6.72 ± 0.65
G3	Clear	Pale Yellow	7.28 ± 2.41	1.014 ± 0.01	6.76 ± 0.41
G4	Clear	Pale Yellow	9.32 ± 2.70	1.023 ± 0.01	7.14 ± 0.38
Female					
	Appearance	**Colour**	**Volume (ml)**	**Specific Gravity**	**pH**
G1	Clear	Pale Yellow	10.44 ± 3.18	0.988 ± 0.01	6.84 ± 0.86
G2	Clear	Pale Yellow	9.97 ± 4.29	0.954 ± 0.02	6.66 ± 0.55
G3	Clear	Pale Yellow	10.85 ± 3.43	0.957 ± 0.02	6.83 ± 0.44
G4	Clear	Pale Yellow	9.44 ± 2.02	0.944 ± 0.01	6.77 ± 0.43

The number of rats in each group was 12 (6 male and 6 female). The results were mean ± SD. The "p" value ≤ 0.05 was considered as significant, statistical test using one-way ANOVA followed by Dunnett's t-test. SD - Standard deviation.

Table 9: Summary of average absolute organ weights (g)

Male						
Group	**Brain**	**Heart**	**Liver**	**Kidneys**	**Adrenals**	**Testis**
G1	1.945 ± 0.074	1.434 ± 0.052	11.271 ± 0.096	2.726 ± 0.089	0.05 ± 0.003	3.385 ± 0.251
G2	1.987 ± 0.082	1.402 ± 0.125	12.03 ± 0.298	2.683 ± 0.426	0.045 ± 0.006	3.226 ± 0.322
G3	2.036 ± 0.057	1.417 ± 0.100	11.1 ± 0.456	2.695 ± 0.290	0.05 ± 0.006	3.412 ± 0.410
G4	1.913 ± 0.051	1.436 ± 0.085	11.084 ± 0.665	2.618 ± 0.296	0.049 ± 0.007	3.366 ± 0.379
Female						
Group	**Brain**	**Heart**	**Liver**	**Kidneys**	**Adrenals**	**Ovaries**
G1	1.761 ± 0.065	0.974 ± 0.036	6.594 ± 0.246	1.745 ± 0.043	0.064 ± 0.003	0.912 ± 0.016
G2	1.777 ± 0.059	0.952 ± 0.043	6.078 ± 0.514	1.753 ± 0.086	0.064 ± 0.008	0.886 ± 0.007
G3	1.78 ± 0.037	0.965 ± 0.030	6.133 ± 0.525	1.846 ± 0.170	0.059 ± 0.004	0.878 ± 0.050
G4	1.788 ± 0.050	0.969 ± 0.027	5.97 ± 0.624	1.7 ± 0.133	0.06 ± 0.004	0.915 ± 0.028

The number of rats in each group was 12 (6 male and 6 female). The results were mean ± SD. The "p" value ≤ 0.05 was considered as significant, statistical test using one-way ANOVA followed by Dunnett's t-test. SD - Standard deviation

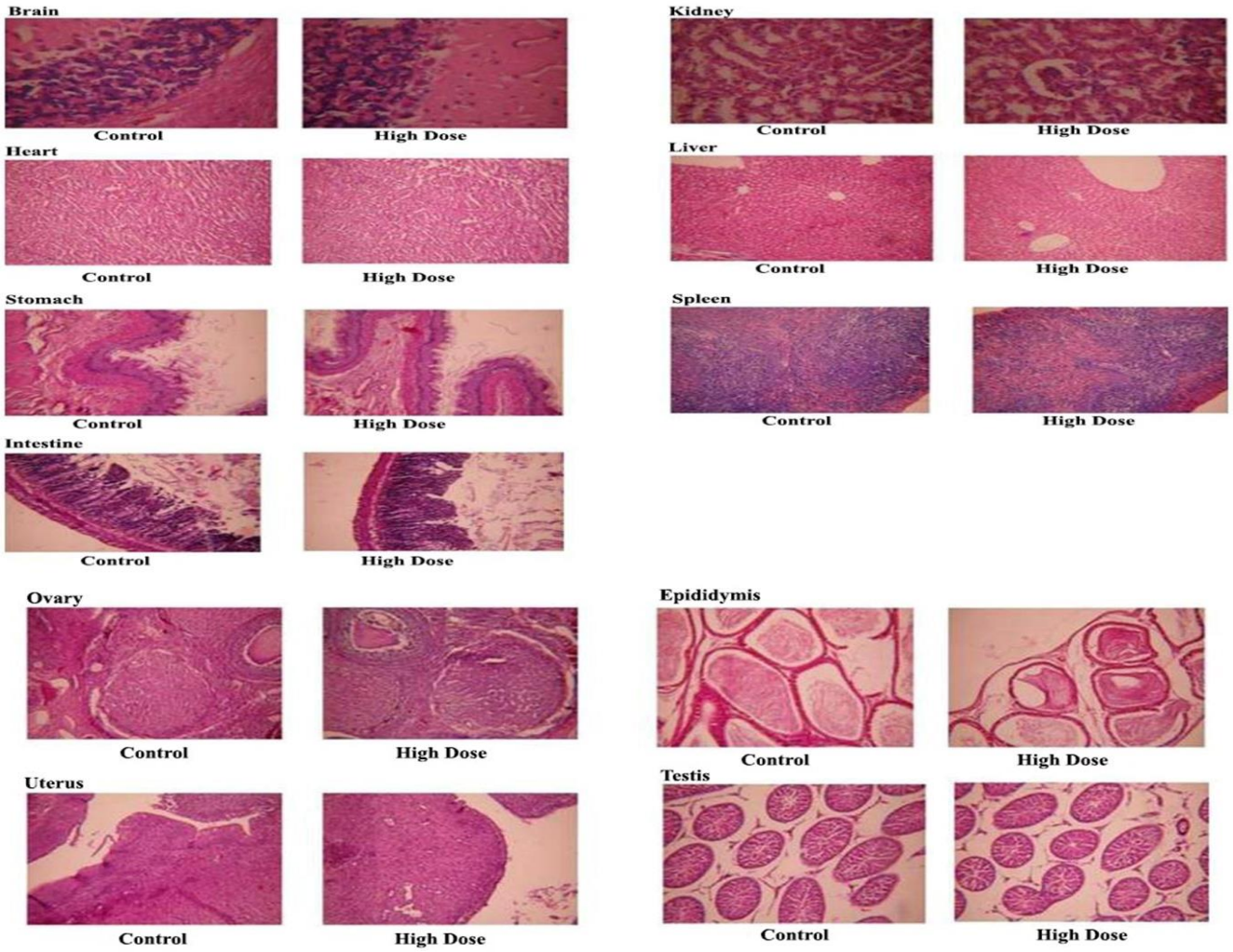

Figure 1: Photomicrograph of tissues (brain, heart, kidney, liver, spleen, stomach, intestine, ovary, uterus, epididymis, and testis) of control and high dose (1000 mg/kg) groups.

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