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Research Article

Toxicological Evaluation of *Ashtabhairava mathirai* Acute and Subacute Safety Assessment

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ABSTRACT

Background/Purpose: *Ashtabhairava mathirai* (ABM), a Traditional Siddha formulation referenced in the Siddha Vaidhiya Thirattu, is traditionally used to treat 64 types of fever. Despite its historical significance, scientific validation of its safety is crucial for its acceptance in modern medicine. This study aimed to evaluate the acute and subacute toxicity of ABM using Wistar albino rats (*Rattus norvegicus*). **Materials and Methods:** Wistar albino rats were used for acute (OECD 423) and subacute (OECD 407) toxicity studies. Behavioral, biochemical, and histopathological parameters were analyzed. Data were statistically evaluated using one-way ANOVA ($p<0.05$). **Results:** The findings of the study demonstrated that all evaluated parameters remained within normal limits, with no mortality observed throughout the experimental period. The acute oral toxicity assessment indicated that the LD_{50} value of *Ashtabhairava mathirai* (ABM) exceeds 2000 mg/kg body weight. Additionally, the repeated dose 28-day oral toxicity study determined No Observed Adverse Effect Level (NOAEL) of more than 150 mg/kg body weight. Histopathological examinations of vital organs provided further reassurance, as no abnormalities or adverse changes were noted, supporting safety of the formulation. **Conclusion:** These results confirm the safety of ABM for further clinical investigations but also validate its ongoing use in traditional practices. This study offers a thorough preclinical safety evaluation of ABM, providing scientific support for its traditional applications and potential as a therapeutic agent in future medical use.

Keywords: Siddha, Fever, *Ashtabhairava mathirai*, Toxicity study, Acute toxicity, Repeated dose 28-day oral toxicity study, OECD.

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INTRODUCTION

The Siddha system of medicine, one of the oldest forms of traditional medicine in India, focuses on addressing the root causes of diseases by harmonizing the three humors known as Vatham, Pitham, and Kabam within the body.[1] The holistic theory of Siddha, places great emphasis on the equilibrium of the three primary humors-Vaatham, which represents the principle of movement and is associated with the air element; Pitham, embodying the principle of transformation and linked to the fire element; and Kabam, symbolizing the principle of structure and related to the water and earth elements. These humors are believed to govern not only the bodily physiological processes but also the psychological functions.[2] *Ashtabhairava mathirai* (ABM) a herbomineral formulation stated in the Siddha Vaidhiya Thirattu textbook.[3] ABM is specifically indicated for 64 various types of Suram, Siddha text Maruthuvam Podhu describes fever, known as Suram, as a distinct disease with 64 different classifications based on humors.[4]

The herbomineral formulations use minerals and metals in compound form, rather than in their elemental state. This results in a different physiological behavior from heavy metals in their elemental form. Through interactions with various organic and inorganic materials of herbal origin, these compounds are transformed into a modified form that can alter the properties of toxic metals.[5] This process leads to therapeutic effects and ensures a very high level of safety. Despite extensive historical usage and anecdotal reports of therapeutic benefits, scientific validation regarding its safety is lacking. With a growing global interest in herbal and traditional medicines, ensuring the safety and efficacy of such formulations is paramount. Preclinical safety evaluations are critical for identifying potential toxicities and adverse effects that may not be evident through historical

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use alone. Regulatory authorities, including AYUSH and global pharmacovigilance agencies, emphasize the need for thorough toxicological assessments before advancing to clinical trials.[6,7] Conducting preclinical safety evaluations is critical to uncover potential toxicities and adverse effects that may not have been previously identified through historical use. This study aims to address this gap by performing the safety assessment of ABM conducted through acute and subacute toxicity studies in animal models, using OECD guidelines (423,407)[8,9] this investigation aims to provide essential data on the formulation's safety profile. The findings will contribute significantly to validating the safe use of this traditional medicine and guide its future therapeutic applications.

MATERIALS AND METHODS

Choice of the drug

Ashtabhairava mathirai is a herbo-mineral formulation used for treating 64 types of suram. It is documented in the "Siddha text Siddha vaithiya thirattu written by Kuppusamy mudhaliyar" on pages 2-3.

Procurement of ABM

The ABM was procured from IMPCOPS pharmacy located in Thiruvanmiyur, Chennai-600041. Batch no S11-087.

Ingredients for *Ashtabhairava mathirai*

The ingredients are depicted in Table 1.

Preparation of *Ashtabhairava mathirai*

Nervalam (*Croton tiglium* Linn.), 20.5 g, along with the remaining ingredients, each 4.2g, should be purified and powdered separately, powdered ingredients should be ground with breast milk, and lemon juice (*Citrus limon* Linn.) for 6 hr respectively, then again triturate with Poduthalai juice (*Phyla nodiflora* Linn.), Karisalai juice (*Eclipta prostrata* Linn.), Navalpattai juice (*Citrus limon* Linn.) for 3 hr, respectively, and made into a pepper-sized pill and dried.[3]

Experimental Animal Sourcing and Care

The toxicity study was conducted after receiving approval from IAEC at the National Institute of Siddha (NIS /IAEC – 24/ RO4/06122022/20). The Wistar albino rats (*Rattus norvegicus*),[10] weighing 140-160 g, were obtained from authorized animal breeders at the animal laboratory in TANUVAS, Madhavaram, Chennai, and were kept in the animal house at the National Institute of Siddha, Chennai. The animals were housed in polypropylene cages at a controlled temperature ranging from 23°C ±3°C, relative humidity of 30-70%, with photoperiod of 12/12 hr and a 7 days acclimatization period was provided *ad libitum* access to RO water and feed was provided. The toxicity study was conducted as per OECD guidelines.[8,9]

Determination of Animal Dose Level

The Siddha text specifies that the therapeutic dose of ABM is 60 mg. The doses were determined from human therapeutic doses based on surface area, utilizing the conversion table of Paget and Barnes.[11] The doses were as follows: Low dose -15 mg/kg b. wt. Mid dose - 75mg/kg b. wt, High dose-150 mg/ kg b.wt.

Acute Toxicity Study

An acute toxicity study was conducted as per OECD guideline 423. Six female animals, all nulliparous were divided into two groups, each consisting of 3 animals. Only the female animals were selected in this study since they exhibit slightly higher sensitivity to toxic effects.[10] The control was administered honey while the treatment group received a single oral dose of 2000 mg/ Kg b. wt. of ABM. The animals were fasted for 12 hr prior to drug administration, while water was available *ad libitum*. Following drug administration, all animals were continuously monitored for the first 30 min, and thereafter at hourly intervals up to 24 hr, for any behavioral changes and mortality.

Body weight was recorded weekly. Behavioral parameters such as abnormal gait, aggressiveness, exophthalmos, ptosis, akinesia, catalepsy, convulsion, excitation, head twitches, lacrimation, loss of corneal reflex, loss of traction, piloerection, reactivity to touch, salivation, scratching, sedation, chewing, head movements, sniffing, straub, tremor and writhes, diarrhoea, leathery, sleep, and coma were monitored across all groups throughout the study period.

Subsequently, the animals were observed for an additional 14 days. After the completion of the study, on the 15th day all animals were weighed and euthanized with excess anaesthesia (Thiopental sodium 1 mg/100 g) to assess any gross pathology. If any gross pathology was noted, organ histopathology was performed.[8]

Repeated Dose 28-day Oral Toxicity

The study involved 20 male and 20 female Wistar albino rats (*Rattus norvegicus*) for a repeated dose 28-day oral toxicity study following OECD 407 guidelines. These animals were categorized into four groups, each consisting of 10 animals (5 males and 5 females). The first group served as the control, second, third and fourth group were treated with ABM. The control animals were administrated honey throughout the study. The doses were as follows: Group 2: Low dose – 15 mg/kg b. wt., Group 3: Mid dose - 75mg/kg b. wt., Group 4: High dose – 150 mg/kg b.wt. The low, mid, and high doses were determined from human therapeutic doses based on surface area, utilizing the conversion table of Paget and Barnes (1964).

Body weight was recorded weekly. At the end of the 28-day treatment period, all animals were euthanized on the 29th day using the intra-peritoneal injection of Thiopental Sodium (1 mg/g). Blood samples were obtained from anesthetized animals

through the abdominal aorta. Haematological parameters, such as Haemoglobin, total red blood cell count, white blood cell count, platelet count, mean corpuscular volume, and mean corpuscular haemoglobin, was assessed using an automated analyzer. Additionally, serum samples were collected and analyzed for urea, creatinine, triglycerides, total cholesterol, HDL, and LDL, as well as the activities of aspartate aminotransferase and alanine aminotransferase, using standard colorimetric methods. Furthermore, all major organs, and selected vital organs such as the brain, heart, lungs, liver, spleen, kidneys, stomach, and

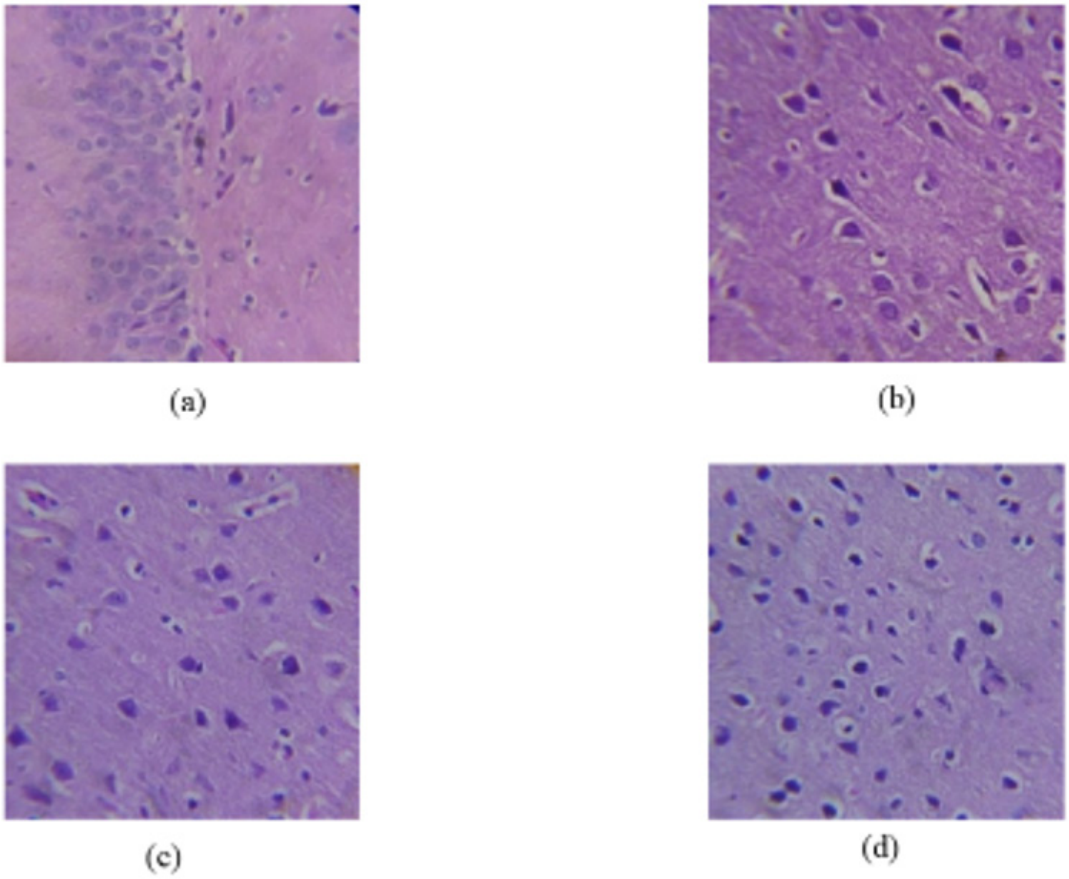

Figure 1: Histopathological examination of the brain (a) control male (b) control female (c) high dose male (d) high dose female.

Table 1: Ingredients of *Ashtabhairava mathirai*.

Sl. No.	Ingredients	Botanical name/Chemical name	Quantity
1.	Purified Gandhagam	Sulphur	4.2 g
2.	Purified Rasam	Hydrargyrum	4.2 g
3.	Purified Lingam	Red sulphide	4.2 g
4.	Purified Pooram	Hydrargyrum subchloride	4.2 g
5.	Purified Veeram	Hydrargyrum perchloride	4.2 g
6.	Purified Nabi	*Aconitum ferox*. Wall	4.2 g
7.	Purified Chukku	*Zingiber officinale*. Roscoe	4.2 g
8.	Purified Milagu	*Piper nigrum* L.	4.2 g
9.	Purified Thippili	*Piper longum* L.	4.2 g
10.	Jathikai	*Myristica fragrans*. Houtt	4.2 g
11.	Jathipathiri	*Myristica fragrans*. Houtt	4.2 g
12.	Lavangam	*Syzygium aromaticum* Linn.	4.2 g
13.	Nervalam	*Croton tiglium* Linn.	17.5g

Table 2: **Behavioral Sign Parameters of rats.**

Sl. No.	Behavioural Signs	Control	2000 mg/Kg b. wt
1.	Alertness	Present	Present
2.	Aggressiveness	Absent	Absent
3.	Pile erection	Normal	Normal
4	Grooming	Normal	Normal
5.	Gripping	Normal	Normal
6.	Touch response	Present	Present
7.	Decreased motor activity	Absent	Absent
8	Tremor	Absent	Absent
9.	Convulsion	Absent	Absent
10	Muscle spasm	Absent	Absent
11	Catatonia	Absent	Absent
12	Muscle relaxant	Absent	Absent
13	Hypnosis	Absent	Absent
14	Analgesia	Absent	Absent
15	Lacrimation	Absent	Absent
16	Exophthalmos	Absent	Absent
17	Diarrhoea	Absent	Absent
18	Writhing	Absent	Absent
19	Dyspnoea	Absent	Absent
20	Mortality	Absent	Absent

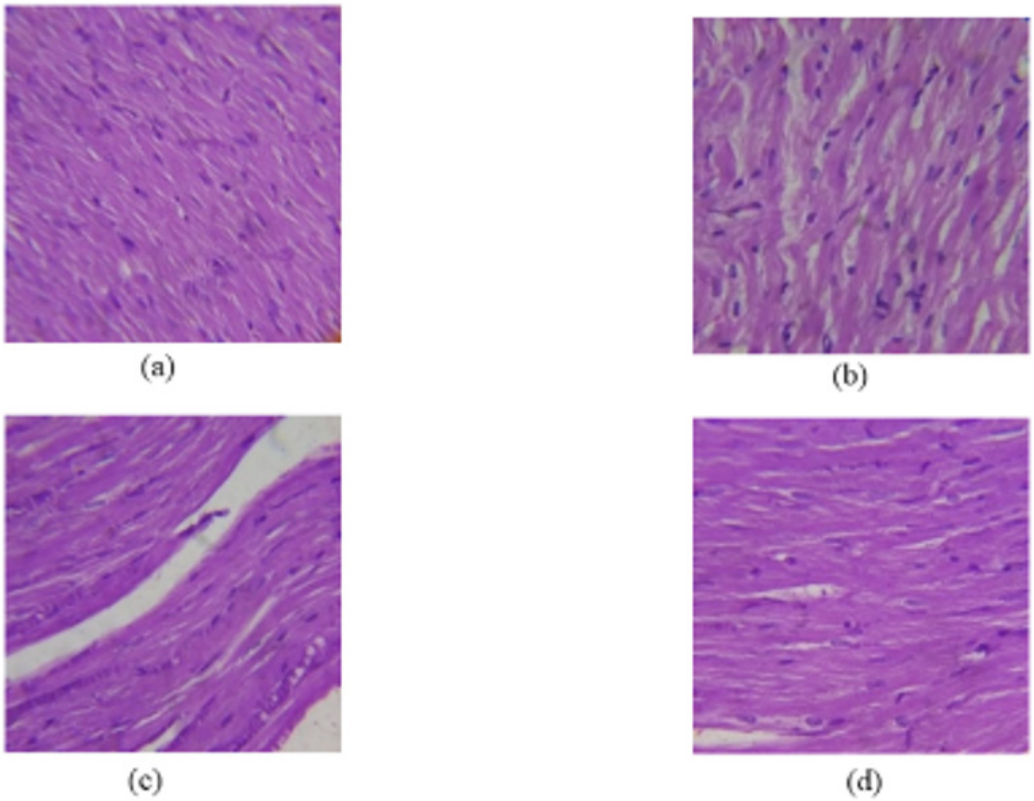

Figure 2: Histopathological examination of the heart (a) control male (b) control female (c) high dose male (d) high dose female.

reproductive organs were preserved in formalin and subjected to histopathological evaluation in both the control and high-dose groups.[9]

Laboratory Investigations

Blood samples were obtained from the animals for hematological and biochemical evaluations. Potassium EDTA, at a concentration of 1.5 mg/mL, served as an anticoagulant for hematological testing, which was performed using an automated analyzer.

Histopathological Examination

All the animals were dissected and the organs were evaluated for any visible histo-morphological changes and all the organs were weighted individually using a digital weighing scale. Histopathological analysis was performed on animals from the control and high-dose groups. Organs including the brain, heart, lungs, liver, kidneys, spleen, stomach, uterus, testes, and ovaries were harvested and fixed in 10% buffered neutral formalin. The samples were sectioned into 5-6 slices, stained with haematoxylin and eosin, and examined microscopically at 40X magnification for any histopathological abnormalities.[12]

Statistical Analysis

Data on body weight changes, feed, and water intake, as well as hematological and biochemical parameters, were meticulously analyzed and statistically compared to the control group using a one-way Analysis of Variance (ANOVA). *Post hoc* analysis was conducted using Dunnett's multiple range test in GraphPad Instat version 3.0, with statistical significance determined at $p<0.05$.

RESULTS

Acute Toxicity

Parameters related to the behavioral signs of Wistar albino rats are summarized in Table 2.

Subacute Toxicity

Tables 3-7 present the mean values of various parameters observed in male and female Wistar albino rats exposed to ABM: Table 3 shows body weight changes, Table 4 shows feed intake, Table 5 shows water intake, Table 6 presents haematological parameters, and Table 7 presents biochemical parameters from the subacute toxicity study group.

Histopathology

The histopathological results of the brain, heart, lung, stomach, liver, kidney, spleen, epididymis, testis, uterus, and ovary from control and high-dose animals are presented in Figures 1-9.

Table 3: Body weight changes in ABM-exposed Wistar rats.

Group	Week 1	Week 2	Week 3	Week 4
Control male	202.8±7.26	226.4±10.69	249.8±20.49	252.4±19.09
Control female	169.8±8.11	180±10.34	180±10.34	183.8±8.35
Low dose male	221.8±7.85*	243.2±8.41	248±15	250.2±13.88
Low dose female	171±2.35	182±7	187.2±6.26	189.8±10.13
Mid dose male	231.6±10.33*	254.4±25.37	250.4±11.91	257.6±15.22
Mid dose female	178.6±6.11	189.2±9.55	193.6±11.13	199.4±8.71*
High dose male	255.4±8.02*	222.8±11.72	279.2±11.03	266±11.34
High dose female	182.4±7.8*	188.4±7.3	193.4±8.76	196.2±9.96

Note: The values are presented as mean±SEM. Statistical significance (P) was determined using a one-way ANOVA Dunnett test (n=5). *$p<0.05$ was calculated by comparing the treated group animals with the control group.

Table 4: Mean feed intake of male and female Wistar albino rats exposed to ABM.

Group	Week 1	Week 2	Week 3	Week 4
Control Male	78.14±6.36	82.43±5.8	89.29±1.38	90.14±2.41
Control Female	82.29±7.85	82.43±5	86.14±3.89	90.29±3.35
Low dose Male	78.29±8.2	82.71±6.21	89.86±1.95	89.29±3.3
Low dose Female	84.86±6.12	86.43±4.89	88.71±1.8	90.86±2.85
Mid dose Male	82.86±6.41	87.57±4.86	91.14±2.61	90.29±1.5
Mid dose Female	82.71±3.35	85.57±3.78	88.29±5.62	91.14±2.41
High dose Male	84.86±5.27	84.57±5	89.29±1.98	89.29±1.6
High dose Female	85.14±3.53	84.43±4.61	89.57±2.88	89.86±1.86

Table 5: Mean water of male and female Wistar albino rats exposed to ABM.

Group	Week 1	Week 2	Week 3	Week 4
Control Male	61.43±5.56	69.14±2.79	71.29±3.9	79±6.48
Control Female	60.43±3.6	68.57±4.69	65.71±6.07	74.29±4.5
Low dose Male	63.57±5.56	70±0.82	71.43±3.05	75±4.32
Low dose Female	63.86±5.64	68.86±3.53	67.14±5.67	72.14±4.88
Mid dose Male	65.43±4.24	71.43±5.8	73.43±3.6	72.71±3.25
Mid dose Female	64.29±3.45	69.14±3.98	72.57±4.24	75±4.08
High dose Male	67.43±3.1	75±4.32	76±4.2	75.71±3.86
High dose Female	64.57±4.93	69.43±3.36	73.43±6.02	74.29±4.64

Table 6: Mean level of Haematological parameters of Subacute toxicity study group.

Group	Control	Low Dose	Mid Dose	High Dose
RBC	7.15±2.17	7.99±0.4	7.95±0.17	8.21±0.3
WBC	13.69±4.43	16.78±4.89	18.55±8.48	18.56±7.21
PLT	835.5±279.05	872.33 ±78.64	875.8±65.47	841.89±80.25
HB	13.33±3.64	14.9±0.49	14.9±0.47	15.23±0.43
N%	10.83±2.53	9.81±2.1	8.53±1.82*	7.89±1.32*
L%	78.12±3.39	79.76±3.12	81.17±3.39	82.83±1.57*
M%	10.2±1.35	9.63±1.39	9.53±2.58	8.4±1.01
E%	0.84±0.3	0.8±0.28	0.75±0.18	0.88±0.19
B%	0.01±0.03	0±0	0.02±0.04	0±0
MCV	87.39±3.27	85.56±3.08	86.4±2.39	85.09±2.48
MCH	19.68±3.91	18.59±0.58	18.73±0.59	18.54±0.46
MCHC	22.43±3.77	21.72±0.33	21.68±0.28	21.81±0.25
HCT (%)	62.12±18.8	68.63±2.75	68.77±2.12	69.84±2.4

Note: The values are presented as mean±SEM. Statistical significance (P) was determined using a one-way ANOVA Dunnett test (n=5). *p<0.05 was calculated by comparing the treated group animals with the control group.

Table 7: Mean level of Biochemical parameters of Subacute toxicity study group.

Parameters	Control	Low Dose	Mid Dose	High Dose
CHOLESTEROL	68.7±18.96	80.43±43.94	72.61±46.94	98.61±30.22
HDL	65.75±20.34	58.33±24.32	45.01±17.47	49.35±18.7
LDL	39.77±15.68	44.03±21.92	46.45±26.86	57.76±17.39
TGL	102.77±39.09	84.45±29.98	48.29±10.21*	82.78±28.82
SGOT	143.84 ±42.79	128.08 ±45.76	132±46.57	133.85±48.12
SGPT	79.15±49.3	56.52±20.62	63.61±26.62	79.17±25.11
UREA	29.23±9.16	31.24±13.04	33.62±15.72	29.27±12.55
GLUCOSE	328.45±167.32	319.43±132.27	280.01±100.43	283.15±133.65
ALBUMIN	2.64±0.92	2.62±0.61	2.86±1.39	3.14±1.02
T. PROTEIN	7.28±3.14	6.17±2.88	7.43±2.64	7.48±1.69
URIC ACID	8.24±3.24	8.56±3.41	8.74±3.57	8.57±4.25

Note: The values are expressed as mean±SEM. Statistical significance (P) was assessed using a one-way ANOVA followed by Dunnett's test (n=5). A significance level of *p<0.05 was determined by comparing the treated group with the control group.

DISCUSSION

The Siddha literature contains numerous remedies for addressing fever. ABM is one of the formulations suggested in the Siddha Vaidhiya Thirattu textbook for treating 64 types of fever and may offer potential benefits for those seeking alternative approaches to fever management.[3,13] However, modern scientific methods are required to validate its safety profile and historical usage in humans. The research aims to explore the safety of ABM and evaluate acute and repeated dose 28-day oral toxicity using the Wistar albino rats (*Rattus norvegicus*) model. In the acute toxicity assessment, female Wistar albino rats (*Rattus norvegicus*) were monitored for 24 hr following a single oral administration of ABM at a dose of 2000 mg/kg body weight. No abnormal behavioral changes, fatalities, or signs of illness were observed during this

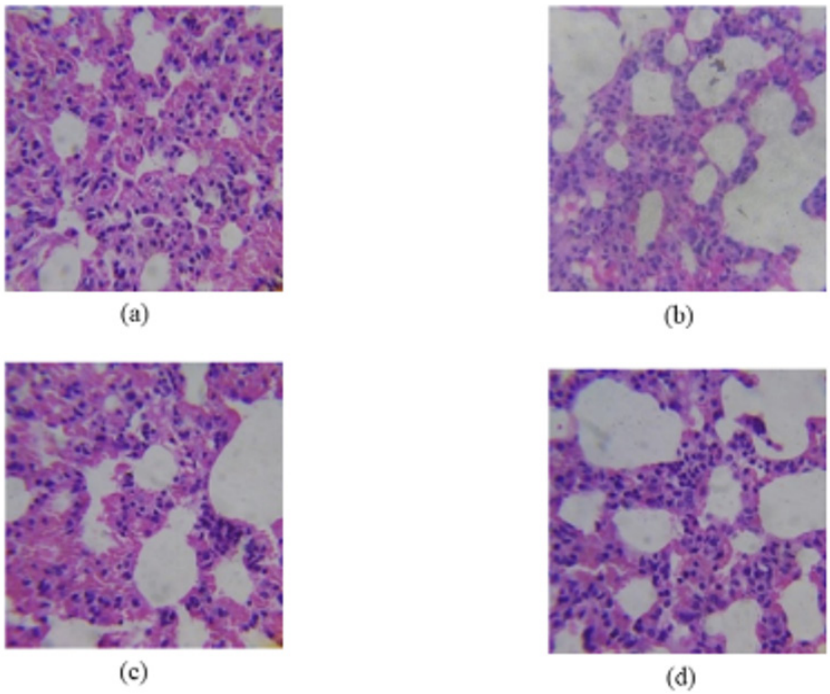

Figure 3: Histopathological examination of the lung (a) control male (b) control female (c) high dose male (d) high dose female.

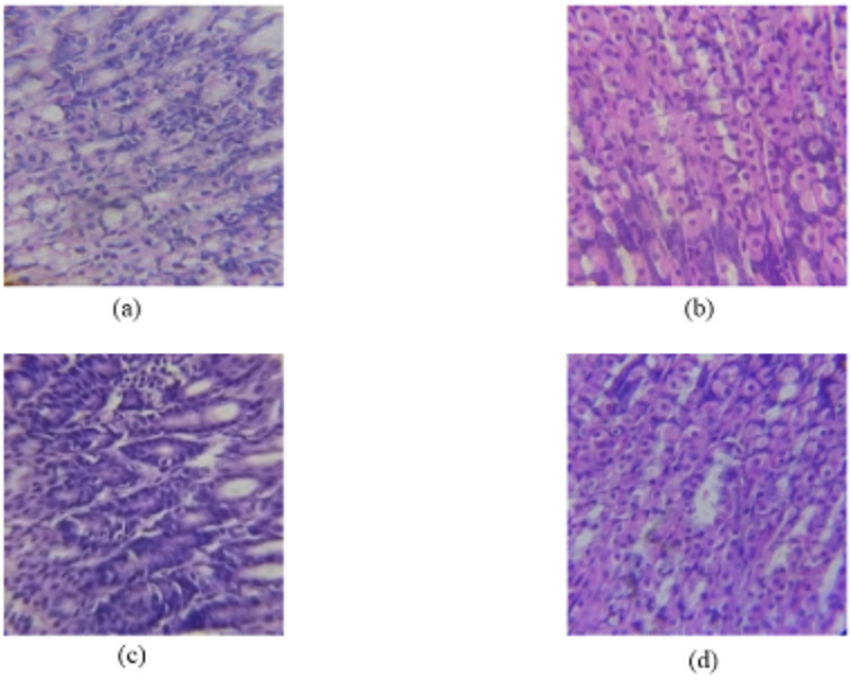

Figure 4: Histopathological examination of the stomach (a) control male (b) control female (c) high dose male (d) high dose female.

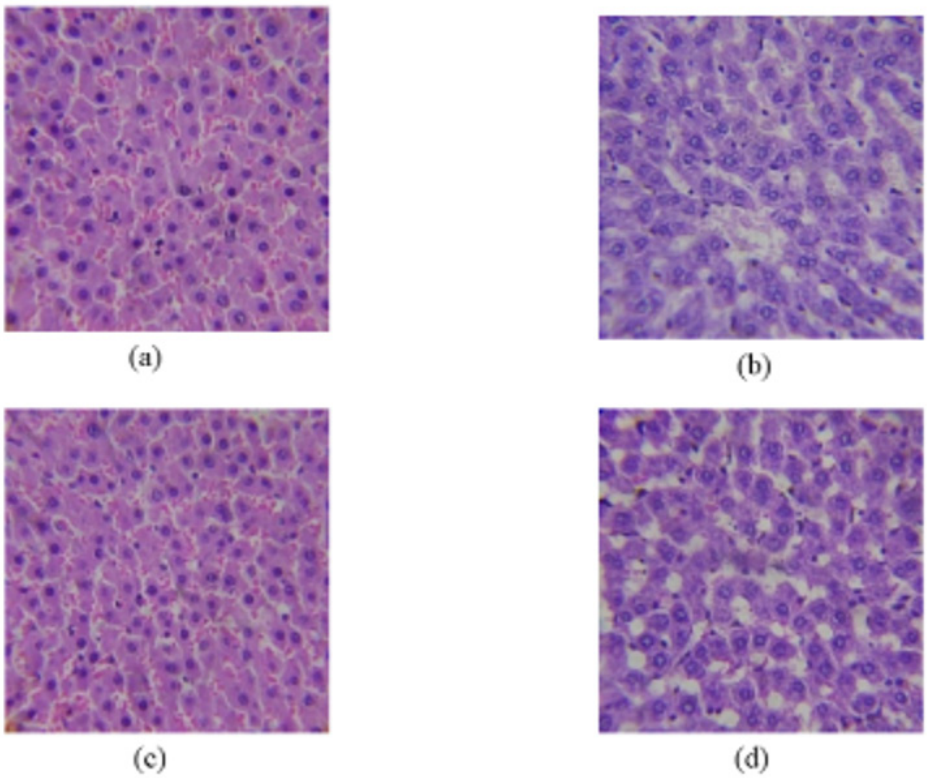

Figure 5: Histopathological examination of the liver (a) control male (b) control female (c) high dose male (d) high dose female.

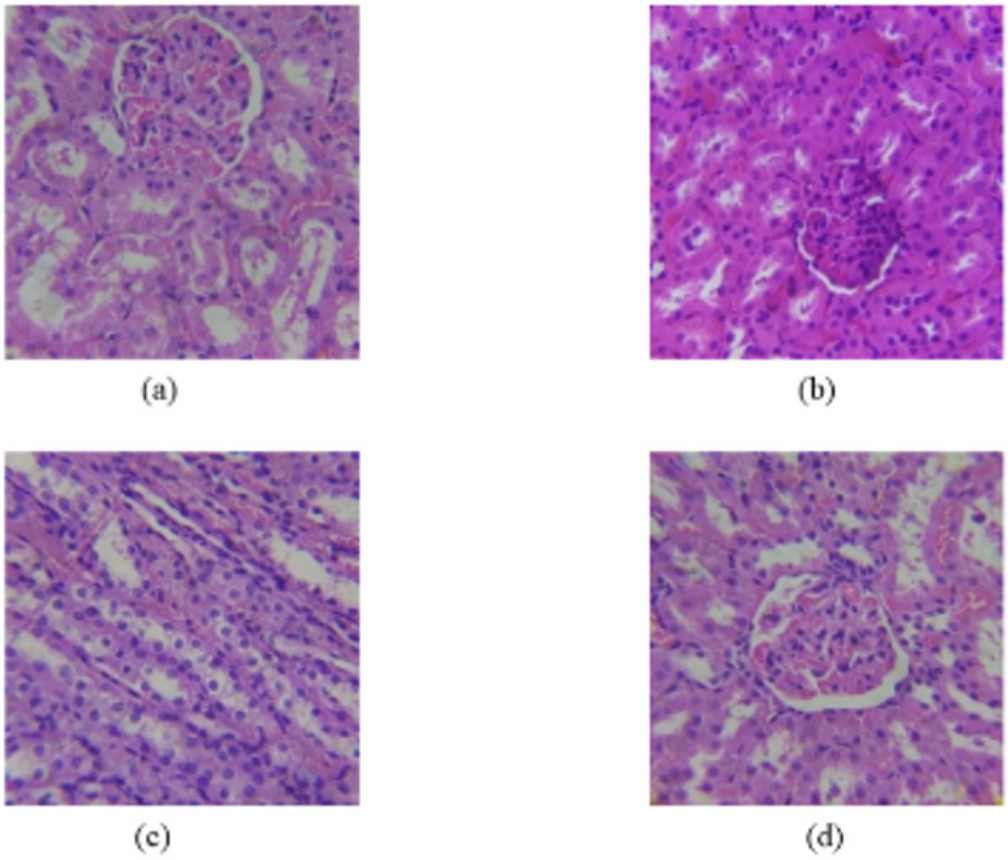

Figure 6: Histopathological examination of the kidney (a) control male (b) control female (c) high dose male (d) high dose female.

period. No pathological changes were detected in the internal organs of the treated animals upon examination. Based on these observations, the Lethal Dose (LD_{50}) of ABM was determined to exceed 2000 mg/kg body weight.

A Repeated Dose 28-Day Oral Toxicity study was conducted by OECD Guideline 407. A total of 10 Wistar albino rats (*Rattus norvegicus* - five males and five females) were divided into four groups. Group I served as the control and received RO-purified water, while Groups II, III, and IV were administered ABM at doses of 15, 75, and 150 mg/kg body weight, respectively. Throughout the 28-day study period, no significant behavioral changes were observed in any of the ABM-treated groups. Body weight gains in both male and female rats were consistent with the control group and remained within the expected physiological range (Table 3). Food and water consumption also showed a gradual increase in the treatment groups compared to controls (Tables 4, 5). The hematopoietic system is an essential marker for assessing toxic compounds and ruling out pathological conditions in both humans and animals.[14] Hematological evaluations revealed no significant differences in total blood counts, with all parameters remaining within normal physiological limits (Table 6). Biochemical parameters serve as important markers due to their response to the clinical signs and symptoms caused by toxicants.

The assessment of renal and hepatic function is critical for evaluating the toxic effects of a drug, as the liver is mainly involved in metabolism and the kidneys are responsible for excretion.[14] The biochemical analysis showed no significant variations, with all biochemical parameters remaining within normal limits (Table 7). The examination of internal cavities and vital organs showed no pathological changes. Histopathological assessments were conducted on the brain, heart, lungs, liver, kidneys, stomach, testes, epididymis, uterus, and ovaries from both control and high-dose groups. No abnormalities or alterations were observed in any of the specimens (Figures 1-9). The findings of this repeated dose 28-day oral toxicity study established that the No Observed Adverse Effect Level of ABM is 150 mg/kg body weight, confirming its safety for human consumption.

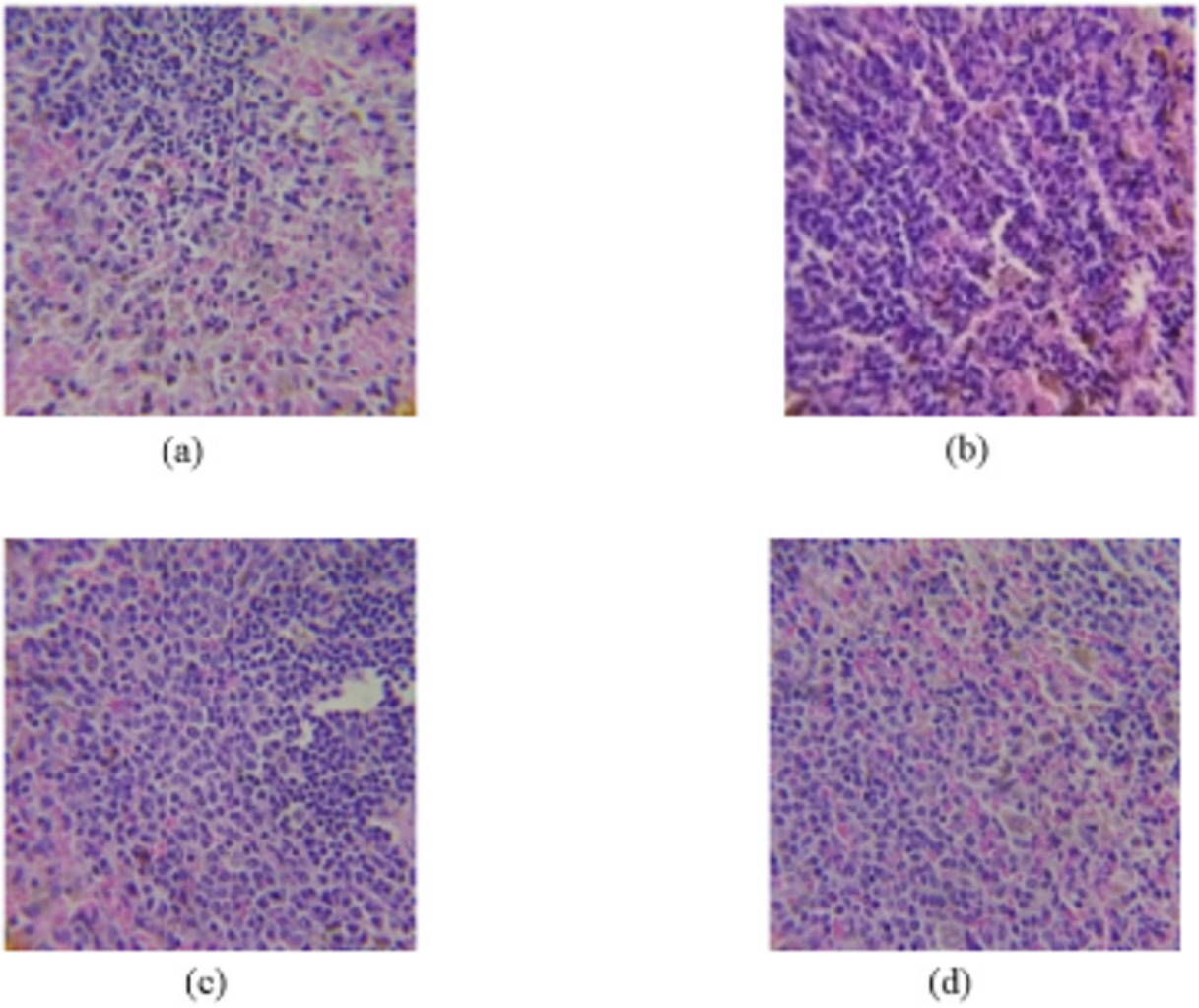

Figure 7: Histopathological examination of the spleen (a) control male (b) control female (c) high dose male (d) high dose female.

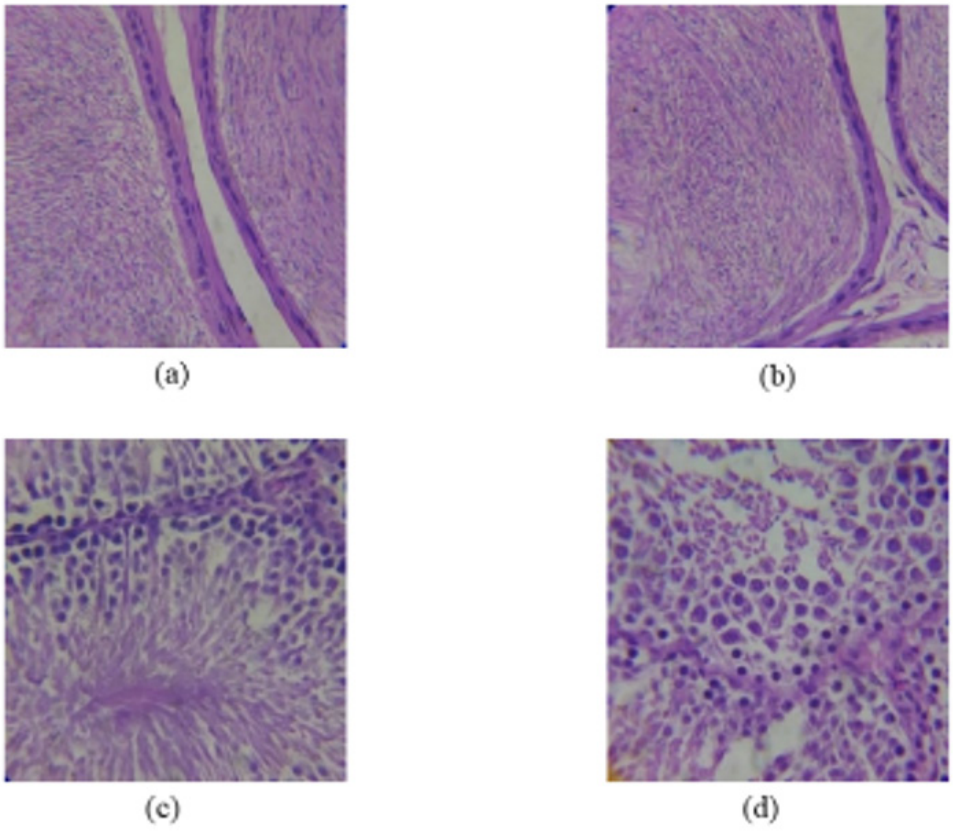

Figure 8: Histopathological examination of the epididymis and testes. (a) Epididymis – control male (b) Epididymis – high-dose male (c) Testes – control male (d) Testes – high-dose male.

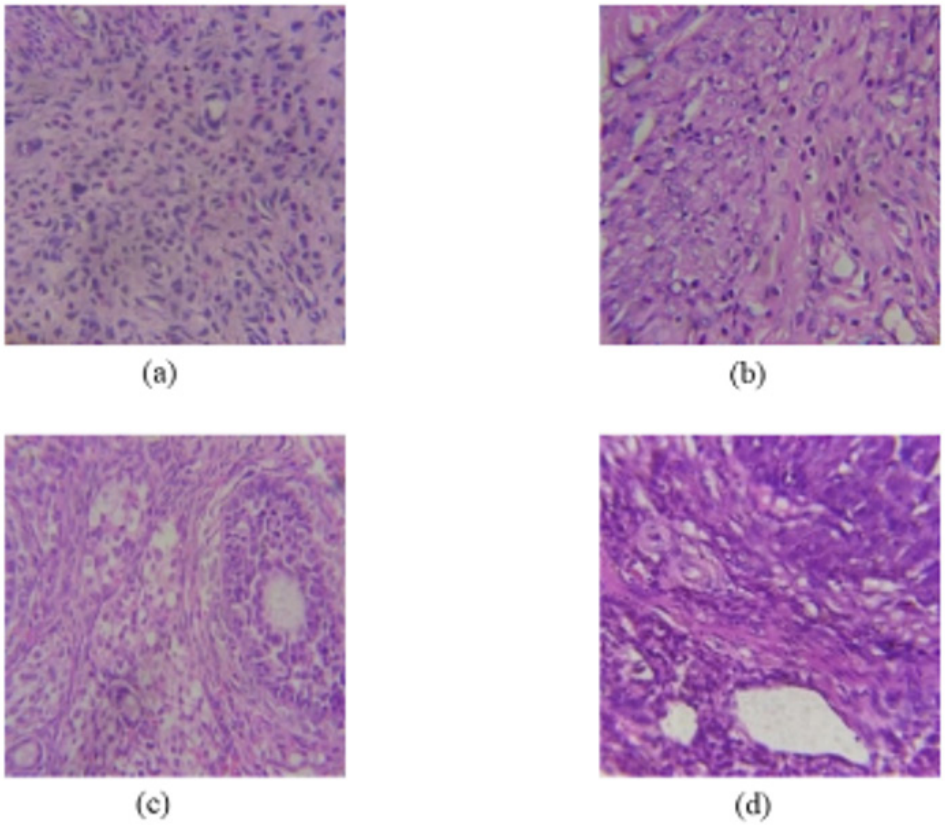

Figure 9: Histopathological examination of the uterus and ovary. (a) uterus – control female (b) uterus – high-dose female (c) ovary – control female (d) ovary – high-dose female.

CONCLUSION

The acute and subacute toxicity studies on Wistar albino rats (*Rattus norvegicus*) revealed no significant alterations in vital organ morphology, hematological profiles, or biochemical parameters. The absence of adverse toxicological effects underscores its safety and aligns with its long-standing usage in Siddha medicine. These findings provide strong evidence for the potential safety of the formulation when used as per traditional guidelines. However, further clinical trials are essential to substantiate its safety in humans, confirm its therapeutic efficacy, and explore its applicability across diverse populations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

NIS: National Institute of Siddha**; ABM:** *Ashtabhairava mathirai*; **OECD:** Organization for Economic Co-operation and Development; **IAEC:** Institutional Animal Ethics Committee; **LD:** Lethal Dose; **RBC:** Red blood cells; **WBC:** White blood cells; **PLT:** Platelet; **HB:** Haemoglobin; **N:** Neutrophils; **L:** Lymphocytes; **M:** Monocytes; **E:** Eosinophil; **B:** Basophil; **MCV:** Mean Corpuscular Volume; **MCH:** Mean Corpuscular Haemoglobin; **MCHC:** Mean corpuscular Haemoglobin concentration; **HCT:** Haematocrit; **HDL:** High Density Lipoprotein; **LDL:** Low Density Lipoprotein; **SGOT:** Serum Glutamic Oxaloacetic Transaminase; **SGPT:** Serum Glutamic Pyruvic Transaminase; **TGL:** Triacylglycerol Lipase; **EDTA:** Ethylene Diamine Tetra acetic Acid; **ANOVA:** Analysis of Variance.

AUTHOR CONTRIBUTION

Conceptualization: RM; Medicine Preparation: RM; Data collection and compilation: RM; Manuscript Writing: RM, AB, KR, SR, SAR, and MR; Proofreading and editing: RM, AB, KR, SR, RR, SAR, and MR.

ETHICS COMMITTEE

The protocol of this study was reviewed and approved by IAEC at the National Institute of Siddha (NIS /IAEC – 24/ RO4/06122022/20).

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