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Acute Oral Toxicity Assessment of *Naath Pottali* (a Traditional Mercurial Formulation) in Female Wistar Albino Rats

[1]Dnyaneshwari Vyawahare, [2*]Prasanna Mathad, [3]Snigdha Das Mandal, [4]Mayur Barve, [5]Shantanu Golde

ABSTRACT:

Background: *Naath Pottali* is a formulation containing *Hingulottha Parada* (mercury extracted from cinnabar), *Shuddha Gandhaka* (purified sulfur), *Rajata Bhasma* (silver ash) and *Swarna Makshika Bhasma* (copper pyrite ash) processed in *Ahiphena Beeja Kashaya* (poppy seeds decoction). Due to potential toxicity of heavy metals, acute oral toxicity study was conducted. The study evaluated acute oral response to a single dose of 2000 mg/kg of *Naath Pottali* in female Wistar rats. **Methods and Materials:** After approval of Institutional Animal Ethics Committee, two groups of female (nulliparous, non-pregnant) Wistar Albino rats (n=3 per group) were selected. Group 1 (vehicle control) received 0.5% carboxymethyl cellulose; Group 2 received single dose of *Naath Pottali* (2000 mg/kg) suspended in 0.5% carboxymethyl cellulose. Animals were observed for body weight, clinical and behavior signs like convulsions or tremors, piloerection, salivation, diarrhea etc. over 14 days. On 15th day, animals were euthanized and the brain, heart, liver, kidney, stomach and intestine were examined for gross and histopathological signs. **Results:** All three animals in trial group as well as in control group survived, and no signs of mortality or severe clinical side effects were observed. Body weight trajectories were normal. On gross necropsy, no macroscopic abnormalities were observed. In histopathological examination of all three treated animals, moderate (grade 3) lesions were noted: periportal inflammation with steatosis in the liver, focal tubular necrosis with hydropic change in the kidney, coronary congestion without ischemic change in the heart, and hypertrophy of mucosal glands in the stomach. Brain and intestine showed no such signs. **Conclusion:** *Naath Pottali*, at the single dose of 2000 mg/kg caused no mortality; this finding cannot be extrapolated to the overall safety of the formulation and histopathological changes in the liver, kidney, heart and stomach indicate potential organ-level stress. Comprehensive dose-response and repeated-dose studies are required to establish safety.

KEYWORDS: Acute oral toxicity, Anticonvulsant, Epilepsy, Histopathology, *Natha Pottali*, *Pottali Kalpana*.

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Corresponding Author Email:

prasanna.mathad86177@paruluniversity.ac.in

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1. INTRODUCTION

Rasashastra vividly demonstrates the preparation and use of herbo-mineral compounds, especially mercurial formulations, for therapeutic purposes. [1] Based on the method of preparation, the medicines prepared from processed mercury are classified into four types, namely *Kharaliya*, *Parpati*, *Kupipakwa* and *Pottali Rasayana*. [2] Among these preparations, *Pottali Rasayana* (a mercurial preparation shaped into a compact bolus, wrapped in cloth and heated) is a specialized preparation in which the ingredients are converted into a compact and comprehensive size and shape that is administered in very small doses. [3] *Naath Pottali* is one such formulation described by Acharya Matsyendranaath and prepared mainly using *Hingulottha Parada* (mercury extracted from cinnabar), *Rajata Bhasma* (ash of silver), *Swarna Makshika Bhasma* (ash of copper pyrite) and *Shuddha Gandhaka* (purified sulfur). [4] At first, *Kajjali* (black mercury sulfide) was prepared from *Hingulottha Parada* (mercury extracted from cinnabar), *Rajata Bhasma* (ash of silver), *Swarna Makshika Bhasma* (ash of copper pyrite) and *Shuddha Gandhaka* (purified sulfur) by levigating a mixture of all these with *Ahiphena Beeja Kashaya* (decoction of poppy seeds). Then this mixture was shaped in the form of *Pottali* (rectangular shape) and the standard *Pottali* preparation method of *Gandhaka-paka* (sulfur-based heating process) was carried out and the final product was *Kurma-Prushthakar* (pentagonal shape) *Pottali*. [5] The metals and minerals present in *Naath Pottali* underwent *Shodhana* (purification) and *Marana* (incineration) beforehand as a part of the traditional preparation process. [6] Although it is believed that this processing makes the formulation safer to use, the effects of these processing steps on toxicity, bioavailability or pharmacological activity were not experimentally evaluated in the present study. As the safety aspect of such formulations, especially of those which contain processed mercury, raises scientific issues and regulatory concerns, an acute oral toxicity study of *Naath Pottali* was needed. There are different methods available to assess the toxicity of a drug such as acute toxicity study (single dose and 14-days observation of effect), sub-acute toxicity study (repeated dose for 14 to 28 days), sub-chronic toxicity study (for 90 days) and chronic toxicity study (for 6 months to lifetime). These methods further are separated according to route of drug administration/ exposure like oral, dermal, inhalation or parenteral route, etc. Each method has its own significance related to toxicity study. Acute oral toxicity establishes the dose that produces lethality or serious toxicity in a short period of time, it determines the median lethal dose (LD50) or its category, provides dose-range data that determine starting doses for subsequent sub-chronic or chronic studies and it identifies target organs and clinical signs early, which helps for further study design. Due to all these advantages acute oral toxicity study method was selected to study toxicity of *Naath Pottali*. Also, there are no sufficient existing *in vivo, in vitro* or other clinical data regarding *Naath Pottali* formulation. OECD (Organisation for Economic Co-operation and Development) test Guideline 423 [7] describes the acute toxic class method, according to which dosing in groups of 3 was done (only the first step was performed in this study).

The aim of this study was to describe the acute oral toxicity findings of *Naath Pottali* following a single dose of 2000 mg/kg in female Wistar rats. The dose-selection rationale was based on the limit-test provision of OECD 423. [7] Before study initiation, available information on constituent materials of *Naath Pottali*, related preparations, and structurally or compositionally related tested materials was considered. OECD 423 permits selection of 2000 mg/kg as the starting dose when available information indicates that mortality is unlikely at that dose; in such circumstances, the lower fixed starting doses of 300, 50 and 5 mg/kg are not required as sequential starting doses. Importantly, toxicity information for individual ingredients or related preparations was used only as supportive evidence for dose selection and was not interpreted as establishing safety of the finished *Naath Pottali* formulation. The present experiment consisted of one three-animal administration at

2000 mg/kg and therefore should not be interpreted as a complete OECD 423 acute toxic class determination or as providing a definitive acute toxicity category or LD50.

In the present study, a dose of 2000 mg/kg body weight was selected as the test dose based on the available pre-study information indicating a low risk of mortality at this dose. Three female rats received the test formulation and were observed for 14 days. All three animals survived without mortality or moribundity. Because the available pre-study information indicated that mortality at this dose was unlikely. [8, 9, 10] Therefore, lower starting doses were not considered necessary under the OECD 423 limit-test approach. The findings therefore represent the observed outcome following administration of 2000 mg/kg in the animals actually tested and should not be interpreted as determination of a precise LD50.

2. MATERIALS AND METHODS:

Preparation of *Naath Pottali*:

Collection of Raw Drugs: The raw materials, namely *Hingula* (cinnabar), *Ashuddha Gandhaka* (impure sulfur), *Rajata* (silver) and *Swarna Makshika* (copper pyrite) were procured from authentic sources, while *Ahiphena Beeja* were procured from a peripheral area of Rajasthan. All the raw drugs were authenticated by the Department of *Rasashastra* and *Bhaishajyakalpana* and by the Department of *Dravyaguna* at the institute with the voucher specimen number PIAR/RDA/2025/50.

Pharmaceutical Preparation of *Naath Pottali*: *Naath Pottali* was prepared according to the standard *Pottali* preparation method as explained in classical Ayurvedic texts. [5]

Parada (mercury) was extracted from *Hingula* (cinnabar); [11] *Shodhana* (purification) of *Gandhaka* (sulfur) [12] *Rajata* (silver) [13] and *Swarna Makshika* (copper pyrite); [14] was done followed by *Marana* (incineration) of *Swarna Makshika* (copper pyrite) [15] and *Rajata* (silver). [16] First *Kajjali* (black mercury sulfide) of *Parada* (mercury) and *Shuddha Gandhaka* (purified sulfur) was prepared and later *Bhasmas* (ashes) of *Swarna Makshika* (Copper pyrite) and *Rajata* (silver) were added and triturated well. To this mixture, *Bhavana* (levigation) of *Ahiphena Beeja Kashaya* (decoction of poppy seeds) [17] was given for seven days. The prepared mass of *Rasakalka* was shaped into small rectangular shape and after it dried completely it was wrapped in *Kshem Vastra* (silk cloth) forming the compact shape of a *Pottali*. This wrapped *Pottali* was then subjected to controlled heating in *Gandhaka* (sulfur) using a sand bath (*Valuka Yantra*). The temperature was gradually increased and maintained at 600°C for a specified duration. After observing signs of complete *Pottali Paka* (digestion), it was allowed to cool naturally. The *Kurma-Prushthakar* (pentagonal shape) *Pottalis* were then collected and stored in airtight containers. The finished product was characterized by physicochemical tests (color, pH, loss on drying, ash value) and advanced analytical parameters (X-ray diffraction [XRD], scanning electron microscopy with energy-dispersive spectroscopy [SEM-EDS], X-ray fluorescence [XRF], inductively coupled plasma mass spectrometry [ICPMS], zeta potential, particle size distribution). [18]

Acute Oral Toxicity Study: The study protocol involving animals was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of the institute, with the project proposal number PIPR 984/2025/01/16, dated August 30, 2025, prior to the initiation of the study. The study was not prospectively registered in a public experimental protocol registry. The study was conducted as per the instructions prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

Experimental Animals:

Selection of Experimental Animals: Certified healthy Wistar albino rats of female sex (nulliparous, non-pregnant), with a body weight of 250 ± 10 g and 8 to 10 weeks old rats were procured from Global Bioresearch Solutions Private Limited, India. The animals were maintained under ideal husbandry conditions mentioned below.

Wistar albino rats were selected for the study because they are one of the recommended species as test system for preclinical toxicity studies. They have been widely used throughout the industry for the evaluation of product safety. Also, extensive data available for comparative purpose.

Experimental Unit: The individual rat was considered the experimental unit because treatment was independently assigned and administered to each animal, and outcome measurements were obtained at the individual-animal level. Thus, n = 3 represents three independent experimental animals in each group.

Husbandry: Animals were kept in standard suspended polypropylene cages (3 animals per cage, matched by group), provided with dust-free paddy husk as bedding material. [7] Bedding was changed regularly to maintain hygienic conditions during study period. The top grill had facilities for pelleted feed and drinking water in polypropylene bottles with stainless steel sipper tubes.

Acclimatization: Animals were kept in a quarantine room for 7 days before starting the study that means prior to dosing. During this time-period they were monitored daily for signs of illness, injury or abnormal behavior to confirm fitness for study inclusion. [7] The animals were certified healthy at the time of procurement and were nulliparous, non-pregnant female Wistar rats. During the 7-day acclimatization/quarantine period, animals were monitored daily for illness, injury or abnormal behavior. No previous experimental procedures, drug administration or other experimental interventions were performed on the animals before their inclusion in the present study.

Feeding and Watering Schedule: The animals had free access to sterile pelleted food of standard composition containing all macro and micronutrients and purified water *ad libitum* in polypropylene bottles with a stainless-steel sipper tube. [10]

Environmental Conditions: The environmental conditions were maintained at 22°C ± 2°C, with 50-60% relative humidity and 10-15 air changes per hour, 12-hour light and 12-hour dark cycle. [7] No additional environmental enrichment was provided.

Inclusion Criteria of Animals: Nulliparous and non-pregnant female rats with 250 ± 10 gram of body weight and 8-10 weeks old, were included in study because, female rats are more sensitive toward physiological changes as explained in OECD guidelines. The predefined inclusion criteria were nulliparous, non-pregnant female Wistar rats aged 8–10 weeks and weighing approximately 250 ± 10 gram, with no signs of illness, injury or abnormal behavior during acclimatization. Animals meeting any predefined exclusion criterion were excluded before allocation to the experimental groups. No animals were excluded after allocation. No animals died, or were removed for humane reasons, or were excluded from the final analysis. No individual observations or data points were excluded from the reported analyses. Therefore, the final analyzed sample included three animals in the vehicle-control group and three animals in the *Naath Pottali* group.

Exclusion of Animals: Pre-study exclusion criteria were established and animals with any signs of injury, illness, abnormal behavior or significant weight variation during acclimatization were excluded from the study.

Groups of Experimental Animals: (Table 1) The acute toxicity study was conducted at a dose of 2000 mg/kg based on pre study information. Animals were kept for overnight fasting before the administration of the drug, but had free access to drinking water. Three hours after drug administration, the animals were allowed to resume eating. [7] OECD 423 specifies fixed starting doses of 5, 50, 300 and 2000 mg/kg body weight and provides for a 2000 mg/kg limit test approach when available information indicates that mortality is unlikely at the highest starting dose. In the present study, pre-study information concerning related preparations and constituent materials was reviewed before dosing and was considered supportive of a low likelihood of mortality at 2000 mg/kg. On this basis, 2000 mg/kg was selected directly as the initial test dose rather than using 300, 50 or 5 mg/kg as lower starting doses. This

decision was based on the anticipated acute lethality at the test dose, not on an assumption that the individual ingredients were intrinsically safe or that their toxicity profile was identical to that of the finished formulation. Because the present experiment included only one group of three animals at 2000 mg/kg, it is reported as a three-animal 2000 mg/kg acute exposure study informed by OECD 423 rather than as a complete TG 423 limit test or definitive acute toxic class determination. [7] The second three-animal step of the TG 423 limit-test sequence was not performed because the IAEC-approved protocol granted permission for limited number of animals as earlier dosages of the individual drugs present in the formulation were found to be safe in previous studies. Further, the available pre-study information was considered sufficient to indicate that mortality of the test material was unlikely at 2000 mg/kg body weight; therefore, 2000 mg/kg was selected as the starting dose in accordance with the limit-test provision of OECD 423. [8, 9, 10] However, these data were used only to support selection of the test dose, the toxicity profile of individual ingredients cannot be considered equivalent to that of the finished *Naath Pottali* formulation because processing, composition, physicochemical characteristics and interactions among constituents may alter its toxicological profile. Therefore, the present study was undertaken specifically to generate acute oral toxicity information for the finished *Naath Pottali* formulation. Animals were allocated to the vehicle-control and *Naath Pottali* groups using body-weight matching to obtain comparable baseline body weights. Formal randomization of animals to treatment groups was not performed. Therefore, the allocation procedure was non-randomized. To minimize potential confounding, animals were of the same sex, age range, strain, comparable body weight and husbandry conditions, and all procedures were conducted under the same environmental and handling conditions. This non-randomized allocation is acknowledged as a methodological limitation of the study and may introduce allocation bias. Trial group (group 2) contained three female rats, with another group of three female rats kept in a vehicle control group (group 1). Female rats were used as explained in OECD guideline due to their more sensitive nature toward physiological changes. Animals of the trial group received a single dose and a 14-day observation period. Following the observation recommendations of the OECD guideline, clinical observations included monitoring for mortality, behavioral abnormalities, effects on the autonomic and central nervous systems, and general indications of toxicity were done.

Group 1 served as the vehicle control group and received 0.5% carboxymethyl cellulose (CMC) alone, administered at the same volume per unit body weight as Group 2 (6 mL/kg; approximately 1.5 mL for a 250 g animal), with animals having a mean body weight of 250 ± 10 gram. Group 2 received a single dose of the test substance at 2000 mg/kg body weight, prepared in 0.5% CMC as the vehicle. Based on a mean body weight of 250 ± 10 gram, the calculated dose administered was 500 mg per animal (0.250 kg x 2000 mg/kg), given at a volume calculated individually from each animal's body weight (6 mL/kg, i.e., approximately 1.5 mL for a 250-gram animal). The drug was administered as a freshly prepared suspension at a concentration of 333.33 mg/mL in 0.5% CMC in distilled water. Individual doses were calculated according to the actual body weight of each animal at 2000 mg/kg body weight.

Table 1: Groups of Wistar Albino Rats for Acute Oral Toxicity Study.

Group	Treatment/Dose	Body weight (g)	Dose (mg)	Volume (mL)	Vehicle
Group 1	Vehicle Control	250 ± 10	-	6 mL/kg (≈1.5 mL for 250 g)	0.5% CMC
Group 2	2000 mg/kg	250 ± 10	0.250 x 2000 = 500 (for a 250 g animal)	6 mL/kg (≈1.5 mL for 250 g)	0.5% CMC

Abbreviations: CMC = carboxymethyl cellulose; g = gram; mL = milliliter; mg/kg = milligram per kilogram of body weight; n = 3 animals per group. Body weight (250 ± 10 g) is the nominal inclusion range; individual weights are given in Table 2.

Outcome Measures and Blinding: The principal acute-toxicity endpoint was mortality within 14 days. Supportive observations included clinical signs, body weight changes, gross pathology, and histopathology. Animal allocation and dose administration were not blinded because the person administering the test substance knew the treatment being administered. Clinical observations, body-weight measurements and gross necropsy assessment were performed by a person blinded to treatment allocation. Histopathological slides were coded before microscopic examination and were assessed by an independent pathologist who was blinded to treatment allocation. The data analyst was also blinded to group identity during data compilation and descriptive analysis; treatment codes were revealed only after completion of the analysis.

Dose Preparation and Administration: [Table 2] *Naath Pottali* was freshly suspended in 0.5% carboxymethylcellulose (CMC) in distilled water on the day of administration. The required dose was calculated individually for each animal according to its actual body weight measured immediately before dosing. The administered dose was calculated using the formula - Individual dose (mg) = individual body weight (kg) × 2000 mg/kg. No body surface area conversion was used for administration of the experimental dose. The dosing volume was adjusted according to the individual body weight (6 mL/kg) while maintaining a constant suspension concentration of 333.33 mg/mL, i.e., individual volume (mL) = individual dose (mg) ÷ 333.33 mg/mL, giving approximately 1.5 mL for a 250-gram animal. Control animals received the vehicle at the same volume per body weight (6 mL/kg). The dose (mg) and volume (mL) actually administered to each animal are listed in Table 2. The volume was below the OECD TG 423 maximum for rodents (1 mL/100 g body weight). [7] Oral administration was performed by gavage using a suitable intragastric cannula following the prescribed fasting period. [19]

Table 2: Individual animal body weights, administered dose and volume

Animal ID	Group	Day 0 Body Weight (g)	Dose Given (mg)	Volume Given (mL)
V1	Vehicle control group	248	NA	1.488
V2	Vehicle control group	250	NA	1.5
V3	Vehicle control group	250	NA	1.5
T1	Trial group (2000 mg/kg)	250	500	1.5
T2	Trial group (2000 mg/kg)	249	498	1.494
T3	Trial group (2000 mg/kg)	250	500	1.5
Mean ± SD	Vehicle control group	249.33 ± 1.15	NA	NA
Mean ± SD	Trial group (2000 mg/kg)	249.67 ± 0.58	NA	NA

Abbreviations: g = gram; mg = milligram; mL = milliliter; mg/kg = milligram per kilogram of body weight; NA = not applicable (vehicle only); SD = standard deviation. n = 3 animals per group; mean ± SD calculated from the three individual values per group (sample SD, n-1). Dose (mg) = individual body weight (kg) x 2000 mg/kg; volume (mL) = dose (mg) ÷ 333.33 mg/mL.

Clinical and Behavioral Observations: Animals were observed individually for clinical signs such as mortality, fur and skin conditions, body weight, salivation and respiratory distress, behavioral changes such as tremors, convulsions, lethargy or hyperactivity, for the first 30 minutes, at the end of one, two and six hours on the day of dosing, then every 12 hours on days two and three, and twice a day thereafter untill 14th day. Throughout the trial period, every animal was checked twice a day for illness and mortality. To identify any sign of toxicity, observations were recorded, including general appearance, skin and fur condition, eyes, respiration, autonomic and central nervous system impacts, and behavioral changes. Clinical observations and body-weight measurements were blinded to group allocation. [7]

Humane Endpoints and Monitoring Criteria: Animals were observed twice a day for the 14-day observation period for indications that would suggest an early termination of the experiment on humane grounds. The humane endpoints, as defined before conducting the study, were: a weight loss of more than 20% from the body weight before dosing; an inability to access food and water; severe or prolonged convulsions; difficulty in breathing or no breathing; lethargic behavior or insensitivity to outside stimulation; self-mutilation or severe ulcers in the skin; and any other indication stated by the veterinarian attending to the animals that indicated irreversible suffering or moribundity. All the animals meeting any of these criteria were to be immediately euthanized by cervical dislocation under diethyl ether anesthesia, instead of being allowed to die naturally. [7] This humane-endpoint euthanasia (unscheduled) was a contingency procedure only; no animal met a humane endpoint, so it was not used in this study.

Gross Pathological Examination: At the end of the 14-day observation period, all surviving animals were euthanized humanely and thorough gross necropsy was performed. Vital organs including the brain, heart, kidneys, intestine, liver, and stomach, were examined for gross pathological changes (color, texture, presence of lesions or hemorrhage).

Euthanasia Procedure: Necropsy was done on the 15th day. Two distinct procedures are described. (i) Humane-endpoint euthanasia was not required, as no animal met a humane endpoint. (ii) Terminal euthanasia was performed on all six animals (three control, three treated) at the end of the 14-day observation period: animals were euthanized by an overdose of Ketamine (200 mg/kg) and Xylazine (20 mg/kg) via intraperitoneal injection, in accordance with CPCSEA guidelines, and death was then confirmed by the absence of respiratory movements and cardiac activity such as no heartbeat on palpation for minimum five minutes, also absence of corneal response on touch and no response to flashlight when applied ; then dissected and the internal organs viz. the brain, liver, kidneys, stomach, heart, and intestine were removed, freed of fat and connective tissue. After observation, organs were transferred to 10% phosphate buffered formalin solution for fixation, subjected to dehydration, wax embedding, sectioning and staining with hematoxylin and eosin for histological evaluation. [21]

Histopathological Examination: (Table 3) Histopathological examination was conducted as a supportive assessment. Observations were done under a trinocular research Carl Zeiss microscope at various magnifications to note down the histopathological changes in the following organs: brain, liver, kidneys, stomach, heart, and intestine. [21] The slides were assessed by a qualified pathologist who was blinded to treatment allocation. Control and treated specimens were collected, fixed, processed (dehydration, paraffin embedding, sectioning at 3-5 µm) and stained with hematoxylin and eosin together, in the same batch and under identical conditions; slides were coded by a person independent of the evaluation, and the pathologist examined all coded slides from control and treated animals in a single session without knowledge of group allocation. Lesions were graded for each animal and organ on a six-level (grade 0-5) severity scale (Table 3), and individual-animal findings are reported in Tables 9 and 10.

Table 3: Histopathological severity grading criteria (six-level scale, grade 0-5)

Grade	Severity	Criterion (approximate extent of tissue affected)
0	Absent	No lesion; within normal limits
1	Minimal	Barely detectable; <10% of tissue/field
2	Mild	10%-25%
3	Moderate	26%-50%
4	Marked	51%-75%
5	Severe	>75%

Abbreviation: % = approximate percentage of tissue/microscopic field affected. Grades 0-5 were assigned per animal for each organ.

Statistical Analysis: Body weight values of animals have been represented using descriptive statistics in the form of mean ± SD (sample SD, n-1, calculated from the individual values in Table 2; with n=3 the mean and SD are imprecise and must be read together with the individual values).

Because of the very small number of animals (n=3 per group) and because only a single three-animal step was performed, no inferential statistical comparison between the groups was carried out but only descriptive analysis was conducted. Testing for the normality or homogeneity of variance was not required as no hypothesis testing was undertaken.

3. OBSERVATION AND RESULTS:

Primary Outcomes:

Clinical Observations and Mortality: (Table 4) Animals showed no signs of treatment related side effects or mortality throughout the 14-day observation period. (Table 4) None of the animals in either treatment group reached the humane endpoints. All three treated animals survived the 14-day observation period following a single administration of 2000 mg/kg, without mortality or moribundity. Although the 2000 mg/kg dose was selected using the dose-selection rationale of OECD TG 423, the present experiment comprised only one three animal step and therefore did not constitute a complete TG 423 acute toxic class determination. Accordingly, the findings are interpreted as the observed acute response to a single 2000 mg/kg administration rather than as an OECD/GHS acute toxicity classification. Because no mortality occurred at the tested dose, an exact LD50 value was not determined.

Secondary Outcome Measures:

Body Weight: (Table 5) No animal showed any signs of abnormal weight loss in the entire span of the study.

Gross Pathological Examination: (Table 6) No signs of abnormal appearance were observed in the gross pathological examination of any experimental animal.

Table 4: Observations of Clinical Findings and Mortality in vehicle control group and trial group

Sr. No.	Observation Parameter	Finding in vehicle control group	Finding in trial group (2000 mg/kg)
1.	Mortality	0/3 (No mortality)	0/3 (No mortality)
2.	Skin and fur	Normal	Normal
3.	Eyes and mucous membrane	Normal	Normal
4.	Convulsions/ tremors	Absent	Absent
5.	Piloerection	Absent	Absent
6.	Salivation/ diarrhea	Absent	Absent
7.	Respiratory distress	Absent	Absent
8.	Body weight trajectory	Normal progressive gain	Normal progressive gain
9.	Food and water intake	Normal	Normal
10.	Gross behavior	Normal	Normal
11.	Gross necropsy findings	No macroscopic lesions	No macroscopic lesions

n=3 animals per group; mortality is expressed as number of deaths/number of animals treated (0/3 = no deaths). No inferential statistics were applied; findings are descriptive.

Table 5: Body weight of animals during 14-day observation study

Animal ID	Animal Groups	Body weight on day of dosing, Day 0 (g)	Body weight on Day 7 (g)	Body weight on day of sacrifice Day 14 (g)
V1	Vehicle control group	248	252	260
V2	Vehicle control group	250	255	264
V3	Vehicle control group	250	254	268
T1	Trial group (2000 mg/kg)	250	254	265
T2	Trial group (2000 mg/kg)	249	255	260
T3	Trial group (2000 mg/kg)	250	255	265

Mean ± SD	Vehicle control group	249.33 ± 1.15	253.67 ± 1.53	264.00 ± 4.00
Mean ± SD	Trial group (2000 mg/kg)	249.67 ± 0.58	254.67 ± 0.58	263.33 ± 2.89

Abbreviation: g = gram; SD = standard deviation. n = 3 animals per group; mean ± SD calculated from the three individual values per group (sample SD, n-1). Day 0 = day of dosing.

Table 6: Gross Pathological Examination of the organs in vehicle control and trial group

Sr. No.	Organs	Vehicle control group	Trial group (2000 mg/kg)
1.	Brain	Normal	Normal
2.	Heart	Normal	Normal
3.	Liver	Normal	Normal
4.	Kidneys	Normal	Normal
5.	Stomach	Normal	Normal
6.	Intestine	Normal	Normal

n = 3 animals per group; all animals were examined at necropsy. Normal = no macroscopic abnormality.

Histopathological Examination: (Tables 7, 8, 9 and 10) Some histopathological changes were observed in the organs of the euthanized treated animals: moderate (grade 3) lesions were present in the liver, kidney, heart and stomach of all three treated animals (3/3), whereas the brain and intestine showed no abnormality (Tables 8 and 10). Representative photomicrographs of the vehicle control and trial groups are shown in Figures 1-12 (brain, Figures 1 and 2; heart, Figures 3 and 4; liver, Figures 5and 6; kidney, Figures 7 and 8; stomach, Figures 9 and 10; intestine, Figures 11 and 12). Coded histopathological slides were examined by an independent pathologist, who was blinded to the treatment groups to minimize bias. Individual animal-level findings and severity grades are given in Tables 9 and 10 (grading criteria in Table 3).

Table 7: Histopathological findings in the vehicle control group animal organs

Organ	Normal Histological Features	Severity	Incidence
Brain	Cortical layering; vesicular neurons	Absent	0/3
Heart	Parallel myofibers; intercalated discs; patent coronaries	Absent	0/3
Liver	Hepatocyte cords; central veins; portal triads	Absent	0/3
Kidney	Normal glomeruli; intact tubular epithelium	Absent	0/3
Stomach	Parietal/chief cells; gastric glands; intact mucosa	Absent	0/3
Intestine	Villi; goblet cells; crypts; normal lamina propria	Absent	0/3

Incidence = number of animals with the finding/number of animals examined (n=3 per group); severity graded per Table 3 (Absent = grade 0).

Table 8: Histopathological findings in the *Naath Pottali*-treated animal organs

Organ	Normal Histological Features	Histopathological Findings (at 2000 mg/kg dose)	Severity	Incidence
Brain	Cortical layering; vesicular neurons	No significant histopathological abnormality	Absent	0/3
Heart	Parallel myofibers; intercalated discs; patent coronaries	Congested coronaries; no ischemic change	Moderate (grade 3)	3/3
Liver	Hepatocyte cords; central veins; portal triads	Periportal inflammation and steatosis	Moderate (grade 3)	3/3
Kidney	Normal glomeruli; intact tubular epithelium	Focal tubular necrosis with hydropic change	Moderate (grade 3)	3/3

Stomach	Parietal/chief cells; gastric glands; intact mucosa	Mucosal gland hypertrophy	Moderate (grade 3)	3/3
Intestine	Villi; goblet cells; crypts; normal lamina propria	No significant histopathological abnormality	Absent	0/3

Severity graded per Table 3 (Moderate = grade 3); incidence = number of animals with the finding/number of animals examined (n=3 per group); mg/kg = milligram per kilogram of body weight.

Table 9: Individual animal histopathological findings and severity grades, vehicle control group

Organ	Finding (vehicle control)	Animal V1 (grade)	Animal V2 (grade)	Animal V3 (grade)
Brain	No abnormality	0	0	0
Heart	No abnormality	0	0	0
Liver	No abnormality	0	0	0
Kidney	No abnormality	0	0	0
Stomach	No abnormality	0	0	0
Intestine	No abnormality	0	0	0

Grade 0 = absent (Table 3); V1-V3 = individual vehicle control animals (n = 3).

Table 10: Individual animal histopathological findings and severity grades in *Naath Pottali*-treated animal organs

Organ	Finding (at 2000 mg/kg dose)	Animal T1 (grade)	Animal T2 (grade)	Animal T3 (grade)
Brain	No significant histopathological abnormality	0	0	0
Heart	Congested coronaries; no ischemic change	3	3	3
Liver	Periportal inflammation and steatosis	3	3	3
Kidney	Focal tubular necrosis with hydropic change	3	3	3
Stomach	Mucosal gland hypertrophy	3	3	3
Intestine	No significant histopathological abnormality	0	0	0

Grade 3 = moderate (Table 3); T1-T3 = individual animals treated with 2000 mg/kg (n = 3); mg/kg = milligram per kilogram of body weight.

Brain

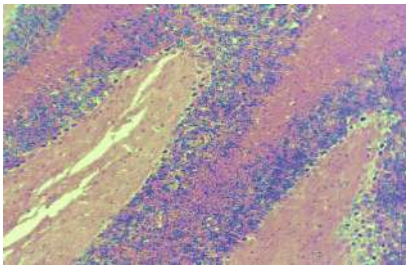


Figure 1: Photomicrograph of Brain (vehicle control group); hematoxylin and eosin (H&E) stain; 10X magnification. (Image courtesy: Dr. Dnyaneshwari Vyawahare)

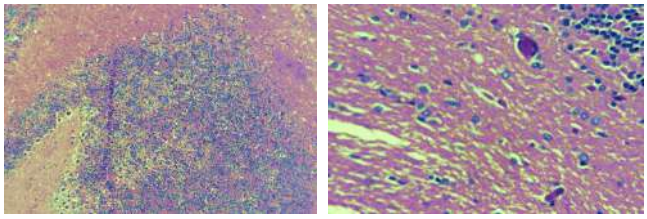


Figure 2: Photomicrograph of Brain (trial group); H&E stain; (Left) 10X magnification; (Right) 40X magnification. Shows no abnormality. (Image courtesy: Dr. Dnyaneshwari Vyawahare)

Heart

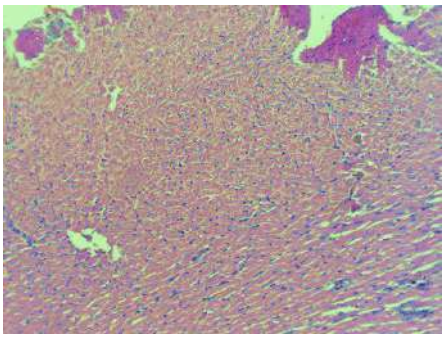


Figure 3: Photomicrograph of Heart (vehicle control group); H&E stain; 10X magnification (Image courtesy: Dr. Dnyaneshwari Vyawahare)

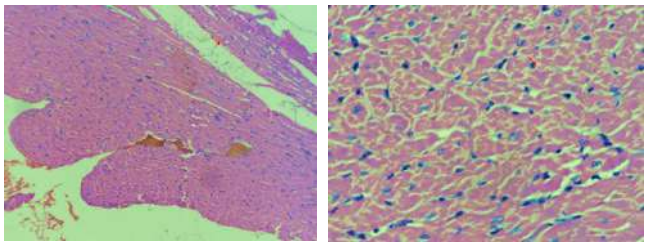


Figure 4: Photomicrograph of Heart (trial group); H&E stain; (Left) 10X magnification ; (Right) 40X magnification. Shows congested coronaries. (Image courtesy: Dr. Dnyaneshwari Vyawahare)

Liver

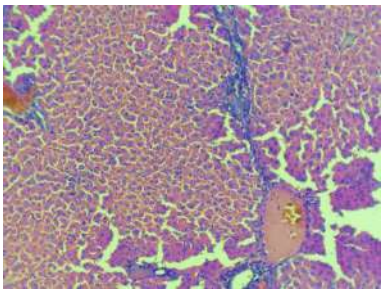


Figure 5: Photomicrograph of Liver (vehicle control group); H&E stain; 10X magnification. (Image courtesy: Dr. Dnyaneshwari Vyawahare)

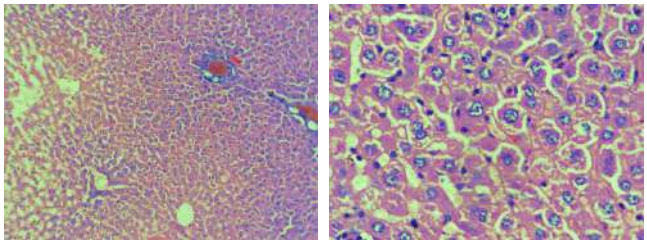


Figure 6: Photomicrograph of Liver (trial group); H&E stain; (Left) 10X magnification ; (Right) 40X magnification. Shows periportal inflammation and steatosis. (Image courtesy: Dr. Dnyaneshwari Vyawahare)

Kidney

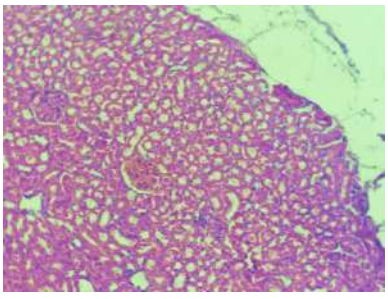


Figure 7: Photomicrograph of Kidney (vehicle control group); H&E stain; 10X magnification. (Image courtesy: Dr. Dnyaneshwari Vyawahare)

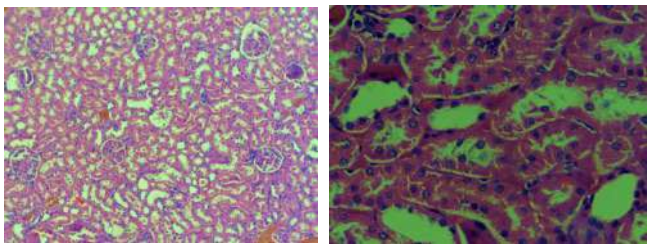


Figure 8: Photomicrograph of Kidney (trial group); H&E stain; (Left) 10X magnification ; (Right) 40X magnification. Shows focal tubular necrosis. (Image courtesy: Dr. Dnyaneshwari Vyawahare)

Stomach

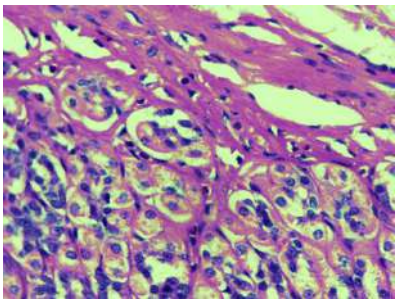


Figure 9: Photomicrograph of Stomach (vehicle control group); H&E stain; 10X magnification. (Image courtesy: Dr. Dnyaneshwari Vyawahare)

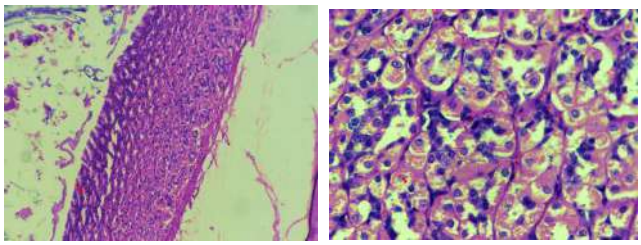


Figure 10: Photomicrograph of Stomach (trial group); H&E stain; (Left) 10X magnification ; (Right) 40X magnification. Shows mucosal gland hypertrophy. (Image courtesy: Dr. Dnyaneshwari Vyawahare)

Intestines

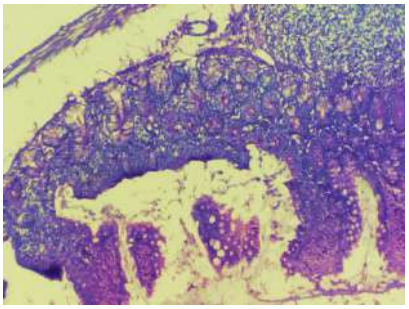


Figure 11: Photomicrograph of Intestine (vehicle control group); H&E stain; 10X magnification. (Image courtesy: Dr. Dnyaneshwari Vyawahare)

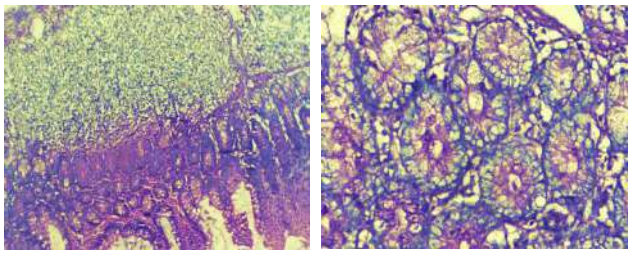


Figure 12: Photomicrograph of Intestine (trial group); H&E stain; (Left) 10X magnification ; (Right) 40X magnification. Shows no abnormality. (Image courtesy: Dr. Dnyaneshwari Vyawahare)

4. DISCUSSION:

This study assessed the acute oral toxicity of *Naath Pottali* in Wistar albino rats. *Naath Pottali* contains *Hingulottha Parada* (mercury extracted from cinnabar), *Shuddha Gandhaka* (purified sulfur), [22] *Swarna Makshika Bhasma* (incinerated copper pyrite ash), and *Rajata Bhasma* (incinerated silver ash); all these ingredients were processed with *Ahiphena Beeja Kashaya* (decoction of opium seeds). All these minerals and metals undergo different purification procedures. After the preparation of *Naath Pottali* as described in the classics, it was subjected to physicochemical tests (color, pH, loss on drying, ash value) and advanced analytical parameters (XRD, SEM-EDS, XRF, ICPMS, zeta potential, particle size distribution). The analytical characterization provides important physicochemical context for interpreting its toxicological profile. *Naath Pottali* exhibited a nanoscale particle size (Z-average: 893 nm; D50: 644.3 nm) with a zeta potential of -9.63 mV. Elemental analysis identified sulfur as the predominant element (58.84%), along with mercury and silver, while XRD indicated α-HgS (cinnabar) as the major crystalline phase. [18] After analytical study *Naath Pottali* was subjected to an acute toxicity study, using the dose-selection approach of OECD Guideline 423 (first three-animal step only), at a single dose of 2000 mg/kg. [23]

Only the first three-animal step was performed in the present experiment. Thus, the absence of mortality in these three animals supports the conclusion that no mortality was observed under the tested conditions, but it does not by itself establish the complete TG 423 limit-test outcome or permit assignment of an acute toxicity category. Lower doses were not administered because 2000 mg/kg was selected as the initial limit-test dose; however, the study did not proceed to the additional same dose step required for a complete 2000 mg/kg limit-test determination.

Compared with previous toxicity studies of formulations containing mercury, sulfur and silver, some toxicity was observed at the given dose. *Rasa Parpati* was evaluated acutely at 2000 mg/kg and repeatedly at 22.5-225 mg/kg for 90 days, and *Purna Cantirotaya Centuram* (PCC) (which contains mercury and sulfur) at 2.5-50 mg/kg for 28 days. *Rasa Parpati* produced dose-related visceral toxicity, while PCC showed only mild gastric hyperplasia. *Swarna Makshika Bhasma* showed no genotoxicity at 4.5 mg/kg. *Rajat Bhasma* caused mortality at 2000 mg/kg, unlike herbal silver nanoparticles but was safe at lower doses. On the other hand, *Naath Pottali* was tested only at a limit dose of 2000 mg/kg, whereas despite the absence of mortality at 2000 mg/kg, *Naath Pottali* showed hepatic, renal, cardiac and gastric lesions. [8, 9, 10]

During the 14-day observation period, no mortality was seen among animals of the trial group. In contrast, histopathological examination showed moderate (grade 3) lesions in the liver, kidney, heart and stomach of all three treated animals (3/3; Table 8).

In the histological study, the brain and intestine did not show any treatment-related histopathological changes. In the examined brain sections no significant histopathological abnormality was observed (Figures 1 and 2). These findings provide no histological evidence of structural brain injury under the conditions of the present study; however, they do not exclude functional or molecular neurotoxicity. Also, there were no signs of villous atrophy, crypt hyperplasia or any other abnormality observed in the intestine (Figures 11 and 12).

In the liver, no hepatocellular necrosis, bile duct proliferation, bridging fibrosis, or cholestasis was observed. However, at higher magnification a proportion of hepatocytes in the periportal zone showed cytoplasmic vacuolation with lipid droplet accumulation (steatosis;

Figures 5 and 6). In the kidney some of the tubular epithelial cells were damaged (Figures 7 and 8). Focal tubular necrosis with hydropic change indicates acute nephrotoxic injury; it suggests a mild to moderate acute tubular injury.

The present findings demonstrate the occurrence of these histopathological alterations in the examined *Naath Pottali*-treated animals but do not establish that all observed lesions were caused by *Naath Pottali*. The findings may represent treatment-associated changes, spontaneous background lesions, or changes influenced by other experimental factors. Additional studies incorporating individual lesion scoring, appropriate clinical biochemistry and larger sample sizes are required to establish treatment-association and toxicological significance. The liver's characteristic toxicological reactions to xenobiotic metabolic stress include periportal hepatic inflammation and steatosis. After oral dosage, the periportal area of the hepatic acinus receives nutritional and xenobiotic-rich portal blood first. This makes the periportal zone more prone to concentrated exposure. A common early hepatic reaction to metabolic excess, fatty alteration (steatosis) in this area shows disturbance of normal lipid metabolism. An immune-mediated or reactive inflammatory response is suggested by the associated mononuclear periportal inflammation. Although not lethal, these alterations suggest hepatic stress at 2000 mg/kg.

These findings were broadly consistent with the prior acute toxicity evaluation studies of other Ayurvedic formulations containing mercurial and heavy-metals, where mortality was absent at the limit dose, with histopathological evidence of hepatic and renal involvement. [8, 9, 23]

A well-known pattern of acute tubular injury (ATI) involves focal tubular necrosis with hydropic change in the kidney. It is typically caused by nephrotoxic chemicals that are concentrated in proximal tubular cells during urine excretion. The proximal tubule segment is more prone to xenobiotic damage because of its many mitochondria, high metabolic activity, and active transporters that help in the reabsorption of filtered solutes. In contrast to broad cortical necrosis, the localized distribution of necrosis suggests that the renal injury is still in its early stages and may be reversible. However, reversibility was not directly assessed in this study, as animals were not maintained beyond the 14-day observation period; confirming recovery or progression of these changes would require a follow-up or dedicated reversibility arm.

Coronary congestion without observed ischemic change was noted in the examined cardiac sections (Figures 3 and 4). Mucosal gland hypertrophy was noted in the stomach (Figures 9 and 10). No mucosal erosion, ulceration, hemorrhage, submucosal edema or acute inflammatory infiltration was observed in the intestine (Figures 11 and 12). Because OECD 423 uses mortality as the main classification parameter, organ-level changes can occur without death; the absence of mortality therefore does not imply the absence of toxicity.

Since the brain is the intended pharmacological target of *Naath Pottali*, the absence of major histopathological changes in brain tissue is noteworthy.

Limitations:

1. The study duration was limited to a single-dose, 14-day acute exposure and does not address the effects of repeated or prolonged administration. Reversibility of the observed hepatic, renal and gastric changes was not tested. Animals were allocated by body-weight matching, not randomization.
2. Serum clinical biochemistry (liver function tests such as alanine aminotransferase [ALT], aspartate aminotransferase [AST], alkaline phosphate [ALP] and bilirubin; kidney function tests such as serum creatinine, blood urea nitrogen and uric acid) and hematological parameters were not evaluated, despite the presence of focal histopathological lesions in the kidney, liver, stomach and heart. Absolute and relative organ weights, and quantitative food and water intake, were not recorded.

3. Only the first three-animal step of TG 423 was performed (n = 3 per group); the second step required for a complete 2000 mg/kg limit test was not performed because the IAEC-approved protocol permitted a limited number of animals. With n = 3, statistical power to detect low-incidence effects is limited; a single animal strongly influences group values, and the absecnce of mortality cannot exclude a low incidence lethal effect. No inferential statistics were performed (body weight is reported descriptively as mean ± SD); the findings apply only to the animals tested.

5. CONCLUSION:

A single oral dose of 2000 mg/kg of Naath Pottali caused no mortality in the three female Wistar rats tested during the 14-day observation period. However histopathological examination showed moderate (grade 3) lesions in all three treated animals (3/3): periportal inflammation with steatosis in the liver, focal tubular necrosis with hydropic change in the kidney, coronary congestion without ischemic change in the heart, and mucosal gland hypertrophy in the stomach; the brain and intestine were unaffected. The absence of mortality therefore must not be equated with absence of toxicity or with safety, and the treatment relation and toxicological significance of these lesions require confirmation. As only the first step using three-animals of OECD TG 423 was performed, no acute toxicity category or LD50 is assigned. Repeated dose studies with biochemical investigations are required to establish the safety profile.

Authors Details:

[1]3rd Year P.G. Scholar, Department of Rasashastra & Bhaishajya Kalpana, Parul Institute of Ayurved & Research, Vadodara, Gujarat, India.

ORCID link: https://orcid.org/0009-0004-1172-1839

[2*]Professor & H.O.D. Department of Rasashastra & Bhaishajya Kalpana, Parul Institute of Ayurved & Research, Parul University,Vadodara, Gujarat, India.

ORCID link: https://orcid.org/0000-0002-5521-9822

[3]Professor, Department of Pharmacology, Parul Institute of Pharmacy and Research, Parul University

ORCID link: https://orcid.org/0000-0001-7437-6535

[4]Professor, Department of Rasashastra & Bhaishajya Kalpana, Parul Institute of Ayurved & Research, Parul University, Vadodara, Gujarat, India.

ORCID link: https://orcid.org/0009-0000-0966-0273

[5]2nd Year P.G. Scholar, Department of Pharmacology, Parul Institute of Pharmacy and Research, Parul University,Vadodara, Gujarat, India.

ORCID link: https://orcid.org/0009-0008-1599-2715

Authors Contribution:

Conceptualization and clinical management: DV

Data collection and literature search: DV

Writing – original draft preparation: DV

Reviewing & editing: PM, SDM, MB, SG

Approval of final manuscript: All authors

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Ethical Statement: The study was conducted in accordance with applicable ethical principles and was approved by the Institutional Animal Ethics Committee (IAEC) of Parul University, Parul Institute of Pharmacy and research, Parul University, Vadodara, Gujarat (Approval No.: PIPR 984/2025/01/16, dated 30/08/2025). All animal procedures were conducted in accordance with applicable institutional and national guidelines for the care and use of laboratory animals.

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