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Research Article

Toxicology for Safe Therapy

Sub-acute Toxicity Study and Toxicity Reversibility of Ayabirungarajakarpamin Wistar Albino Rats

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Abstract: Toxicological screening is a paramount need for developing new drugs or providing a safety profile for existing traditional medicine. Metals and minerals that are transformed into drugs must have excellent therapeutic efficacy and safety. It is essential to evaluate the margin of safety between the dose level that exerts the therapeutic effect and adverse effect to provide benefit to risk assessment. The present study aims to establish the safety of the Ayabrungaraja karpam (ABK), a herbometallic Siddha drug by 28 days repeated dose sub-acute toxicity study. In this study, 70 healthy young wistar albino rats (Rattusnorvegicus Sp.) of both sex between 8-12 weeks of age and weighing 150 -175g were studied. Among them 30 rats were divided into 3 groups of n=10 and administered with ABK at the dose of 250, 500, and 1000 mg/kg body weight respectively. The control group n=10, vehicle control group n=10, satellite vehicle control n=10, satellite high dose group n=10 received distilled water and lime juice and ABK at the dose of 1000mg/kg b.w respectively. Satellite group was pre set to determine the reversibility or recovery from toxic effect. All the groups received same water and food. No observation made on behavioural changes. All the wistar albino rats were alive until the study ended. Prominent pathological changes were not observed during 28 days repeated dose and reversal toxicity study in haematological, biochemical and histopathological investigations in both sexes of wistar albino rats treated with Ayabhringraja Karpam. Ayabhringraja Karpam in different concentrations was found to be completely safe and non-toxic under sub-acute toxicity studies.It is recommended to do further study on subchronic or chronic toxicity study.

Keywords: Mortality, Animals, Toxicology, Screening, Adverse effect, Safety

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1. INTRODUCTION

Toxicological screening is a paramount need for the development of new drugs or to provide a safety profile for existing traditional systems of medicine. The US Food and Drug Administration (FDA) states animal experimentation is the only means through which this evaluation can be made to screen the new molecule for pharmacological activity or toxicity potential.[1] Advances in medical technology have encouraged the development and novel use of biological resources locally and internationally in drug development.[2] Although natural products are popular with some rural areas, natural substances are potentially toxic and may be harmful to human health. Therefore, systematic safety studies are essential for compounds that are natural-based medicines.[3,4] Many people depend on traditional medicine usage which is an important role of peoples' culture and their tradition.[5] Since ancient times, herbs were widely used as the main treatment strategy for treating diseases [6]. Medicinal plants have shown promising potential therapeutic effects of high global demand, but even the concerns about drug efficacy and safety still being considered [7].People believe herbal drugs are very safe and devoid of adverse effects which is not true but also misleading. However, researches have indicated that the most medicinal plants show adverse effects. [8] In Siddha Medicine, the metals and minerals are very often used especially in the form of *parpam and Chenduram* preparations which are always under attentive observations since they contain highly toxic inorganic elements such as lead, mercury, arsenic and others.[9] These metallic preparations have distinct procedures of preparations involving purification and/or detoxification. It also involves impregnation and/or levigation which enhances bioavailability and improves content uniformity via active substance which is absorbed into the drug particles or the active substance permeates the porous structure of the drug particles and forms a solid solution on the surface of the drug particles. Incineration and/or calcinations methods are being practiced till now since ancient pharmaceutical era.[10] These methods were developed by traditional practitioners of this science to detoxify the raw material by natural chemical transformations and thus modifies the properties of therapeutic materials to enhance their potential.[11,12]. Hence their use cannot be denied just because of the heavy metal content but unfortunately some metals and minerals have the potential to produce adverse effects.[13]In this era, scientific validation and good manufacturing practices plays a major role in standardizing traditional drugs. Siddha System is one of the oldest traditional systems of medicine in India [14]. This system is enriched with herbal, metal, mineral and animal resources [15]. Ayabirungaraja karpam(ABK), is one of the herbo-metallic, anti-anemic siddha formulation. The present study investigated the sub-acute toxicity of ABK which is the need of the hour due to its long historical and prevalent use in siddha system of medicine without any scientific safety profile. ABK has been used as a kaya karpam in siddha medicine to treat or prevent anaemia, grey hair, fatigue and senility. The reputed Siddha pharmacology text "siddha vaithiyathirattu" in the form of verse mentioned ingredients of ABK including Ayam-*Karumponn*(iron), Manduram-*Ayomalam* (iron oxide), *Karisalai-*(*Wedelia chinensis)* and *Sathapala-lemon* (*Citrus lemon*)[16].

2. MATERIALS AND METHODS

2.1 Preparation of ABK

2.1.1 *Authentication*

This study aims to find out the safety profile of ABK by evaluating subacute toxicity and reversal toxicity to provide scientific validated information to traditional therapist.The two main ingredients such as raw ore iron(*Ayam)*andraw cast iron (*Manduram*) were purchased from Trichy local market and obtained authentication from Dr.KKadirvelu, retired Professor of Geology, V.O.Chidambaram College, Tuticorin. *Wedeliachinensis*(Osbeck) Merr (Voucher specimen No CARISM 109) was collected from local herbal garden, Thanjavur and *Citrus limon*L(Voucher specimen No CARISM 110) fruit was purchased from local market, Thanjavur. Both herbal raw materials were authenticated by Dr Ravichandran,Asst.Professor Botany, CARISM, Sastra, University, Thanjavur.

2.1.2 Process of Preparation

The preparation technique was mainly drenching metals in herbal juices and then drying under solar treatment. Purified Ayam powder 140g and purified Mandura powder 210g were mixed together well and then they were drenched in *Wedelia chinensis* juice at the volume of 1.3L corresponds to this ratio and the mixture was kept under sunlight heat (*Sooryapudam*) for drying. Once it was dried up completely, the lime juice (*Citrus limon*) 1.3L of volume was added and drenched for complete drying under solar heat. Again, the drying process was repeated by adding only *Wedeliachinensis* juice at the half of the volume of 1.3Luntil become waxy consistency.Themixture was stirred by spatula in regular interval during drying process period. Then the waxy end product was transferred into herbal cup (*thonnai*) which was made by dry leaves of *Ficus bengalensis,* for complete drying under solar heat. The grey colour ash powder was obtained as the final product after grinding the dried product.[16]

2.1.3 Animal Study

Seventy healthy Wistar albino rats (Rattus norvegicus) of both sexes) between 8-12 weeks of age and weighing 150 - 175 gm were used in this experiment.. The experiment was conducted at the central animal facility, SASTRA University, Thanjavur, registered (No 817/PO/ReRc/S/04/CPCSEA) for breeding and experiments of animals by the committee for the purpose of control and supervision of experiments on animals by the Ministry of Forest and Environment, Govt. of India. During the study period, the animals were housed in a room maintained at a temperature maintained at 22±3°C and relative humidity of 50 ± 20%, with artificial lighting from 07:00 to 19:00 at 150 to 300 Lux of luminous intensity and 10 to 15 air changes per hour. The animals were acclimatized to laboratory conditions for 7 days prior to commencement of the experiments. Not more than 3 animals were housed in a stainless steel wire mesh cage (260 ×350 × 210 mm) during the quarantine and acclimation, and one animal was placed per cage during the dosing periods. Standard rodent pellet feed supplied by M/s ATNT Laboratories, Mumbai, India and Reverse Osmosis (RO) water were provided to the animals ad libitum. All animals were fasted overnight about 18h prior to the initial dosing and in the end stage of terminal necropsy.

2.1.4 Protocol and Ethical clearance

The experimental protocol was conducted in accordance with internationally accepted principles for laboratory animal use and care, the Economic Co-operation and Development (OECD) guidelines for the testing of chemicals "sub acute toxicity -407" (OECD/TG-407, 2008). The study was conducted after approval by the Institutional Ethical Committee, Sastra University, Thanjavur. (No 522/SASTRA/IAEC/RPP).

2.1.5 Dose fixation and schedule

The dose for experimental study of the test drug ABK was calculated by extrapolating the human dose 1000 mg/day to animal dose 100 mg/kg (considered as therapeutic equivalent dose, TED) based on the body surface area ratio. Among various factors, body weight and body surface area are considered as two major approaches to determine the dose for toxicity study. Body surface area (BSA) scaling has been used for prescribing individualized dosages of various drugs with predicting equivalent dosages for humans from biological observations in laboratory animals. Drug suspension was prepared in 100mg/mL in lime water.The lower, medium and higher doses were set at the 2.5, 5 and 10 time concentration to the therapeutic dose such as 250mg/kg b.w, 500 mg/kg b.w, 1000 mg/kg b.w. respectively.

2.1.6 Toxicity study- Animal Group : I - V

This study was divided into two phases viz toxicity study and recovery study. The animals were divided into seven groups each contains 10 wistar albino rats(n=5 male, n=5 female).In toxicity study, the five groups that were involved are Group I,(served as normal control and was administered with 1 mL distilled water in oral gavage), Group II, (orally received lime water 1mL/100g body weight as vehicle control), Groups III, IV and V (daily administered with the Ayabirungaraja karpam preparation in oral gavage for 28 days at 250 mg/kg as low dose, 500mg/kg as medium dose and 1000mg/kg as higher dose respectively).

2.1.7 Toxicity reversal study-Animal Group: VI - VII

In recovery study, two satellite groups were set in order to determine the reversibility or recovery from toxic effect. Group VI served as satellite control and received 1mL lime water. Group VII served as a satellite treatment group received ABK preparation at the high dose of 1000mg/kg body weight for 28 days, followed by a 14 days post treatment observation period without any drug administration.

2.1.8 Treatment Regime

The weight of each rat was recorded on day 1^{st} ,7^{th} ,14^{th} ,21^{st},28^{th} for groups I-V, but in addition to this 35^{th} and 42^{nd} day, the weight were taken for satellite group VI &VII. All experimental animals were observed for signs of morbidity such as changes in food consumption and weight gain, running nose, salivation, diarrhoea, cough, behavioural changes intoxication and visual monitoring for mortality twice a day, till the completion of treatment. Clinical observations were made once daily to detect signs of toxicity as changes in posture, skin, fur, eye, nasal mucous membrane, autonomic sign like piloerection, lacrimation, salivation, perspiration, urination and defecation, central nervous system signs tremor, drowsiness, gait and convulsion preferably at the same time one hour after vehicle or ABK administration. At the end of 28 days, they were fasted overnight and the 50 animals from group I-V was euthanized with carbon dioxide, 1mL blood samples were collected from the retro-orbital plexus into two tubes: one with EDTA for immediate analysis of haematological parameters, the other without any anticoagulant and were centrifuged at 4000 rpm at 4 °C for 10 minutes to obtain the serum. Serum was stored at 4°C for one day. Then the animals belong to toxicity study groups were sacrificed and dissected to carry out the necropsy, weight of the organs viz liver, kidneys, spleen were recorded and histopathological investigation of these three organs were done. On the 42^{nd} day, the same procedures were performed to satellite groups VI & VII for haematological, biochemical and histopathological investigations.[17,18]Inorder to determine the effect of ABK on body , the organ weight of animals, the gross body weight, before and after drug administration were recorded and compared with control group.[19] Haematological parameters like RBC, HB, HCT, MCV, MCH, MCHC, WBC count of the control and ABK treated group were determined and compared with control group using an automatic haematology analyser.[20] Biochemical analysis was carried out by clinical biochemistry analyser on serum and parameters like albumin, total bilirubin, direct bilirubin, cholesterol, creatinine, glucose ,protein, ALP,AST.ALT, urea and uric acid were calculated for all animal groups were compared to determine the safety profile of ABK [21]The diagnosis and study of the effect of ABK on histopathology of selected organs such as liver, kidney and spleen were removed after sacrificing the animals, then the organ pieces (3-5µm thick) of the highest dose level of 1000 mg/kg were preserved and were fixed in 10% formalin for 24 h and washed in running water for 24 h. Embedding was done by passing the cleared samples through three cups containing molten paraffin at 50°C and then in a cubical block of paraffin made by the "L" moulds and the slides were stained with Haematoxylin-eosin for microscopical examination.Then the treated and satellite groups were compared with control group to determine the safety and reversal toxicity of the ABK.[22]

3. STATISTICAL ANALYSIS

Data is expressed in Mean ± standard error mean(SEM). Mean difference between the control and treatment groups were analyzed by one-way ANOVA followed by post-hoc Tukey's multiple comparison as post hoc test for 28 day repeated dose toxicity. n=10 Statistically significant variation was derived by comparing Normal Control versus Vehicle Control, Low Dose, Medium Dose and High Dose). Value was analysed by independent t-test using SPSS version 20, comparing two groups (Satellite Lime and Satellite High Dose), and significant level alpha 0.05 for two-tilled.
The significant level was set at $P>0.05$ for all test.

4. RESULTS AND DISCUSSION

4.1 Effect of ABK on gross body weight

Recording body weight on day 1, once a week during the dosing and recovery periods were made. Data is précised below (Table 1a & 1b)). In male and female rats,statistically significant change was not observed between the gross weight of the ABK treatment group and the normal control group.

Table: 1a . Animal gross body weight-Toxicity group					
Days	Normal Control	Vehicle Control	Low Dose	Medium Dose	High Dose
0th Day	146.35±19.99	146.62±19.82^{NS}	146.64±19.27^{NS}	147.07±18.20^{NS}	146.97±18.26^{NS}
7th Day	173.50±28.48	175.02±30.09^{NS}	174.29±31.39^{NS}	178.04±29.43^{NS}	175.01±27.96^{NS}
14th Day	190.15±31.59	188.84±34.26^{NS}	190.92±36.44^{NS}	195.23±37.35^{NS}	191.69±32.41^{NS}
21st Day	202.62±38.76	198.28±36.97^{NS}	202.03±38.39^{NS}	204.60±41.51^{NS}	201.88±38.53^{NS}
28th Day	214.19±41.89	211.73±40.66^{NS}	212.91±43.72^{NS}	219.58±47.32^{NS}	217.60±44.78^{NS}

Value expressed as Mean ± SD; n= 10 Multiple Comparisons (Turkey HSD). 'p' value - P>0.05 compared to normal control.(NS- Non significant)

Table: 1b Animal gross body weight of satellite group				
Days	Satellite Lime	Satellite High Dose	*P* value	(Two-tailed)
35th Day	223.52±49.13	225.82±44.74	0.913^{NS}	
42nd Day	226.78±49.34	234.95±47.90	0.711^{NS}	

Value expressed as Mean ± SD; n= 10 independent t-test two group comparison 'p' value - P>0.05 compared to vehicle control for two - tailed. (NS- Non significant)

4.2 Absolute organ weight and relative organ weight (ROW)

Absolute terminal organ weight and percentage of relative organ weight are the indicators of test compound that caused the changes in function of target organs, in metabolism, fluctuations of enzymes, hormones, hyper or hypoplasia and tissue necrosis.[23] In this study, the absolute organ weight and relative organ weight (ROW) of selected organs such as Liver, Kidney and Spleen statistically was not significant in ABK treated group and satellite group compare with control in both male and female rats. These findings demonstrated a non-significant difference in the body weight of rats which were treated with the different doses of ABK and reversal toxicity satellite group.(table 2a & b)

Table.2a: Animal organ weight Toxicity group					
Organs	Normal Control	Vehicle Control	Low Dose	Medium Dose	High Dose
Liver	8.50±1.41	8.42±1.57^{NS}	8.28±1.60^{NS}	8.67±1.98^{NS}	8.65±1.99^{NS}
Kidney	1.55±0.34	1.56±0.33^{NS}	1.53±0.31^{NS}	1.59±0.36^{NS}	1.59±0.34^{NS}
Spleen	1.41±0.28	1.60±0.66^{NS}	1.46±0.37^{NS}	1.43±0.49^{NS}	1.54±0.41^{NS}

. Value expressed as Mean ± SD; n= 10 Multiple Comparisons (Tukey HSD). 'p' value - P>0.05 compared to normal control. (NS- Non significant)

Table.2 b:Animal Organ weight Satellite group				
Organs	Satellite Lime	Satellite High Dose	*P* value	(Two-tailed)
Liver	8.73±1.90	8.87±2.10	0.878^{NS}	
Kidney	1.70±0.37	1.71±0.40	0.968^{NS}	
Spleen	1.46±0.44	1.40±0.36	0.732^{NS}	

Value expressed as Mean ± SD; n= 10 independent t-test two group comparison 'p' value - P>0.05 compared to vehicle control for two - tailed.

4.3 Effect of ABK on haematological parameters

The results of the haematology parameter were summarized in the table (3a&3b). All the tested haematological parameters including WBC/DC, Hb, RBC, HCt, MCV, MCH, MCHC, were within the normal range of rats. No significant differences (p. <0.05) between treated animals and control groups were found. The hemopoietic system serves as important susceptible target for toxic substances which is considered as typical scientific model of patho-physiological studies in human as well as animal.[24] In this study, haematological parameter of ABK treated group and recovery satellite groups were within the normal range of rat species used, which demonstrate that it has no adverse effect on circulating blood cells and its formation.[25] The red pulp of the spleen is a blood filter that removes foreign material and effete erythrocytes. It is also a storage site for iron, erythrocytes, and platelets. In rodents, it is a site of haematopoiesis, particularly in foetal and neonatal animals. The spleen is also the largest secondary lymphoid organ containing about one-fourth of the body's lymphocytes and initiates immune responses to blood-borne antigens.[26]The red pulp macrophages are actively phagocytic and remove old , damaged erythrocytes and blood-borne particulate matter. Extra modularly haematopoiesis is common in rodent red pulp, especially in foetal and neonatal animals. Any combination of erythroid, myeloid, and megakaryocytic cells may be evident. [27]

Table. 3a: Haematology Parameters of 28 days treated group					
Parameters	**Normal Control**	**Vehicle Control**	**Low Dose**	**Medium Dose**	**High Dose**
WBC ($\times10^3$/µl)	18.30±4.65	20.21±5.67^{NS}	17.98±3.51^{NS}	15.89±3.01^{NS}	18.18±3.21^{NS}
RBC ($\times10^6$/µl)	6.51±0.58	6.67±0.83^{NS}	6.64±0.37^{NS}	6.79±0.52^{NS}	6.52±0.67^{NS}
Hb(g/dL)	13.21±1.05	13.21±1.35^{NS}	13.44±0.53^{NS}	13.55±0.90^{NS}	13.23±0.93^{NS}
HCT(%)	43.91±3.68	44.81±4.07^{NS}	44.44±2.34^{NS}	45.10±3.76^{NS}	43.77±3.30^{NS}
MCV (fL)	66.99±3.07	66.84±6.62^{NS}	66.48±4.39^{NS}	66.11±2.01^{NS}	66.90±3.07^{NS}
MCH (pg)	20.14±0.70	19.86±1.00^{NS}	20.11±0.98^{NS}	19.85±0.59^{NS}	20.20±1.04^{NS}
MCHC (g/dl)	30.06±0.90	27.19±8.36^{NS}	30.29±1.17^{NS}	30.09±0.81^{NS}	30.24±0.76^{NS}

Value expressed as Mean ± SD; n= 10 Multiple Comparisons (Tukey HSD). 'p' value - P>0.05 compared to normal control.

Table. 3b: Haematology parameters of satellite group				
Parameters	**Satellite Lime**	**Satellite High Dose**	**P Value**	**(Two-tilled)**
WBC ($\times10^3$/µl)	19.15±6.06	19.14±3.60	0.996^{NS}	
RBC ($\times10^6$/µl)	7.34±0.50	7.16±0.27	0.336^{NS}	
Hb (g/dL)	13.98±0.58	14.06±0.28	0.700^{NS}	
HCT(%)	45.48±2.61	45.00±1.48	0.620^{NS}	
MCV (Fl)	61.69±2.09	62.50±2.35	0.427^{NS}	
MCH (pg)	18.99±1.04	19.51±0.68	0.205^{NS}	
MCHC (g/dL)	30.71±0.99	31.26±0.91	0.215^{NS}	

Value expressed as Mean ± SD; n= 10 independent t-test two group comparison 'p' value - P>0.05 compared to vehicle control for two - tailled.

4.4 Effect of ABK on biochemical parameters

The liver and kidney are important organs, which are responsible for the metabolism, detoxification, storage, and excretion of toxic substance and their metabolites which are susceptible to damage by external substances.[28] Once the hepatic cell membrane gets damaged, the cytosol enzymes are released into the blood, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), serum enzymes that have been shown to be the most effective and sensitive indicators of hepatocellular injury.[29] However, both AST and ALT are intracellular enzymes of which appearance in the blood is an indicative of cellular damage. [30]Therefore, the determination of these enzymes in blood serum indicate as universal marker for the detection of organic damage.[31]Unfortunately, AST also can exist in many organs including the heart and muscles; therefore, its release is not specific for acute liver diseases.[32]Some naturally occurring chemicals (endobiotics) become toxic chemicals when present in the environment at excessive concentrations. The liver is the main metabolizing and detoxifying organ for drugs which make the liver as common target of toxic chemical damage and thus producing severe liver related adverse reactions. [33]

Table. 4a: Biochemical Parameters of 28 days treated group					
Parameters	**Normal Control**	**Vehicle Control**	**Low Dose**	**Medium Dose**	**High Dose**
Albumin (g/dL)	2.67±0.21	2.60±0.36^{NS}	2.64±0.33^{NS}	2.63±0.39^{NS}	2.36±0.40^{NS}
ALP (U/L)	328.70±94.96	367.80±93.46^{NS}	373.20±138.17^{NS}	297.30±71.21^{NS}	376.30±123.63^{NS}
Total bilirubin (mg/dL)	0.87±0.50	0.62±0.25^{NS}	0.47±0.16^{NS}	0.60±0.29^{NS}	0.49±0.15^{NS}
Direct bilirubin (mg/dL)	0.50±0.40	0.29±0.17^{NS}	0.26±0.14^{NS}	0.29±0.12^{NS}	0.24±0.12^{NS}
Cholesterol(mg/dL)	62.00±18.56	64.90±21.38^{NS}	58.20±22.06^{NS}	61.80±18.42^{NS}	53.20±18.98^{NS}
Creatinine(mg/dL)	0.52±0.05	0.51±0.05^{NS}	0.48±0.05^{NS}	0.46±0.05^{NS}	0.46±0.04^{NS}
Glucose (mg/dL)	119.60±15.95	115.10±13.01^{NS}	110.60±16.16^{NS}	110.50±14.48^{NS}	113.80±11.74^{NS}
Protein (g/dL)	6.35±0.54	6.09±0.58^{NS}	6.17±0.60^{NS}	5.96±0.31^{NS}	6.40±0.51^{NS}
AST(U/L)	140.30±14.46	153.60±18.08^{NS}	150.30±17.55^{NS}	138.80±22.09^{NS}	151.70±26.06^{NS}
ALT(U/L)	86.40±41.51	104.40±59.27^{NS}	96.40±30.57^{NS}	93.30±43.45^{NS}	100.70±45.78^{NS}
TG(mg/dL)	91.27±45.30	64.09±29.97^{NS}	93.79±36.44^{NS}	77.07±42.64^{NS}	83.72±54.95^{NS}
Urea (mg/dL)	42.56±7.85	42.78±8.22^{NS}	37.60±6.20^{NS}	40.20±4.05^{NS}	40.01±5.72^{NS}
Uric acid (mg/dL)	1.10±0.57	1.01±0.57^{NS}	1.04±0.56^{NS}	0.66±0.60^{NS}	0.76±0.41^{NS}

Value expressed as Mean ± SD; n= 10 Multiple Comparisons (Tukey HSD). 'p' value - P>0.05 compared to normal control.(NS- Non significant)

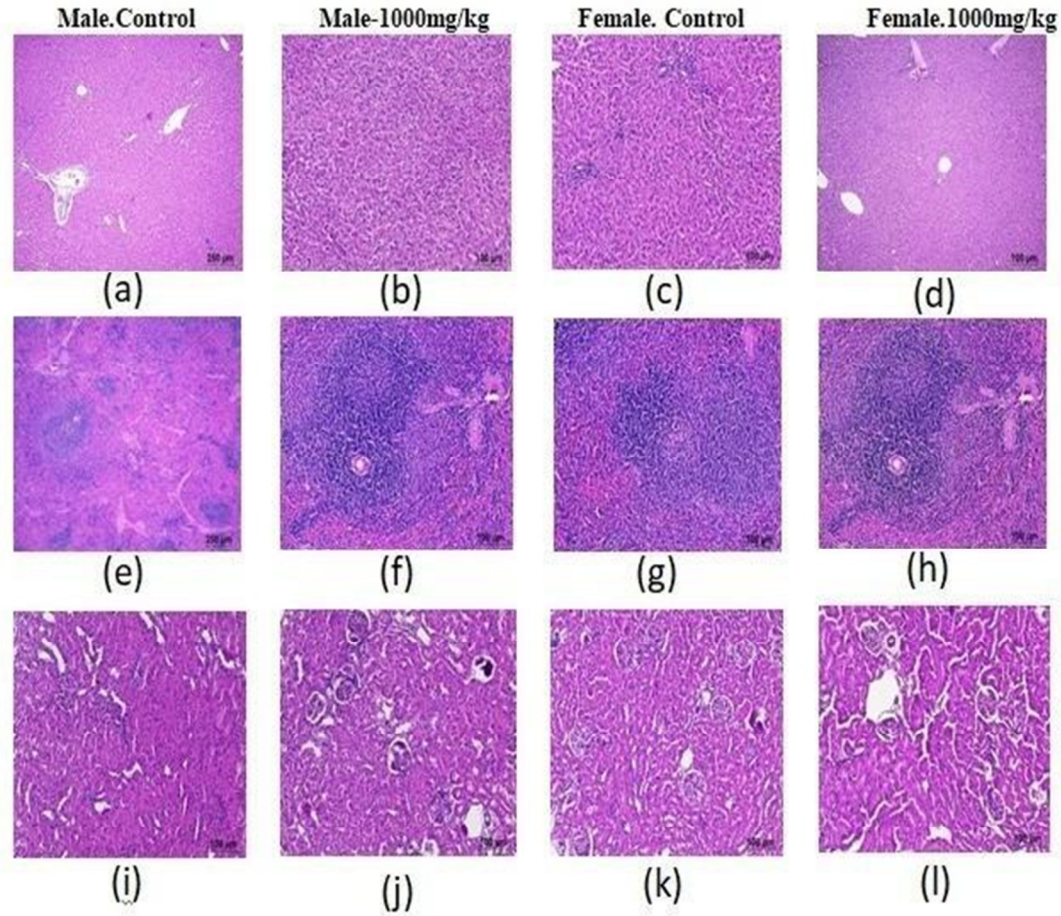
Table.4 b: Biochemical Parameters of Satellite group		
Parameters	**Sattelite Lime**	**Sattelite High Dose**
Albumin (g/dL)	2.18±0.55	2.21±0.53 ^{NS}
ALP (U/L)	276.10±50.17	314.70±89.42 ^{NS}
Total bilirubin(mg/dL)	0.52±0.29	0.56±0.31 ^{NS}
Direct bilirubin(mg/dL)	0.18±0.12	0.29±0.12 ^{NS}
Cholesterol (mg/dL)	64.10±21.04	69.70±25.39 ^{NS}
Creatinine(mg/dL)	0.43±0.05	0.48±0.10 ^{NS}
Glucose (mg/dL)	101.40±8.79	106.90±9.05 ^{NS}
Protein (g/dL)	5.82±0.77	6.58±1.39 ^{NS}
AST(U/L)	166.30±14.57	192.20±47.95 ^{NS}
ALT(U/L)	72.90±7.50	76.60±20.43 ^{NS}
TG(mg/dL)	85.03±60.47	87.39±45.98 ^{NS}
Urea (mg/dL)	38.82±2.93	44.96±12.33 ^{NS}
Uric acid (mg/dL)	0.73±0.35	0.87±0.42 ^{NS}

Value expressed as Mean ± SD; n= 10 independent t-test two group comparison 'p' value - P>0.05 compared to vehicle control for two - tailled.(NS- Non significant)

The results of various biochemical tests of control, ABK treated and satellite groups are summarized in table (4a& b). The oral administration of ABK at the dose of 250,500,1000 mg/kg body wt did not cause any significant changes in serum biochemical parameter like albumin ,total bilirubin, direct bilirubin, cholesterol, creatinine, glucose, protein, AST, ALT, TG, urea and uric acid. Clinical biochemistry and haematological detection plays critical role to ascertain the toxicity induced by the drug.[34]Transaminase (AST and ALT) are the main biochemical markers for liver function and to predict the hepatic toxicity. Any elevation regards to these enzymes indicate their out flow into bloodstream due to liver damage of parenchymal cells.[35].Elevation of bilirubin level in serum or plasma is a clue for hepatocellular damage and dysfunction. Increase serum creatinine is the indicator for renal insufficiency.[36]kidney performs three main functions including elimination of toxic substances that are produced during metabolism, regulation of internal liquid medium haemostasis, and production of hormones which could be used to assess the renal status. In acute or chronic renal failure, the end product of nitrogen metabolism builds up, increasing non protein nitrogen levels by which blood urea nitrogen and serum creatinine level will be elevated. Accordingly, renal function in blood can be evaluated through the determination of urea as the end product of protein metabolism which is formed in the liver from ammonia and later eliminated by the kidney.[37]The present study findings indicated that serum urea and creatinine levels showed no significant difference in test group animals in all tested doses of ABK as compared with those of rats in the normal control group.In this study, ABK did not alter the liver and kidney function because the levelmarker enzymes ALT, AST and renal function indicator creatinine were found to be within the normal range[38]Satellite group did not produce delayed toxic effect in high dose.

4.5 Effect of ABK on histopathological study

Histopathological results of the micrographs of liver, spleen and kidney tissue samples of control and high dose treated groups are shown in figure 1 as follows : 1(a) Liver of control male, 1(b) Liver of high dose male,1(c) liver of control female1(d) liver of high dose female(1e) spleen of control male(f)spleen high dose male(g)spleen of control female (h)spleen of high dose female(i)kidney of control male(j)kidney of high dose male(k) kidney of control female. (l) Kidney of high dose female Histopathological study is usually carried out under 40X magnification using the microscope for degeneration, fatty changes, necrotic changes and evidence for hepatotoxicity.[39]. The below mentioned micrographs of tissues revealed the normal cytoarchitecture of spleen in both male and female control group (Fig: 1e,g), whereas treated group also found to be the same.(Fig: 1f,h). Microscopic examination of liver and kidney obtained from control group exhibited normal cytoarchitecture with mild changes (fig: 1a,c,i, and k) where as high dose treated group both male and female showed, mononuclear, peribiliary/random, multifocal, mild and Microgranuloma minimal. (Fig: 1c, d,j,l). Since these changes are minimal the histopathology study for low dose, medium dose and satellite group were not performed. The evaluation of histopathological changes play vital role in toxicity assessment.[40]



Monograph of histopathology findings in selected organs tissues taken at ×100 magnification.(a) Male liver .control (b)Male liver treated with ABK. TED×10 (c) Female liver .control (d) Female liver treated with ABK. TED×10 (e) Male spleen .control (f) Male spleen treated with ABK. TED×10 (g) Female spleen .control (h) Female spleen treated with ABK. TED×10 (i) Male Kidney .control (j) Male kidney treated with ABK. TED×10(k) Female Kidney .control (l) Female kidney treated with ABK. TED×10

Fig: 1 Monographs of Histopathology findings

The purified Mandurum and Ayam(Louha) are the main ingredients of the tested drug ABK. The recent past research findings on mandura bhasma has shown hepatoprotective action based on the biochemical and histopathological findings respectively. SGOT,SGPT and ALT were found to be reduced and the 80-90%necrosis caused by paracetamol was reduced by 30-40% with the treatment of mandura bhasma, by then it is proven to have cytoprotective action.[41]LauhaBhasma and mandura bhasma were found to be safe at 5 times therapeutic effective dose in Charles foster albino rats.[42]Mandurabhasma(ash)showed hepato protective activity on CCl_4 induced hepatic injury.[43] No significant toxic effect was found in Lauha Bhasma(ash) treated group Only mild impairment of hepatic function and hepatic architecture,mild fatty changes and sinusoidal dilatation was observed in the Mandura Bhasma(ash) treated group. Other vital organs like spleen, heart, lungs, kidney, brain, etc. exhibited almost normal cytoarchitecture in all the groups. Microscopic examination of sections of liver from both the recovery groups showed almost normal cyto architecture with mild micro-fatty changes in hepatic cells .[44,45]The previous study of single dose oral acute toxicity in wistar albino rats was conducted at the dose of 2000mg/kg b.w while no visible signs of toxicity on behavioural pattern changes, respiration, circulation, autonomic and central nervous system were observed. Necropsy changes and mortality also were not noticed.in all of 5/5 female wistar albino rats ABK treated group throughout the observation period.[46]

5 CONCLUSION

The present sub-acute toxicity study for 28 days with repeated dose at 250mg/kg, 500mg/kg and 1000mg/kg revealed no remarkable toxicity signs and no mortality was recorded. No significant haematological and biochemical changes were observed in both sexes treated with ABK. The satellite group did not show any reversal toxicity in vehicle control and high dose ABK treated group. The histology examination revealed no remarkable changes in the selected internal organs like kidney, liver, spleen, in both control and treated groups. Hence, ABK 1000mg/kg body wt can be considered as safe for oral administration for stipulated period as per reputed siddha text since ABK did not produce prominent detrimental toxicity signs at the ten times concentration (1000mg/kg) of the therapeutic dose(medium) (100mg/kg). Therefore, the present investigation is substantiating the safety of the ABK which was found to be in line with the long history of its use in Siddha system of Medicine. The clinical uses of metal and mineral drugs were limited due to toxicity concern among the scientific community. Hence, the sub acute toxicity profile of ABK has been produced as evidence in this study to support the therapeutic use of ABK in the siddha medicine for the treatment of Iron deficiency anaemia in future. It is further recommended on chronic toxicity study.

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7 AUTHORS' CONTRIBUTION STATEMENT

All Authors contributed to the acquisition of data, analysis and interpretation, conception and design of the study and also provided critical revision of the article and final approval of the version to publish. Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

8 CONFLICT OF INTEREST

Conflict of interest declared none

9 REFERENCE

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