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Biosafety Assessment of Shagandha, Standardized Extract From the Roots of *Withania somnifera*

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ABSTRACT

Withania somnifera, commonly known as Ashwagandha, is a widely recognized medicinal plant in India, belonging to the family Solanaceae, used in Ayurveda due to its diverse therapeutic properties. The roots of Ashwagandha are considered the most active part of the plant, for its biological and pharmacological effects. However, very little scientific evidence regarding its safety assessment has been published. Thus, the objective of the present study was to assess the safety of the standardized extract of Ashwagandha, known as Shagandha, which is prepared from the roots of Ashwagandha containing 2.5% Withanolides, analysed using a USP method (HPLC). The GLP studies for acute, subacute, subchronic, reproductive, bacterial reverse mutation assay, and mammalian erythrocyte micronucleus test were conducted following the test guidelines established by the Organization for Economic Cooperation and Development (OECD). Treatment with Shagandha (Ashwagandha Root Extract-ARE) did not result in any toxicologically significant changes regarding abnormal clinical signs or behavioral changes, body weight, reproductive and developmental parameters, or gross and histopathological changes. Additionally, the results of genotoxicity as evaluated by the in vitro reverse mutation assay and in vivo micronucleus test in mice demonstrated that ARE did not induce any genotoxic effects. These findings indicate that the oral administration of ARE is safe in rodents, non-mutagenic, with no adverse effects under experimental conditions.

1 | Introduction

In recent times, research into naturally derived herbal products has shown significant promise for managing various chronic disorders, including neurodegenerative and neurological conditions. Among the most well-known Indian medicinal plants, *Withania somnifera*, commonly known as Ashwagandha, Indian Ginseng, or winter cherry, has held a prominent place in Ayurvedic preparations since the Vedic era, dating back 3000–4000 years. In Ayurveda, *Ashwagandha* is classified as a Rasayana, meaning a rejuvenating tonic, and is widely used by people of all ages for various imbalances.

Ashwagandha has been used as an adaptogen, an anti-stress agent, a memory enhancer, and a remedy for cognitive deficits (Dafni and Yaniv 1994; Andallu and Radhika 2000). The phytochemical constituents in *Withania somnifera* have been extensively studied and include alkaloids, steroidal lactones (withanolides, withaferins), saponins, glycol withanolides, and tannins. Withanolides, the steroidal lactones, with 28-carbons of triterpenoid backbone, are the primary chemical constituents and most studied pharmacologically active constituents in the plant (Mirjalili et al. 2009; Sharma et al. 2011; Singh et al. 2010). Numerous studies, both in vitro and animal models, have demonstrated that

W. somnifera offers various pharmacologically beneficial effects, including anxiolytic, antidepressant, immunomodulatory, anti-inflammatory, and anti-stress effects (Kumar and Kalonia 2008; Deshpande et al. 2018; Ven Murthy et al. 2010; Verma and Kumar 2011).

Shagandha (Ashwagandha root extract [ARE]), a standardized extract prepared from the roots containing 2.5% Withanolides, was analyzed by HPLC. Shagandha is a registered trademark of Sami-Sabinsa Group Limited. ARE has been clinically proven to improve depression and anxiety in individuals with mild to moderate symptoms by increasing serotonin levels, suggesting a mechanism of action through monoamine neurotransmitters (Majeed et al. 2024). In another study, ARE effectively induced positive effects on stress and anxiety, improving the quality of life for healthy individuals with mild and moderate stress. ARE improves stress and anxiety by reducing cortisol levels and increasing serotonin levels in healthy individuals, indicating its effects through the hypothalamic–pituitary axis (Majeed et al. 2023). However, the toxicological evaluation of ARE has not been performed. Thus, the purpose of the present study was to investigate the safety assessment of ARE using in vivo experimental models for acute, subacute, subchronic, and reproductive/developmental studies. Additionally, the genotoxic potential of ARE was assessed using an in vitro reverse mutation assay and an in vivo mammalian erythrocyte micronucleus test, conducted according to OECD test guidelines and Good Laboratory Practice.

2 | Materials and Methods

2.1 | Preparation and Standardization of Shagandha

Shagandha (Ashwagandha root extract—ARE) is prepared from the roots of *Withania somnifera* through a solvent extraction process. The extract is standardized for 2.5% Withanolides by HPLC, analyzed using the USP Monograph (USP 2020) (Figure 1).

2.2 | Chemicals

In the reverse mutation assay, chemicals used as a positive control for assays included 2-Nitrofluorene, Sodium Azide, Mitomycin-C, and 9-Aminoacridine. The tester strains used were *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537. All the chemicals and solvents used in the present study were of analytical grade.

2.3 | Maintenance of Experimental Animals

Wistar rats were used for acute, subacute, subchronic, and reproductive/developmental studies, and Swiss Albino mice were used for the mammalian bone marrow micronucleus test. Rats were procured from Global Bioresearch Solution Private Limited (Registration No.: 1899/PO/RcNRcBt/S/16/CCSEA). Mice for the mammalian erythrocyte micronucleus test were procured from Cadila Pharmaceuticals Ltd. (Registration No.: 161/PO/RcBiBt/S/99/CPCSEA).

Animals were housed in polycarbonate cages, at a temperature (19.2°C–24.9°C), and relative humidity (32%–69%) with a 12 h light and 12 h dark cycle maintained. Throughout the study period, all animals were provided with a pelleted rodent diet and purified filtered/reverse osmosis (RO) water *ad* libitum.

2.4 | Ethics

The individual experimental protocols for acute (JDM/IAEC/2024/01/016), subacute (JDM/IAEC/2022/03/119), subchronic (JDM/IAEC/2022/03/124), reproduction/developmental (JDM/IAEC/2023/01/145) toxicity studies, and mammalian erythrocyte micronucleus test (JDM/IAEC/2024/01/250) were approved by the Institutional Animal Ethics Committee (IAEC) of JDM Scientific Research Organization Private Limited, Gujarat.

Experimental protocols were conducted following the recommendation of the Committee for Control and Supervision of

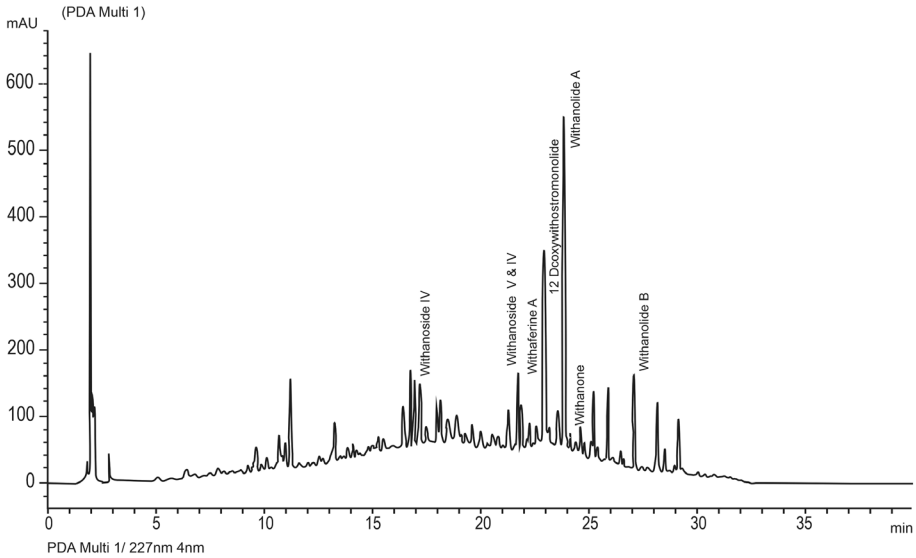

FIGURE 1 | HPLC chromatogram of Shagandha.

Experiments on Animals (CCSEA) guidelines for laboratory animal facilities published in the Gazette of India, on December 15th, 1998. Under OECD principles of Good Laboratory Practice, the studies were performed to ensure and promote consistency, quality, safety, and reliability during non-clinical and laboratory testing. The standard operating procedures described in the guidelines for testing of chemicals no. 423, 407, 408, 421, 471, and 474 were followed.

2.5 | Acute Oral Toxicity in Female Wistar Rats

The study to assess acute toxicity was conducted per the protocol outlined in OECD Guideline 423 (OECD 2002). Six healthy young adults female Wistar rats (3 animals per step), aged 10–11 weeks and weighing between 218.82 g and 237.43 g, were used. The animals were fasted overnight for 16–18 h before administration of ARE, and feed was provided 3 to 4 h after dosing. Based on the body weight, on Day 0, the ARE was dissolved in water (vehicle) and administered to the animals by oral gavage using a suitable intubation cannula.

For step 1, the animals were administered an initial dose of 2000 mg/kg body weight, with no mortality observed; additional dosing was discontinued as per the test guidelines. All the animals were acclimatized for 7 days before Step 1. Following dosing, animals were individually monitored for signs of toxicity at 30 min, 1, 2, 3, and 4 h, and observed once-daily for 14 days and twice-daily for mortality. Body weights were recorded before dosing (Day 0), on the 7th and 14th days after administration. After 14 days of observation, the animals were euthanized using an overdose of carbon dioxide and subjected to external and internal gross necropsy examinations.

2.6 | Subacute Toxicity in Wistar Rats

The study to assess subacute toxicity was conducted per the protocol outlined in OECD Guideline 407 (OECD 2008). A total of 60 Wistar rats (30 males and 30 females), distributed into six groups (5 animals/sex/group, 7–8 weeks old), weighing approximately 213.79–271.52 g for males and 184.02–236.11 g for females, were used in the study. As per the dose range-finding study, the dose levels were as follows: low dose (G2) 500 mg/kg body weight (b.w.), mid dose (G3) 1000 mg/kg b.w., and high dose (G4) 2000 mg/kg b.w. The grouping of animals and respective doses is detailed in Table 1.

The animals in the treatment groups were administered three different doses of ARE (500, 1000, 2000 mg/kg b.w.) for 28 consecutive days through oral gavage using a suitable intubation cannula. The maximum volume for administration was 1 mL/100 g of b.w. The control group received only the vehicle (G1 water). The animals in the control (G1R) and high-dose (G4R) recovery groups were observed for an additional 14 days after treatment. At the end of the treatment period, reversibility of treatment-related changes or delayed test item-related toxic effects was observed. Throughout the study, all animals were monitored for mortality twice daily, observed for clinical signs once daily, assessed for body weight changes, and feed consumption once a week. Blood samples for hematological and biochemical analyses were collected from all groups on Day 28—main groups (G1-G4) and Day 42—recovery groups (G1R & G4R). Blood samples were drawn from the retro-orbital plexus using a micro-hematocrit heparinized glass capillary tube. For biochemical analyses, the blood samples were centrifuged, and plasma was separated. Hematological analyses were conducted using blood samples collected in centrifuge tubes containing dipotassium ethylene di-amine tetra acetic acid (K2 EDTA) for hematological analyses. Urine samples were collected and analyzed on Day 28 (for main groups) and Day 42 (for recovery groups). After the experimental period, all animals from the main and recovery groups were sacrificed by exsanguination under carbon dioxide. During necropsy, tissues and organs from all animals were collected. The organ/tissue samples were fixed in a 10% neutral-buffered formalin solution except for the testes, which were preserved in Bouin's fluid. The preserved organs and tissue samples from each animal in both the control and high-dose groups were embedded, sectioned, stained, and examined under a microscope. All gross lesions were thoroughly examined and recorded.

TABLE 1 | Dose grouping for the subacute toxicity study of ARE.

Group no.	Treatment groups	Dose (mg/kg b.w.)	No. of rats	
			Male	Female
G1	Vehicle control	0	5	5
G2	Low dose	500	5	5
G3	Mid dose	1000	5	5
G4	High dose	2000	5	5
G1R[a]	Control recovery	0	5	5
G4R[a]	High dose recovery	2000	5	5

[a]Recovery groups with a 14-day recovery period.

2.7 | Subchronic Toxicity in Wistar Rats

The experiment on subchronic toxicity was conducted under the protocol guidelines OECD 408 (OECD 2018). One hundred Wistar rats (50 males + 50 females), 8–9 weeks old, weighing 226.07–285.21 g for males and 189.64–221.13 g for females, were selected and distributed into six groups as 40 each (males and females) for the treatment group and 10 each (males and females) for recovery group. The grouping of animals and respective doses is explained in Table 2.

In the treatment groups, animals were administered ARE at varying dose levels: low dose of 500 mg/kg b.w. (G2), mid dose of 1000 mg/kg b.w. (G3) and high dose of 2000 mg/kg b.w. (G4) once daily by oral gavage for 90 consecutive days using a suitable intubation cannula, for the control group (G1), vehicle (water) was administered. The maximum dose volume for administration was 1 mL/100 g of body weight. Animals in the recovery group, which included the control (G1R) and high dose (G4R) groups, were

TABLE 2 | Dose grouping for the subchronic toxicity study of ARE.

Group no.	Treatment groups	Dose (mg/kg b.w.)	No. of rats	
			Male	Female
G1	Vehicle control	0	10	10
G2	Low dose	500	10	10
G3	Mid dose	1000	10	10
G4	High dose	2000	10	10
G1R[a]	Control recovery	0	5	5
G4R[a]	High dose recovery	2000	5	5

[a]Recovery groups with a 28-day recovery period.

TABLE 3 | Dose grouping for the reproductive/developmental toxicity study of ARE.

Group no.	Treatment groups	Dose (mg/kg b.w.)	No. of rats	
			Male	Female
G1	Vehicle control	0	10	10
G2	Low dose	100	10	10
G3	Mid dose	500	10	10
G4	High dose	1000	10	10

observed for an additional 28 days to assess any reversibility of treatment-related changes/delayed ARE-related toxic effect.

All animals were monitored for mortality twice daily and clinical signs of toxicity once daily. A detailed clinical observation was performed once before the start of treatment and weekly throughout the treatment and recovery period. The animals were subjected to neurological and ophthalmological examinations, and their body weight and feed consumption were recorded weekly. At the end of the 90-day treatment period and during the recovery period, hematological, biochemical, and urine analyses were noted.

After the experimental period, all the animals were sacrificed by exsanguination under carbon dioxide, necropsy was performed, and the weight of various organs was recorded. After completion of gross pathology examination, tissues and organs were collected from all animals. The organs/tissue samples were fixed in 10% buffered neutral formalin solution, except for the testes and eyes, which were preserved in Bouin's fluid. A histopathological examination was then performed on the preserved organs and tissues of all animals in the control and high-dose groups. Since there were no treatment-related changes in the high-dose group, examinations were not extended to animals of other dose groups and recovery groups. All gross lesions were examined and recorded.

2.8 | Reproductive/Developmental Toxicity in Wistar Rats

The experiment on reproductive/developmental toxicity was conducted as per the protocol described in OECD guidelines 421 (OECD 2016). In the reproductive study, 8 Wistar rats [40 males and 40 females (nulliparous and non-pregnant)] were divided into four groups of 10 animals/sex/group, administered diets with ARE at graded doses of 0, 100, 500, and 1000 mg/kg b.w. (Table 3).

The Wistar rats of 13 to 14 weeks old weighing male: 325.99–459.76 g and female: 234.01–314.06 g were used in the study. In the study, animals received water, which was used as a vehicle (G1), and other groups (G2-G4) were treated with 100, 500, and 1000 mg/kg b.w. ARE by oral route through intubation cannula. Male rats were treated for 2 weeks before mating, during mating, and up to the day before sacrifice during the post-mating period (28-day treatment period). Female rats were treated for 2 weeks pre-mating, during mating, during the gestation period, and up to lactation Day 13. Pups and females (dams) were sacrificed on lactation Day 14. The dose volume of 1 mL/100 g of b.w. was maintained in all the groups.

All experimental animals have been examined for mortality twice daily and clinical signs once daily during the study. Food consumption was recorded weekly for males and females during pre-mating, gestation, and lactation. Body weight of all animals was measured weekly (including females on Day 0, Day 7, Day 14, and Day 20 post-partum and on lactation Day 1, 4, 7, 14). All females were observed pre- and post-treatment for oestrous cyclicity, and vaginal smears were observed every day until the successful mating period. Oestrous cycles were divided into four stages (i.e., Estrus, Metestrus, Diestrus, and Proestrus), and the frequency of a 4–5-day cycle was the same in all treatment group rats.

After the mating period, all females showed a 100% fertility index and 100% gestation index (all gave live birth). On the litter day (lactation Day 1), the number of pups (dead or alive), sex, and gross abnormalities were examined. Live pups' body weight was weighed on Days 1, 4, 7, and 14 post-partum. The anogenital distance of each pup was measured on postnatal Day 4, and the number of nipples/areolae in male pups was counted and recorded on postnatal Day 13. Blood samples were collected from all male animals on Day 28, females on Day 13 post-partum, pups on Day 4 after birth, and at termination on Day 13. Blood samples were assessed for serum levels of thyroid hormones (T4 and TSH). All the animals were weighed and sacrificed by exsanguination under carbon dioxide, and a necropsy was carried out. The number of implantation sites was recorded, and vaginal smears were examined in the morning on the day of the necropsy to determine the stage of the estrous cycle and to allow correlation with the histopathology of the ovaries. Dead pups and pups killed at Day 4 post-partum or 13, were examined externally for gross abnormalities, especially external reproductive genitals for signs of altered development. For animals of both high dose and control groups, a detailed histological examination was performed on the ovaries, uterus with cervix, testes, and epididymis. The preserved organs, including the thyroid from pups, were also examined.

2.9 | Genotoxicity Studies

2.9.1 | Bacterial Reverse Mutation Test—In vitro

As per the OECD test guidelines 471 (OECD 2020), a bacterial reverse mutation assay was conducted to evaluate the ability of ARE to induce reverse mutations in the presence or absence of mammalian microsomal enzymes at the histidine locus in the genome of *Salmonella typhimurium* tester strains (TA98, TA100, TA1535, TA1537, and TA102). A preliminary cytotoxicity assay was conducted to establish the dose concentration for the mutagenicity assay. Using the tester strain TA100, a cytotoxicity assay was conducted, with eight concentrations of the test item, ranging from 125 to 5000 μg/plate, in both the presence and absence of 5% v/v S9 mix. Normal bacterial background lawn and no reduction in the number of revertant colonies was observed at concentrations 125–5000 μg/plate in both the presence and absence of the metabolic activation system when compared with the vehicle control.

Five different tester strains of *Salmonella typhimurium* were used in the mutagenicity assay. The assay was conducted both in the presence and absence of the S9 mix along with concurrent vehicle and strain-specific positive controls using triplicate plates for each concentration. The concentrations tested in the mutagenicity assay (Trial I) were 50, 150, 500, 1500, and 5000 μg/plate, with both presence and absence of metabolic activation system (5% v/v S9 mix), separated by approximately half-log.

2.9.2 | Mammalian Erythrocyte Micronucleus Test–In Vivo

The experiment on mammalian erythrocyte micronucleus test was conducted under the protocol guidelines OECD 474 (OECD 2014). The objective was to evaluate the induction of micronucleus polychromatic erythrocytes (PCE) in the bone marrow using Swiss albino mice. Forty animals (20 male and 20 female), aged 7–8 weeks, were selected for a dose range finding study with five animals/sex/group, and the doses were selected as 500, 1000, and 2000 mg/kg b.w. They were administered at 10 mL/kg b.w. for two consecutive days, separated by 24 h. As per the results of the dose range finding study, no toxicity was observed in either sex; thus, only female animals were selected for the main study. Twenty-five female animals (8–9 weeks old) were distributed into five groups: A vehicle control group (received water), three test groups (500, 1000 & 2000 mg/kg b.w. respectively), and a positive control group (cyclophosphamide monohydrate at 50 mg/kg b.w.).

The ARE was administered on two consecutive days, separated by about 24 h, by oral route to respective groups of animals. The animals were observed for clinical signs at intervals of 1 h, 2 h after, and before each treatment, also before necropsy of the animals. After treatment, all animals were sacrificed by CO_2 asphyxiation post-treatment and bone marrow samples were collected from their femurs. Fetal bovine serum was inserted through the opening at the lower end of the femur bone to rinse the marrow into pre-labeled tubes. The samples were centrifuged at 1500 rpm for 10 min, and the supernatant was removed. A small amount of re-suspended cell pellet was smeared onto pre-labeled slides, resulting in two slides per animal, which were air dried, fixed, and stained. To determine the proportion of immature erythrocytes among total erythrocytes, at least 500 erythrocytes were counted for each animal. Additionally, parameters like clinical observations, mortality, body weight, P/E ratio, and micronucleus induction were evaluated.

3 | Statistical Analysis

The data have been subjected to statistical analysis for the significance of test item-induced changes and their interpretation of potential for toxicity using Sigma Plot statistical software. One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test or student "*t*" test (as appropriate) was performed for different treatment and recovery groups compared with the respective control group data. All analyses and comparisons were evaluated at the 95% level of confidence ($p < 0.05$).

4 | Results

4.1 | Acute Oral Toxicity

In an acute oral toxicity study, after a single dose of ARE at 2000 mg/kg b.w., there was no mortality and no clinical signs of toxicity in female Wistar rats. No treatment-related changes were observed in mean body weight and percent change in body weight in any of the animals. A physiologically normal increase in body weight was recorded on Day 7 and Day 14, as compared to Day 0, as indicated in Table 4. No gross pathological changes were observed internally or externally in any of the animals at a dose level of 2000 mg/kg b.w., and thus, histopathological evaluation was not performed.

4.2 | Subacute Toxicity

In subacute toxicity, the animals neither showed any clinical signs of toxicity nor mortality at doses up to 2000 mg/kg in any of the groups throughout the study period. Additionally, there were no significant changes in body weight, body weight gain percentage (Figure 2A,B), or feed consumption in any of the groups.

The hematological (Table 5), biochemical (Table 6), and urine analysis showed no control-related effects in animals of either sex in any dose group when compared to their respective groups. Similarly, absolute (Table 7) and relative organ weights did not indicate any treatment-related effects in any of the treatment groups. Also, no treatment-related findings were observed during the gross and microscopic examinations.

4.3 | Subchronic Toxicity

The subchronic toxicity study demonstrated no signs of toxicity or mortality in any of the groups. There were no significant changes in body weight or percentage body weight change (Figure 3A,B), feed consumption, hematological, biochemical

TABLE 4 | Effect of single-dose oral gavage to ARE on body weight and body weight change (%) in female Wistar rats.

Study type	Animal no.	Dose (mg/kg b.w.)	Body weight (g) on day			Body weight change (%) on day	
			0	7	14	0–7	0–14
Step 1	1	2000	183.40	201.8	226.11	10.03	23.29
	2		194.64	227.34	239.18	16.80	22.88
	3		179.11	200.51	218.46	11.95	21.97
Mean ± SD			185.72 ± 8.02	209.88 ± 15.13	227.92 ± 10.48	12.93 ± 3.49	22.71 ± 0.68
Step 2	4	2000	190.40	212.19	224.31	11.44	17.81
	5		183.20	202.43	213.09	10.50	16.32
	6		197.77	224.15	236.03	13.34	19.35
Mean ± SD			190.46 ± 7.29	212.92 ± 10.88	224.48 ± 11.47	11.76 ± 1.45	17.83 ± 1.52

Note: Values are expressed as mean ± SD.
Abbreviations: b.w., body weight; SD, standard deviation.

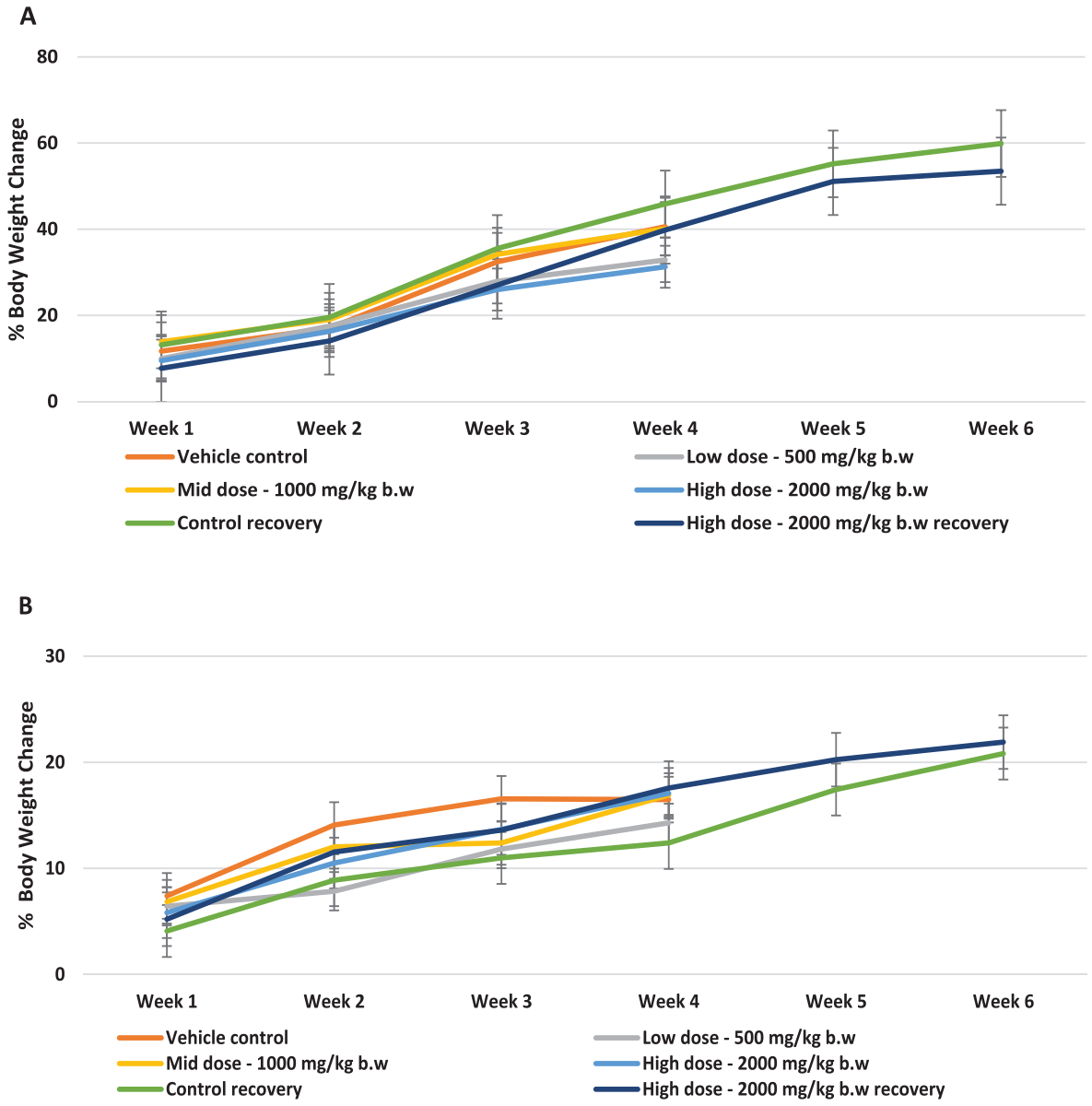

FIGURE 2 | Effect of ARE on the percentage of body weight change of (A) male and (B) female rats in subacute toxicity.

TABLE 5 | Effect of 28 days of exposure to ARE on hematological parameters in Wistar rats.

Parameters	Vehicle control	Treated			Control recovery	High dose 2000 mg/kg b.w. recovery
		Low dose—500 mg/kg b.w.	Mid dose—1000 mg/kg b.w.	High dose—2000 mg/kg b.w.		
Male						
WBC ($\times 10^3$ cells/μL)	9.31 ± 2.36	10.98 ± 3.79	7.69 ± 1.24	8.62 ± 2.82	7.35 ± 3.09	9.81 ± 4.02
RBC ($\times 10^6$ cells/μL)	7.30 ± 0.25	7.51 ± 0.45	7.71 ± 0.52	7.84 ± 0.39	7.62 ± 0.60	8.10 ± 0.25
Hemoglobin (g/dL)	13.06 ± 0.26	12.68 ± 0.73	13.52 ± 0.92	13.08 ± 0.90	13.02 ± 0.95	13.62 ± 0.67
Hematocrit (%)	44.62 ± 1.36	43.52 ± 1.91	45.72 ± 3.14	45.36 ± 2.30	43.62 ± 3.05	46.34 ± 2.11
MCV (fL)	61.20 ± 1.98	57.96 ± 1.55	59.32 ± 0.78	57.88 ± 1.78	57.32 ± 1.24	57.22 ± 2.64
MCH (pg)	17.88 ± 0.77	16.86 ± 0.36	17.54 ± 0.35	16.66 ± 0.51	17.12 ± 0.36	16.82 ± 0.91
MCHC (g/dL)	29.26 ± 0.54	29.08 ± 0.74	29.56 ± 0.23	28.78 ± 0.79	29.84 ± 0.34	29.40 ± 0.52
Platelet count ($\times 10^3$ cells/μL)	1047.40 ± 212.17	905.00 ± 183.66	885.00 ± 108.73	824.60 ± 74.19	819.00 ± 231.83	968.20 ± 125.17
Neutrophils ($\times 10^3$ cells/μL)	1.20 ± 0.19	2.01 ± 0.69	1.56 ± 0.35	1.97 ± 0.16	1.67 ± 0.79	2.59 ± 0.58
Lymphocytes ($\times 10^3$ cells/μL)	7.48 ± 2.18	8.49 ± 3.15	5.79 ± 0.97	6.12 ± 2.55	4.65 ± 2.03	5.76 ± 3.97
Monocytes ($\times 10^3$ cells/μL)	0.39 ± 0.15	0.26 ± 0.12	0.17 ± 0.06	0.20 ± 0.09	0.33 ± 0.17	0.38 ± 0.13
Eosinophils ($\times 10^3$ cells/μL)	0.11 ± 0.09	0.11 ± 0.05	0.09 ± 0.03	0.09 ± 0.04	0.61 ± 0.36	0.96 ± 0.15
Basophils ($\times 10^3$ cells/μL)	0.06 ± 0.03	0.06 ± 0.03	0.04 ± 0.02	0.04 ± 0.03	0.03 ± 0.02	0.04 ± 0.02
Reticulocyte count ($\times 10^9$ cells/L)	125.98 ± 74.31	84.80 ± 7.42	84.34 ± 18.06	104.08 ± 18.26	83.94 ± 14.00	76.46 ± 14.66
Prothrombin time (sec)	10.34 ± 0.48	11.06 ± 0.40	10.80 ± 0.32	10.66 ± 0.27	10.60 ± 0.27	10.72 ± 0.42
APTT (sec)	18.96 ± 1.59	18.46 ± 0.61	18.42 ± 0.97	18.96 ± 1.26	17.98 ± 1.10	19.08 ± 1.17
Female						
WBC ($\times 10^3$ cells/μL)	6.72 ± 1.88	7.62 ± 2.59	9.47 ± 4.35	8.90 ± 3.40	6.65 ± 2.11	7.60 ± 3.78
RBC ($\times 10^6$ cells/μL)	7.69 ± 0.35	7.46 ± 0.51	7.23 ± 0.73	7.11 ± 0.49	7.40 ± 0.43	7.64 ± 0.79
Hemoglobin (g/dL)	13.48 ± 0.23	13.28 ± 1.23	12.68 ± 1.23	12.32 ± 0.68	12.84 ± 0.36	13.38 ± 0.99
Hematocrit (%)	45.00 ± 0.96	44.02 ± 2.73	43.16 ± 3.70	42.58 ± 2.25	43.38 ± 1.41	44.72 ± 2.85
MCV (fL)	58.58 ± 2.01	59.10 ± 2.85	59.82 ± 1.13	59.86 ± 1.41	58.74 ± 1.98	58.76 ± 2.71
MCH (pg)	17.58 ± 0.63	17.78 ± 1.20	17.56 ± 0.55	17.32 ± 0.39	17.40 ± 0.66	17.58 ± 0.85
MCHC (g/dL)	29.98 ± 0.71	30.06 ± 1.09	29.38 ± 0.75	28.96 ± 0.48	29.62 ± 0.77	29.90 ± 0.47
Platelet count ($\times 10^3$ cells/μL)	776.40 ± 402.89	894.80 ± 146.81	938.20 ± 120.78	872.60 ± 79.45	1012.20 ± 129.46	967.00 ± 135.14
Neutrophils ($\times 10^3$ cells/μL)	1.63 ± 0.62	1.93 ± 0.80	2.02 ± 0.79	3.06 ± 3.65	2.88 ± 1.20	2.82 ± 1.31

(Continues)

TABLE 5 | (Continued)

Parameters	Vehicle control	Treated				
		Low dose—500 mg/kg b.w.	Mid dose—1000 mg/kg b.w.	High dose—2000 mg/kg b.w.	Control recovery	High dose 2000 mg/kg b.w. recovery
Lymphocytes (×10^3 cells/μL)	4.65 ± 1.75	5.31 ± 1.78	7.01 ± 3.98	5.02 ± 2.22	2.37 ± 1.65	3.58 ± 3.00
Monocytes (×10^3 cells/μL)	0.24 ± 0.03	0.20 ± 0.09	0.22 ± 0.10	0.66 ± 1.05	0.24 ± 0.09	0.33 ± 0.24
Eosinophils (×10^3 cells/μL)	0.08 ± 0.05	0.10 ± 0.06	0.11 ± 0.08	0.06 ± 0.05	1.06 ± 0.50	0.74 ± 0.31
Basophils (×10^3 cells/μL)	0.03 ± 0.02	0.03 ± 0.01	0.05 ± 0.04	0.05 ± 0.03	0.02 ± 0.01	0.03 ± 0.02
Reticulocyte count (×10^9 cells/L)	43.42 ± 22.33	96.76 ± 21.16	68.66 ± 21.91	75.58 ± 31.35	74.02 ± 16.52	67.98 ± 42.41
Prothrombin time (sec)	10.42 ± 0.60	10.84 ± 0.94	10.64 ± 0.57	10.62 ± 0.30	10.52 ± 0.49	10.64 ± 0.50
APTT (sec)	18.04 ± 0.64	18.94 ± 1.41	18.82 ± 1.04	19.14 ± 1.30	18.28 ± 1.20	18.76 ± 1.26

Note: Values are expressed as mean ± SD.
Abbreviations: APTT, activated partial thromboplastin time; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RBC, Erythrocyte count; WBC, total leukocyte count.

parameters (Tables 8 and 9), urine analysis or organ weight (both absolute and relative, Table 10). Additionally, there were no findings in gross and microscopic examinations up to 2000 mg/kg b.w. in any of the groups (treatment or control).

4.4 | Reproductive/Developmental Toxicity Study

In the reproductive/developmental toxicity study, none of the animals in any of the treatment groups exhibited any clinical signs of toxicity or mortality. In females, during the study period, no significant changes were observed in body weight (Table 11), percentage change in body weight, and feed consumption during the premating, gestation, and lactation periods.

There were no significant changes between control and treatment groups in biochemical parameters, necropsy, and absolute organ weights of males and females (Table 12). No treatment-related effect was observed in the reproductive parameters of the experimental animals.

4.5 | Genotoxicity Studies

4.5.1 | Bacterial Reverse Mutation Test

The results from the initial mutagenicity experiment, Trial I, were confirmed in Trial II using the same ARE concentrations as Trial I, using the pre-incubation method with and without a metabolic activation system (5% v/v S9 mix). In Trials I and II, no reduction in the number of revertant colonies was observed and no cytotoxicity to the bacterial background lawn at a concentration of 5000 μg/plate was noted when compared with the concurrent vehicle control, both in the absence and presence of the metabolic activation system. There was no evidence of a 2-fold increase (or a 3-fold increase in the case of TA1537 and TA1535) in revertant colonies at any concentration tested up to 5000 μg/plate, in comparison with concurrent vehicle control in both trials. A more than 2-fold increase (or 3-fold in case of TA1537 and TA1535) in revertant colonies compared to concurrent vehicle controls was observed in the strain-specific positive controls in the presence and absence of the metabolic activation system in both trials, indicating the sensitivity of the assay. Therefore, based on these results, the ARE did not cause a positive increase in the mean number of revertants per plate with any of the tester strains, both in the presence and absence of the metabolic activation system (S9).

4.5.2 | Mammalian Erythrocyte Micronucleus Test

In the mammalian erythrocyte micronucleus test, no mortalities or abnormal clinical signs were reported in both the dose range finding and the main study. The ARE-related animals at different doses were normal throughout the mammalian erythrocyte micronucleus study. In the main study, the %P/E ratio observed in animals was 60.96, 58.04, 55.40, 52.20, and 47.20 at vehicle, test item dose levels of 500, 1000, 2000 mg/kg body weight, and 50 mg/kg body weight of cyclophosphamide monohydrate, respectively. The % reduction in P/E ratio observed in animals was 4.79, 9.12, 14.37, and 22.57 at test item dose levels of 500, 1000,

TABLE 6 | Effect of 28 days ofexposure to ARE on biochemical parameters in Wistar rats.

Parameters	Vehicle control	Treated			Control recovery	High dose—2000 mg/kg b.w. recovery
		Low dose—500 mg/kg b.w.	Mid dose—1000 mg/kg b.w.	High dose—2000 mg/kg b.w.		
Male						
AST (U/L)	143.32 ± 25.27	138.51 ± 12.80	123.91 ± 15.43	172.96 ± 23.65	145.67 ± 13.83	140.68 ± 5.92
ALT (U/L)	63.02 ± 22.58	55.41 ± 7.95	53.46 ± 12.49	51.29 ± 4.07	64.61 ± 10.59	55.31 ± 6.04
Albumin (g/dL)	3.63 ± 0.12	3.62 ± 0.15	3.88 ± 0.16	3.66 ± 0.09	3.47 ± 0.16	3.47 ± 0.21
ALP (U/L)	210.82 ± 34.08	210.56 ± 32.86	227.56 ± 63.60	174.62 ± 25.63	173.66 ± 46.02	179.48 ± 35.42
Total bilirubin (mg/dL)	0.10 ± 0.02	0.10 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.09 ± 0.02	0.11 ± 0.01
Cholesterol (mg/dL)	63.89 ± 6.19	73.72 ± 10.68	72.24 ± 16.52	76.91 ± 18.14	77.77 ± 10.06	73.54 ± 13.89
Creatinine (mg/dL)	0.33 ± 0.04	0.31 ± 0.03	0.29 ± 0.01	0.31 ± 0.02	0.32 ± 0.02	0.30 ± 0.05
Calcium (mg/dL)	10.37 ± 0.50	10.56 ± 0.28	11.12 ± 0.10	10.80 ± 0.18	10.55 ± 0.26	10.75 ± 0.45
Glucose (mg/dL)	106.15 ± 15.51	103.51 ± 9.62	115.48 ± 33.64	99.18 ± 12.55	95.76 ± 15.79	96.79 ± 9.50
Phosphate (mg/dL)	8.13 ± 1.78	7.66 ± 0.55	7.16 ± 0.56	7.86 ± 0.36	7.17 ± 0.63	7.40 ± 0.29
Total protein (g/dL)	6.25 ± 0.26	6.30 ± 0.22	6.70 ± 0.19	6.62 ± 0.25	6.16 ± 0.28	6.33 ± 0.25
Urea (mg/dL)	34.38 ± 3.86	35.50 ± 4.80	37.50 ± 7.31	35.58 ± 1.98	34.98 ± 6.01	31.30 ± 2.79
Globulin (g/dL)	2.61 ± 0.20	2.68 ± 0.24	2.82 ± 0.18	2.96 ± 0.23	2.69 ± 0.21	2.85 ± 0.28
Sodium (mmol/L)	141.24 ± 2.27	144.94 ± 3.46	149.34 ± 3.72	146.85 ± 3.31	143.60 ± 1.85	143.76 ± 4.37
Potassium (mmol/L)	5.81 ± 1.06	5.43 ± 0.42	5.43 ± 0.35	5.42 ± 0.25	4.97 ± 0.31	5.16 ± 0.28
Chlorine (mmol/L)	149.12 ± 6.16	143.74 ± 3.43	147.69 ± 2.37	145.52 ± 2.80	146.18 ± 3.84	144.81 ± 3.26
Bile acids (μmol/L)	11.63 ± 5.59	17.96 ± 11.52	10.08 ± 4.95	16.05 ± 9.26	7.87 ± 6.69	7.22 ± 4.29
Blood urea nitrogen (mg/dL)	16.07 ± 1.80	16.59 ± 2.24	17.52 ± 3.42	16.63 ± 0.93	16.35 ± 2.81	14.63 ± 1.30
Female						
AST (U/L)	238.56 ± 226.14	151.04 ± 34.66	127.14 ± 15.92	115.12 ± 9.57	133.63 ± 27.90	128.12 ± 20.27
ALT (U/L)	171.89 ± 270.36	58.07 ± 13.19	52.57 ± 8.12	41.00 ± 2.20	57.52 ± 12.64	54.17 ± 10.61
Albumin (g/dL)	3.72 ± 0.52	3.80 ± 0.32	4.00 ± 0.25	3.96 ± 0.27	3.82 ± 0.20	3.73 ± 0.31

(Continues)

TABLE 6 | (Continued)

Parameters	Vehicle control	Treated			Control recovery	High dose—2000 mg/kg b.w. recovery
		Low dose—500 mg/kg b.w.	Mid dose—1000 mg/kg b.w.	High dose—2000 mg/kg b.w.		
ALP (U/L)	118.26 ± 26.14	100.20 ± 24.73	102.06 ± 21.38	126.75 ± 27.57	135.06 ± 31.36	115.12 ± 47.30
Total bilirubin (mg/dL)	0.14 ± 0.05	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.12 ± 0.02	0.14 ± 0.03
Cholesterol (mg/dL)	75.45 ± 26.63	75.85 ± 12.73	92.92 ± 12.10	91.34 ± 7.37	80.76 ± 19.42	85.90 ± 14.88
Creatinine (mg/dL)	0.38 ± 0.04	0.38 ± 0.04	0.36 ± 0.04	0.37 ± 0.03	0.36 ± 0.06	0.45 ± 0.13
Calcium (mg/dL)	10.45 ± 0.52	10.35 ± 0.30	10.93 ± 0.39	10.91 ± 0.44	10.63 ± 0.47	10.66 ± 0.23
Glucose (mg/dL)	97.64 ± 11.75	101.27 ± 7.98	101.89 ± 9.82	102.59 ± 10.66	98.80 ± 9.10	98.05 ± 16.25
Phosphate (mg/dL)	6.80 ± 1.25	6.39 ± 1.37	6.69 ± 1.33	7.22 ± 0.54	6.53 ± 0.75	6.22 ± 1.15
Total protein (g/dL)	6.69 ± 0.84	6.90 ± 0.30	7.19 ± 0.34	6.87 ± 0.58	6.97 ± 0.32	6.50 ± 0.31
Urea (mg/dL)	42.06 ± 5.75	39.45 ± 4.63	32.69 ± 7.44	39.32 ± 6.73	40.70 ± 6.08	41.40 ± 8.62
Globulin (g/dL)	2.97 ± 0.36	3.10 ± 0.17	3.19 ± 0.31	2.90 ± 0.53	3.16 ± 0.30	2.77 ± 0.20
Sodium (mmol/L)	141.33 ± 3.54	142.94 ± 2.00	145.79 ± 2.36	144.27 ± 1.74	139.74 ± 3.96	142.59 ± 2.89
Potassium (mmol/L)	5.13 ± 0.65	5.19 ± 0.24	5.21 ± 0.53	5.56 ± 0.32	5.14 ± 0.21	4.76 ± 0.29
Chlorine (mmol/L)	134.46 ± 13.35	133.89 ± 3.60	135.27 ± 1.89	135.48 ± 2.69	133.10 ± 5.38	129.93 ± 4.44
Bile acids (μmol/L)	20.28 ± 17.73	8.77 ± 1.29	9.39 ± 3.36	7.32 ± 1.21	12.91 ± 6.32	9.46 ± 3.98
Blood urea nitrogen (mg/dL)	19.65 ± 2.69	18.43 ± 2.16	15.28 ± 3.48	18.38 ± 3.14	19.02 ± 2.84	19.35 ± 4.03

Note: Values are expressed as mean ± SD.
Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

TABLE 7 | Effect of 28 days of exposure to ARE on absolute organ weights (g) in Wistar rats.

Organs	Vehicle control	Treated			Control recovery	High dose—2000 mg/kg b.w. recovery
		Low dose—500 mg/kg b.w.	Mid dose—1000 mg/kg b.w.	High dose—2000 mg/kg b.w.		
Male						
Liver	11.7103 ± 1.1915	10.8629 ± 2.1340	11.7893 ± 1.9774	10.7803 ± 0.9827	12.4870 ± 0.5434	12.5936 ± 2.3257
Kidneys	2.4969 ± 0.1133	2.3722 ± 0.3597	2.5105 ± 0.1838	2.2708 ± 0.2178	2.8250 ± 0.0985	2.8957 ± 0.2830
Heart	1.1203 ± 0.0425	1.2608 ± 0.2070	1.2871 ± 0.1185	1.1460 ± 0.1436	1.3644 ± 0.0646	1.3210 ± 0.1631
Spleen	0.7152 ± 0.1770	0.7936 ± 0.1822	0.7800 ± 0.1655	0.8264 ± 0.1501	0.7287 ± 0.2136	0.7999 ± 0.1026
Brain	1.7509 ± 0.1026	2.9661 ± 2.6760	1.7716 ± 0.1050	1.8035 ± 0.1832	1.9288 ± 0.1046	1.9127 ± 0.0660
Thymus	0.2954 ± 0.0440	0.4234 ± 0.1570	0.3508 ± 0.0584	0.3799 ± 0.0672	0.3334 ± 0.0294	0.4241 ± 0.0274
Adrenals	0.0566 ± 0.0218	0.0594 ± 0.0095	0.0596 ± 0.0090	0.0450 ± 0.0088	0.0512 ± 0.0077	0.0548 ± 0.0077
Testes	2.8974 ± 0.1591	2.6693 ± 0.6145	3.0509 ± 0.4681	3.0427 ± 0.1634	3.1493 ± 0.1286	3.1623 ± 0.2106
Epididymis	0.9306 ± 0.0560	1.0280 ± 0.0616	1.2034 ± 0.1362	1.0251 ± 0.1116	1.1835 ± 0.0554	1.1643 ± 0.0926
Prostate plus seminal vesicles with coagulation gland	1.7327 ± 0.3029	1.9168 ± 0.4073	1.9050 ± 0.1233	1.6490 ± 0.2568	1.9799 ± 0.5263	2.0070 ± 0.3108
Thyroid with parathyroid	0.0225 ± 0.0019	0.0247 ± 0.0023	0.0211 ± 0.0033	0.0220 ± 0.0017	0.0208 ± 0.0038	0.0216 ± 0.0039
Female						
Liver	7.6735 ± 0.7639	7.4731 ± 1.3678	7.7195 ± 0.7150	8.9069 ± 2.2887	7.6888 ± 1.9164	8.8520 ± 1.7151
Kidneys	1.6747 ± 0.1024	1.5753 ± 0.2242	1.6794 ± 0.2356	1.5753 ± 0.2795	1.7400 ± 0.4258	1.9933 ± 0.2816
Heart	0.8965 ± 0.1477	0.9457 ± 0.1428	0.9489 ± 0.1301	0.9808 ± 0.1162	0.9465 ± 0.1229	1.1742 ± 0.3648
Spleen	0.5399 ± 0.0607	0.5775 ± 0.0596	0.6554 ± 0.1269	0.7586 ± 0.3922	0.5313 ± 0.0897	0.6889 ± 0.2414
Brain	1.5371 ± 0.4632	1.7621 ± 0.1049	1.7420 ± 0.0844	1.6865 ± 0.0818	1.7071 ± 0.1352	1.8501 ± 0.1070
Thymus	0.2848 ± 0.0643	0.2809 ± 0.0985	0.3228 ± 0.0169	0.3474 ± 0.0704	0.2942 ± 0.0406	0.3450 ± 0.0859
Adrenals	0.0687 ± 0.0084	0.0759 ± 0.0131	0.0747 ± 0.0146	0.0749 ± 0.0073	0.0660 ± 0.0131	0.0732 ± 0.0135
Uterus with cervix	0.4767 ± 0.1278	0.6192 ± 0.0546	0.6611 ± 0.3448	0.6462 ± 0.2642	0.6611 ± 0.2014	0.6573 ± 0.2381
Ovaries	0.0695 ± 0.0207	0.0699 ± 0.0124	0.0737 ± 0.0175	0.0700 ± 0.0154	0.0679 ± 0.0172	0.0848 ± 0.0160
Thyroid with parathyroid	0.0181 ± 0.0031	0.0184 ± 0.0020	0.0192 ± 0.0025	0.0195 ± 0.0032	0.0170 ± 0.0023	0.0221 ± 0.0030

Note: Values are expressed as mean ± SD.

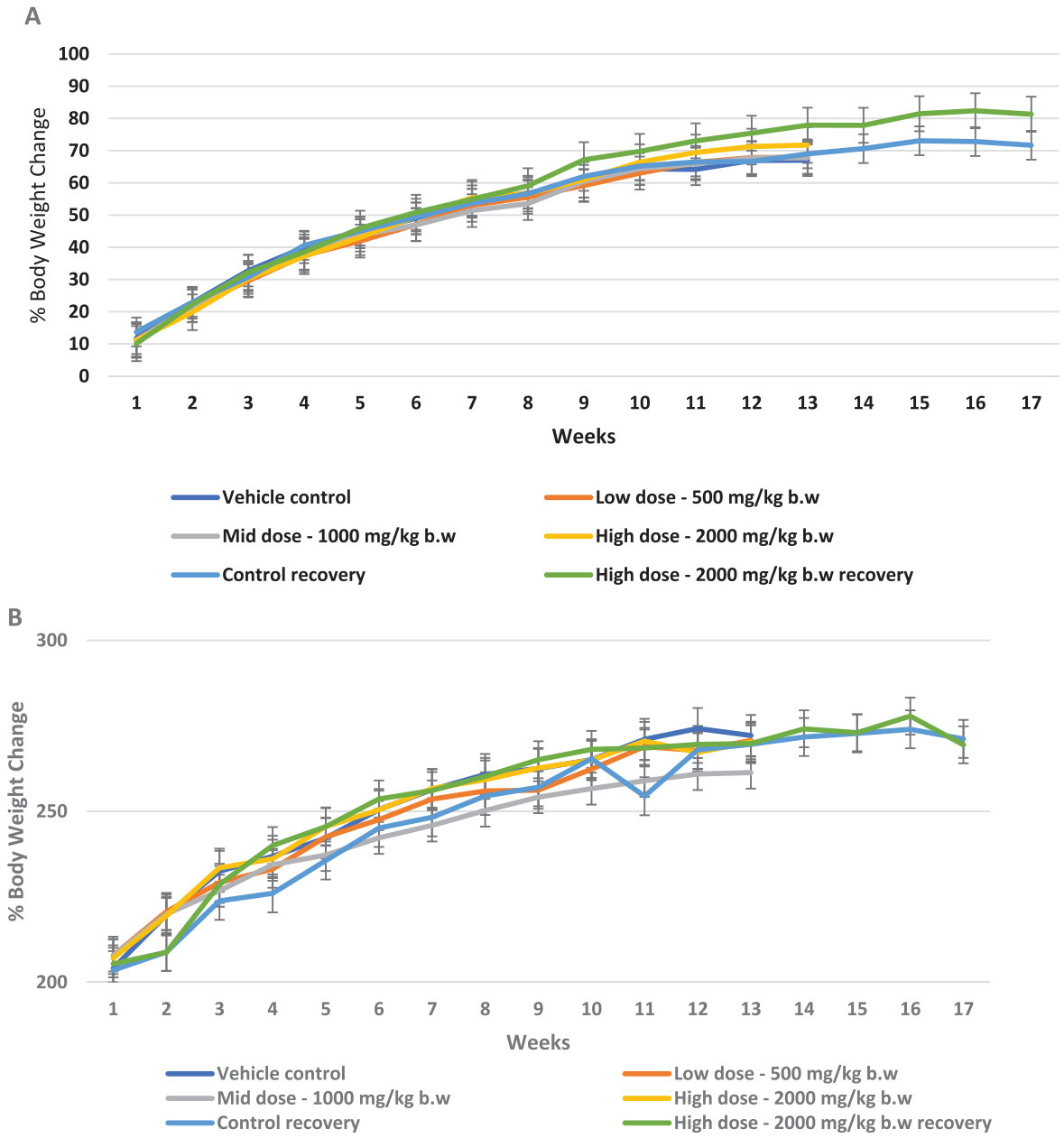

FIGURE 3 | EFFECT of ARE on the percentage of body weight change of (A) male and (B) female rats in subchronic toxicity.

2000 and 50 mg/kg b.w. for cyclophosphamide monohydrate, respectively, as compared to the concurrent vehicle control group. The %MNPCE observed was 0.120, 0.115, 0.110, 0.105, and 1.270 at vehicle, test item dose levels of 500, 1000, 2000 mg/kg body weight and 50 mg/kg body weight of cyclophosphamide monohydrate, respectively. None of the animals treated with the test item exhibited any increase in the numbers of MNPCE compared to concurrent vehicle control. The observed increase ($p < 0.05$) in MNPCE frequency in the positive control group indicated the sensitivity of the test system and was considered biologically significant (Tables 13A and 13B).

5 | Discussion

The present study aimed to evaluate the safety profile of ashwagandha root extract (ARE) derived from the roots of *Withania somnifera*. A prominent member of the Solanacea family, *Withania somnifera*—commonly known as ashwagandha has been used traditionally in Indian medicine for centuries. Recognized as a Rasayana herb in Ayurveda, it is valued for its rejuvenating properties and in promoting health and longevity. It is widely regarded for its adaptogenic, anti-stress, anxiolytic, cognitive-enhancing, and immune-modulatory effects (Ven Murthy et al. 2010; Verma and Kumar 2011). Its pharmacological activity is attributed to a diverse range of phytochemicals, including alkaloids, steroidal lactones (withanolides and withaferins), saponins, and glycol withanolides. Due to its extensive traditional use and therapeutic potential, this study aimed to assess the safety and potential toxicity of ARE to establish a comprehensive toxicological profile. In acute toxicity, throughout the study period, single-dose treatment of 2000 mg/kg b.w. of ARE to female Wistar rats did not show any clinical signs of toxicity and mortality. The body weight gain, and gross pathology

were found to be normal during the study period. Further, no toxic effects were observed during the 14-day observation period. In a subacute toxicity study, ARE was found to be safe up to 2000 mg/kg with no mortality and abnormal clinical signs observed in animals. During both the treatment and recovery period, there were no significant changes in body weight gain and feed consumption. Additionally, no treatment-related changes were observed in hematological or biochemical parameters, except for some changes in a few animals that cannot be attributed to the test item as these changes were neither consistent nor in a dose-dependent manner, and were not observed in both sexes. In males, there was a decrease in MCV & MCH (low and high dose), monocytes (mid and high dose), and an increase in neutrophils (low and high dose). In females, an increase in reticulocytes (low dose) was observed. Similarly, biochemical analysis revealed that male animals showed increased levels of albumin, calcium, and total protein (mid dose), as well as sodium (mid and high dose), whereas in the female animals, a decrease in total bilirubin (low dose), total protein, globulin, and potassium (during high dose recovery). Furthermore, there were no treatment-related changes observed in organ weight (both absolute and relative) or urine analysis when compared to the control group. Except for a few changes in male animals, such as increased absolute organ weight of epididymis (mid dose), thymus (high dose recovery), relative organ weight of thymus (low dose and high dose recovery), whereas in female animals, an increase in absolute organ weight of the thyroid with parathyroid was noted at the high dose recovery. Further, external and internal (gross) examination of animals from both treatment and control groups showed no abnormalities of gross or histopathological significance. Microscopic examination of sacrificed male animals revealed few changes in various organs—the liver, kidneys, prostate (which showed infiltrates and inflammatory cells), and lungs (which exhibited inflammation and non-significant increase in macrophages). In contrast, female animals showed changes in the liver (with infiltrates and inflammatory cells). (See Figure S1 for the histopathological findings of the liver in both control and high dose groups). The microscopic changes observed in the high-dose group male and female animals did not reveal any treatment-related lesions and were almost comparable to those in the control group. Thus, ARE can be considered safe in subacute toxicity, with a no observed adverse effect level (NOAEL) of 2000 mg/kg b.w. in both male and female Wistar rats.

In a subchronic toxicity study, no mortality or clinical signs of abnormalities were observed at doses up to 2000 mg/kg b.w. in Wistar rats. No significant changes were noticed in the body weight, feed consumption and ophthalmological examination in both treatment and recovery groups. No treatment-related changes were observed in hematological and biochemical parameters, except for some changes in a few animals that cannot be attributed to the ARE, as these changes were not consistent, did not follow a dose-dependent pattern, or were not observed in both sexes. Further, organ weight, external and internal gross examinations of animals, their clinical and neurological observations, and urine analysis did not reveal any treatment-related changes compared to respective control groups.

There were a few changes in feed consumption (increase at week 8, 11, and 12) in the recovery high dose group male animals when compared with the recovery control group animals. However, the effect was not considered treatment-related, as it lacks consistency between sexes and was not observed in subsequent weeks during the study period. The hematological parameters in the male group compared to the control showed a significant decrease in MCHC (mid and high dose males), an increase in platelets (high dose, females), WBC, and lymphocyte (high dose recovery females). The changes in biochemistry parameters showed a decrease in gamma glutamyl transferase (low and mid dose) and calcium (high dose) in the male group and total bilirubin (mid and high dose) in both males and females. In the recovery group, there was a decrease in alkaline aspartate aminotransferase & total protein (high dose- male group), and albumin, aspartate aminotransferase & alanine aminotransferase (high dose) in the female group. There were no significant difference in functional observation battery which includes neuro behavioral, sensory observation, foot splay measurement, grip strength assessment and activity meter, except a few changes in the treatment and recovery group were comparable to those in the control group that is a decrease in neurobehavioral urination (high dose), increase in grip strength in the hind limbs (low dose-female) which was considered incidental and not attributed to the test item. A decrease was observed in absolute and relative organ weight of adrenals (high dose) in female animals. Further external and internal (gross) examination of animals from both treatment and control groups showed no abnormalities of gross or histopathological significance. The microscopic findings observed in the high dose were almost comparable to control group and did not reveal any treatment-related lesions however, a non-significant change was observed in both the control and high dose groups. In both male and female animals, a few exhibited lesions in the lungs, liver, and kidneys, all showing infiltrates and inflammatory cells. Additionally, the mandibular lymph node showed dilatation, increased cellularity and the uterus displayed lumenal dilatation (See Figure S1 for histopathological findings of the liver in both control and high dose groups).

In the reproductive and developmental toxicity study, ARE at doses of up to 1000 mg/kg b.w. showed no clinical signs of toxicity or mortality during the study. In females, during the study period, no significant changes were observed in body weight, percentage change in body weight, and feed consumption. Estrous cycle evaluation through vaginal smear analysis was consistent with histopathological findings. No treatment-related changes were observed in maternal and developmental toxicity, including number of corpora lutea, implantations, litter size, number of live and dead foetus, weights, sex ratio, external anomalies, anogenital distance or nipples/areolae in male pups. No ARE-related changes in hormone levels or organ weight, except minor, non-dose-dependent increase in absolute weight of testes (low dose) and Cowper's gland (mid dose) in males. In gross pathology, no significant abnormalities in pups or parent animals, except a few male animals showed a change in size of testes and epididymis, and a female animal showed bilateral ovarian cysts, which were not dose or treatment-related. In the male group, microscopic evaluation revealed minimal to moderate degeneration of seminiferous tubules (low, mid and high dose) in testes, decreased sperm in the lumen (mid and high dose), and the presence of sperm granuloma (mid dose) in epididymis, and active supportive inflammation (control) in ovaries in female. These changes were of minimal to moderate severity

TABLE 8 | Effect of 90 days exposure to ARE on hematological parameters in Wistar rats.

Parameters	Vehicle control	Treated			Control recovery	High dose—2000 mg/kg b.w. recovery
		Low dose—500 mg/kg b.w.	Mid dose—1000 mg/kg b.w.	High dose—2000 mg/kg b.w.		
Male						
WBC ($\times 10^3$ cells/μL)	11.62 ± 2.13	10.89 ± 2.37	10.03 ± 2.18	10.89 ± 2.75	4.92 ± 1.74	5.53 ± 1.70
RBC ($\times 10^6$ cells/μL)	7.99 ± 0.48	8.22 ± 0.38	7.97 ± 0.46	7.84 ± 0.47	5.00 ± 0.46	4.87 ± 0.39
Hemoglobin (g/dL)	13.32 ± 0.72	13.35 ± 0.81	13.14 ± 0.65	12.92 ± 0.65	10.02 ± 0.86	9.84 ± 0.75
Hematocrit (%)	46.28 ± 2.45	46.93 ± 2.22	46.69 ± 1.81	46.68 ± 1.70	38.00 ± 2.88	37.74 ± 2.26
MCV (fL)	57.95 ± 1.61	57.07 ± 1.23	58.68 ± 1.52	59.64 ± 1.86	76.08 ± 1.46	77.54 ± 2.35
MCH (pg)	16.69 ± 0.44	16.21 ± 0.47	16.49 ± 0.63	16.50 ± 0.50	20.08 ± 0.43	20.24 ± 0.73
MCHC (g/dL)	28.78 ± 0.39	28.42 ± 0.57	28.12 ± 0.71	27.65 ± 0.56	26.34 ± 0.55	26.06 ± 0.46
Platelets ($\times 10^3$ cells/μL)	843.40 ± 107.67	853.30 ± 70.28	826.60 ± 76.60	817.60 ± 70.69	896.20 ± 107.81	929.40 ± 102.35
Neutrophils ($\times 10^3$ cells/μL)	2.07 ± 0.34	2.58 ± 0.84	2.66 ± 2.22	1.90 ± 0.61	1.03 ± 0.16	1.13 ± 0.33
Lymphocyte ($\times 10^3$ cells/μL)	8.92 ± 2.07	7.64 ± 1.88	6.68 ± 2.92	8.33 ± 2.30	3.58 ± 1.51	4.02 ± 1.34
Monocyte ($\times 10^3$ cells/μL)	0.33 ± 0.12	0.33 ± 0.15	0.48 ± 0.57	0.40 ± 0.41	0.22 ± 0.10	0.28 ± 0.11
Eosinophils ($\times 10^3$ cells/μL)	0.18 ± 0.07	0.20 ± 0.14	0.12 ± 0.07	0.15 ± 0.09	0.06 ± 0.02	0.06 ± 0.01
Basophils ($\times 10^3$ cells/μL)	0.05 ± 0.02	0.06 ± 0.03	0.04 ± 0.01	0.05 ± 0.02	0.01 ± 0.01	0.02 ± 0.01
Reticulocytes ($\times 10^9$ cells/L)	87.32 ± 19.04	99.68 ± 23.06	100.96 ± 29.76	81.71 ± 11.01	259.12 ± 78.64	271.96 ± 74.58
Prothrombin time (second)	10.89 ± 0.35	10.70 ± 0.26	10.65 ± 0.46	10.56 ± 0.47	10.74 ± 0.34	11.14 ± 0.25
APTT (second)	18.62 ± 0.88	18.38 ± 1.35	18.45 ± 1.32	18.44 ± 0.80	18.88 ± 0.67	19.04 ± 1.20
Female						
WBC ($\times 10^3$ cells/μL)	7.30 ± 1.13	7.49 ± 2.67	7.50 ± 2.36	6.81 ± 1.69	4.67 ± 1.54	6.66 ± 0.54
RBC ($\times 10^6$ cells/μL)	6.74 ± 2.04	7.32 ± 0.19	7.42 ± 0.42	7.56 ± 0.41	6.70 ± 0.80	6.75 ± 0.36
Hemoglobin (g/dL)	12.59 ± 1.57	12.68 ± 0.59	12.63 ± 0.64	13.02 ± 0.62	11.56 ± 1.45	11.70 ± 0.47

(Continues)

TABLE 8 | (Continued)

Parameters	Vehicle control	Treated			Control recovery	High dose—2000 mg/kg b.w. recovery
		Low dose—500 mg/kg b.w.	Mid dose—1000 mg/kg b.w.	High dose—2000 mg/kg b.w.		
Hematocrit (%)	40.34 ± 12.21	44.08 ± 1.05	43.67 ± 1.83	44.75 ± 1.61	40.54 ± 3.91	41.68 ± 1.43
MCV (fL)	59.70 ± 1.14	60.26 ± 0.84	58.94 ± 2.11	59.26 ± 1.56	60.62 ± 1.68	61.82 ± 2.59
MCH (pg)	24.49 ± 21.62	17.32 ± 0.49	17.05 ± 0.51	17.24 ± 0.60	17.28 ± 0.59	17.32 ± 0.69
MCHC (g/dL)	41.22 ± 37.07	28.75 ± 0.76	28.93 ± 0.42	29.10 ± 0.84	28.52 ± 1.08	28.02 ± 0.29
Platelets ($\times 10^3$ cells/μL)	760.80 ± 236.17	866.00 ± 132.01	950.00 ± 203.72	987.10 ± 136.42	834.20 ± 85.33	758.80 ± 91.39
Neutrophils ($\times 10^3$ cells/μL)	1.68 ± 0.50	1.62 ± 0.56	1.78 ± 0.38	1.60 ± 0.75	1.37 ± 0.79	1.75 ± 0.44
Lymphocyte ($\times 10^3$ cells/μL)	5.11 ± 0.64	5.34 ± 2.10	5.17 ± 1.96	4.71 ± 1.13	2.88 ± 0.85	4.46 ± 0.25
Monocyte ($\times 10^3$ cells/μL)	0.28 ± 0.10	0.30 ± 0.14	0.31 ± 0.18	0.26 ± 0.13	0.22 ± 0.11	0.27 ± 0.05
Eosinophils ($\times 10^3$ cells/μL)	0.16 ± 0.17	0.15 ± 0.08	0.17 ± 0.13	0.18 ± 0.07	0.16 ± 0.10	0.12 ± 0.05
Basophils ($\times 10^3$ cells/μL)	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.00
Reticulocytes ($\times 10^9$ cells/L)	69.57 ± 31.80	71.59 ± 21.08	85.05 ± 24.78	71.90 ± 23.49	48.86 ± 22.44	49.72 ± 21.20
Prothrombin time (second)	10.59 ± 0.23	10.94 ± 0.40	10.72 ± 0.42	10.89 ± 0.21	10.82 ± 0.19	10.74 ± 0.57
APTT (second)	18.60 ± 0.75	18.83 ± 1.17	19.10 ± 0.90	19.23 ± 1.48	18.74 ± 1.24	18.68 ± 1.11

Note: Values are expressed as mean ± SD.
Abbreviations: APTT, activated partial thromboplastin time; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RBC, erythrocyte count; WBC, total leukocyte count.

TABLE 9 | Effect of 90 days exposure to ARE on biochemical parameters in Wistar rats.

Parameters	Vehicle control	Treated			Control recovery	High dose—2000 mg/kg b.w. recovery
		Low dose—500 mg/kg b.w.	Mid dose—1000 mg/kg b.w.	High dose—2000 mg/kg b.w.		
Male						
AST (U/L)	148.84 ± 14.13	136.52 ± 23.84	133.50 ± 18.72	143.50 ± 20.69	181.05 ± 16.96	197.83 ± 59.44
ALT (U/L)	72.79 ± 17.84	66.19 ± 9.45	59.38 ± 9.44	62.91 ± 13.36	83.76 ± 15.30	94.43 ± 20.00
GGT (U/L)	4.81 ± 4.38	1.83 ± 0.46	1.76 ± 0.89	2.60 ± 2.24	0.84 ± 0.64	1.24 ± 0.81
Albumin (g/dL)	3.05 ± 0.09	3.09 ± 0.23	3.09 ± 0.13	3.16 ± 0.14	3.02 ± 0.25	2.95 ± 0.18
ALP (U/L)	164.68 ± 42.60	137.00 ± 26.71	133.32 ± 26.22	127.43 ± 38.28	183.12 ± 36.19	119.59 ± 24.05
Total bilirubin (mg/dL)	0.13 ± 0.05	0.10 ± 0.01	0.09 ± 0.03	0.09 ± 0.02	0.17 ± 0.02	0.16 ± 0.02
Cholesterol (mg/dL)	66.95 ± 9.28	66.48 ± 15.46	70.22 ± 17.10	75.20 ± 14.23	78.16 ± 15.49	66.72 ± 15.04
Creatinine (mg/dL)	0.35 ± 0.14	0.34 ± 0.04	0.32 ± 0.04	0.34 ± 0.05	0.35 ± 0.02	0.35 ± 0.04
Calcium (mg/dL)	9.75 ± 0.14	9.64 ± 0.47	9.94 ± 0.24	10.15 ± 0.32	9.56 ± 0.40	9.68 ± 0.43
Glucose (mg/dL)	83.82 ± 9.32	85.45 ± 12.09	94.34 ± 22.93	90.00 ± 12.34	100.03 ± 4.06	89.19 ± 10.12
HDL (mg/dL)	36.96 ± 4.91	36.42 ± 9.62	36.97 ± 9.18	41.26 ± 6.55	41.11 ± 10.05	36.49 ± 8.83
LDL (mg/dL)	8.38 ± 2.29	7.21 ± 1.50	8.44 ± 2.98	8.48 ± 2.14	10.06 ± 2.05	9.08 ± 2.86
Phophaste (mg/dL)	5.94 ± 0.53	5.65 ± 0.30	5.78 ± 0.64	6.05 ± 0.42	5.91 ± 0.85	6.03 ± 0.70
Total protein (g/dL)	6.93 ± 0.36	6.97 ± 0.23	6.84 ± 0.18	6.94 ± 0.27	7.24 ± 0.09	6.86 ± 0.17
Urea (mg/dL)	37.11 ± 12.14	36.63 ± 4.20	37.64 ± 6.79	36.01 ± 6.35	43.50 ± 1.69	45.12 ± 7.52
Globulin (g/dL)	3.89 ± 0.35	3.89 ± 0.14	3.76 ± 0.23	3.78 ± 0.25	4.22 ± 0.32	3.91 ± 0.28
Sodium (mmol/L)	145.67 ± 4.09	144.70 ± 2.37	147.39 ± 4.67	143.50 ± 2.57	150.78 ± 3.31	150.52 ± 2.72
Potassium (mmol/L)	5.31 ± 0.30	5.12 ± 0.24	5.18 ± 0.23	5.19 ± 0.28	5.68 ± 0.23	5.81 ± 0.81
Chlorine (mmol/L)	145.39 ± 2.43	144.01 ± 6.23	144.32 ± 4.11	145.69 ± 5.51	144.67 ± 4.37	140.17 ± 1.78
Blood urea nitrogen (mg/dL)	17.34 ± 5.67	17.12 ± 1.96	17.59 ± 3.17	16.83 ± 2.97	20.33 ± 0.79	21.08 ± 3.52
Female						
AST (U/L)	144.49 ± 30.38	146.27 ± 42.36	139.75 ± 32.08	117.68 ± 15.77	126.25 ± 13.58	107.21 ± 9.42

(Continues)

TABLE 9 | (Continued)

Parameters	Vehicle control	Treated			Control recovery	High dose—2000 mg/kg b.w. recovery
		Low dose—500 mg/kg b.w.	Mid dose—1000 mg/kg b.w.	High dose—2000 mg/kg b.w.		
ALT (U/L)	61.04 ± 10.10	70.57 ± 21.75	53.80 ± 13.23	51.03 ± 7.13	65.11 ± 7.96	59.71 ± 11.51
GGT (U/L)	1.89 ± 0.69	3.89 ± 4.98	1.97 ± 0.51	2.05 ± 0.95	0.55 ± 0.18	1.06 ± 0.69
Albumin (g/dL)	3.46 ± 0.29	3.45 ± 0.19	3.43 ± 0.25	3.62 ± 0.11	3.79 ± 0.24	3.28 ± 0.30
ALP (U/L)	78.35 ± 36.73	78.27 ± 38.33	73.87 ± 21.57	76.82 ± 26.08	66.77 ± 24.77	62.38 ± 11.25
Total bilirubin (mg/dL)	0.14 ± 0.03	0.12 ± 0.02	0.11 ± 0.02	0.10 ± 0.01	0.13 ± 0.04	0.11 ± 0.02
Cholesterol (mg/dL)	82.91 ± 15.14	85.29 ± 11.63	73.23 ± 12.52	86.14 ± 13.36	95.00 ± 26.73	76.62 ± 5.33
Creatinine (mg/dL)	0.41 ± 0.06	0.41 ± 0.04	0.37 ± 0.03	0.41 ± 0.06	0.39 ± 0.06	0.42 ± 0.04
Calcium (mg/dL)	9.86 ± 0.35	9.94 ± 0.38	9.98 ± 0.33	10.11 ± 0.34	10.15 ± 0.22	9.90 ± 0.19
Glucose (mg/dL)	99.20 ± 14.00	99.49 ± 17.45	99.21 ± 15.04	99.07 ± 15.02	96.78 ± 10.68	89.49 ± 7.69
HDL (mg/dL)	54.23 ± 10.34	55.21 ± 8.65	47.63 ± 8.24	56.07 ± 9.28	63.01 ± 17.26	50.94 ± 7.12
LDL (mg/dL)	5.86 ± 1.80	5.90 ± 1.29	4.73 ± 1.03	6.15 ± 1.16	6.59 ± 1.96	4.61 ± 1.04
Phosphate (mg/dL)	4.99 ± 0.71	4.73 ± 0.55	5.14 ± 0.58	4.92 ± 0.64	4.50 ± 0.59	5.01 ± 0.36
Total protein (g/dL)	7.55 ± 0.52	7.54 ± 0.48	7.39 ± 0.38	7.51 ± 0.35	7.80 ± 0.47	7.33 ± 0.26
Urea (mg/dL)	41.54 ± 4.90	39.35 ± 4.98	39.92 ± 8.36	40.72 ± 8.27	46.92 ± 9.75	47.59 ± 8.64
Globulin (g/dL)	4.09 ± 0.38	4.09 ± 0.36	3.96 ± 0.24	3.90 ± 0.36	4.01 ± 0.25	4.05 ± 0.24
Sodium (mmol/L)	146.46 ± 3.63	144.37 ± 2.67	148.70 ± 2.14	148.81 ± 3.07	131.47 ± 2.47	132.53 ± 1.64
Potassium (mmol/L)	4.93 ± 0.37	4.91 ± 0.25	4.94 ± 0.33	5.06 ± 0.34	4.72 ± 0.40	4.79 ± 0.22
Chlorine (mmol/L)	134.53 ± 4.13	132.43 ± 3.22	136.16 ± 1.69	135.45 ± 4.21	138.05 ± 3.88	135.49 ± 2.97
Blood urea nitrogen (mg/dL)	19.41 ± 2.29	18.39 ± 2.33	18.66 ± 3.91	19.03 ± 3.87	21.93 ± 4.55	22.24 ± 4.04

Note: Values are expressed as mean ± SD.
Abbreviations: ALP: alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase.

TABLE 10 | Effect of 90 days exposure to ARE on absolute organ weights (g) in Wistar rats.

Organs	Vehicle control	Treated			Control recovery	High dose-2000 mg/kg b.w. recovery
		Low dose—500 mg/kg b.w.	Mid dose—1000 mg/kg b.w.	High dose—2000 mg/kg b.w.		
Male						
Liver	12.2994 ± 1.1462	13.8215 ± 2.4124	13.7673 ± 1.8566	13.5032 ± 1.6961	13.1253 ± 3.3972	14.7399 ± 1.2116
Kidneys	2.7253 ± 0.1746	2.9039 ± 0.3161	3.0681 ± 0.4196	3.0077 ± 0.3176	2.9164 ± 0.4346	3.1547 ± 0.2972
Heart	1.4438 ± 0.1557	1.4599 ± 0.1555	1.4912 ± 0.2103	1.4792 ± 0.1956	1.3782 ± 0.2173	1.5164 ± 0.2146
Spleen	0.8089 ± 0.1189	0.8903 ± 0.1227	0.9122 ± 0.0935	0.8611 ± 0.1228	0.7415 ± 0.0698	0.9810 ± 0.2538
Brain	1.8786 ± 0.0936	1.9078 ± 0.1527	1.8373 ± 0.1493	1.9033 ± 0.0924	1.9518 ± 0.1360	1.9690 ± 0.1618
Thymus	0.3547 ± 0.0922	0.3037 ± 0.0523	0.3323 ± 0.0748	0.3371 ± 0.0902	0.2890 ± 0.0419	0.2973 ± 0.0355
Adrenals	0.0619 ± 0.0098	0.0592 ± 0.0070	0.0605 ± 0.0111	0.0636 ± 0.0140	0.0844 ± 0.0125	0.0723 ± 0.0164
Testes	3.2064 ± 0.3484	3.3669 ± 0.3788	3.3988 ± 0.2316	3.4288 ± 0.2918	3.2281 ± 0.4449	3.4482 ± 0.3370
Epididymis	1.3209 ± 0.2405	1.4511 ± 0.3018	1.3941 ± 0.2441	1.3957 ± 0.1700	1.3988 ± 0.2036	1.3737 ± 0.2172
Prostate plus seminal vesicles with CG	2.0158 ± 0.4151	2.5450 ± 0.4917	2.3102 ± 0.4558	2.3907 ± 0.7020	2.3587 ± 0.5798	2.1948 ± 0.4138
Thyroid with parathyroid	0.0243 ± 0.0015	0.0268 ± 0.0038	0.0245 ± 0.0037	0.0268 ± 0.0044	0.0220 ± 0.0038	0.0244 ± 0.0050
Pituitary gland	0.0122 ± 0.0011	0.0122 ± 0.0015	0.0117 ± 0.0011	0.0131 ± 0.0020	0.0130 ± 0.0016	0.0128 ± 0.0014
Female						
Liver	9.1531 ± 0.7442	8.7459 ± 1.3771	8.6591 ± 1.4958	8.8673 ± 1.1668	8.8686 ± 1.9024	8.2358 ± 0.4706
Kidneys	1.9359 ± 0.1633	1.9050 ± 0.2339	1.8913 ± 0.2417	1.8887 ± 0.2217	2.0272 ± 0.3278	1.8563 ± 0.1239
Heart	1.0231 ± 0.0947	1.0121 ± 0.1331	0.9696 ± 0.1260	0.9869 ± 0.1229	1.0462 ± 0.1066	0.9522 ± 0.0679
Spleen	0.5949 ± 0.1149	0.5770 ± 0.1270	0.6038 ± 0.0790	0.5390 ± 0.1159	0.4891 ± 0.0876	0.5293 ± 0.1174
Brain	1.8063 ± 0.0602	1.8715 ± 0.1010	1.7546 ± 0.1322	1.8222 ± 0.1554	1.7870 ± 0.0608	1.8107 ± 0.0758
Thymus	0.2433 ± 0.0591	0.2742 ± 0.0663	0.2190 ± 0.0535	0.2177 ± 0.0422	0.2266 ± 0.0357	0.2354 ± 0.0607
Adrenals	0.0746 ± 0.0117	0.0729 ± 0.0188	0.0786 ± 0.0160	0.0744 ± 0.0134	0.0828 ± 0.0118	0.0662 ± 0.0068
Uterus with cervix	0.6403 ± 0.1522	0.5761 ± 0.1119	0.6005 ± 0.2644	0.7039 ± 0.2945	0.6581 ± 0.1024	0.6429 ± 0.1248
Ovaries	0.1343 ± 0.0327	0.1119 ± 0.0329	0.1150 ± 0.0342	0.1116 ± 0.0224	0.1176 ± 0.0204	0.1009 ± 0.0208
Thyroid with parathyroid	0.0160 ± 0.0036	0.0168 ± 0.0021	0.0172 ± 0.0030	0.0159 ± 0.0020	0.0170 ± 0.0021	0.0177 ± 0.0029
Pituitary gland	0.0168 ± 0.0023	0.0164 ± 0.0012	0.0159 ± 0.0021	0.0158 ± 0.0028	0.0169 ± 0.0056	0.0172 ± 0.0031

Note: Values are expressed as mean ± SD.

TABLE 11 | Effect of body weight (g) of female animals during the gestation and lactation periods in reproductive/developmental toxicity study of ARE.

	Gestation period (days)				Lactation period (days)			
Treatment groups	**0**	**7**	**14**	**20**	**1**	**4**	**7**	**14**
Vehicle control	277.26 ± 22.04	304.15 ± 20.74	339.16 ± 31.41	408.62 ± 2.48	317.49 ± 33.13	325.12 ± 35.31	332.14 ± 37.25	344.52 ± 34.80
Low dose—100 mg/kg b.w.	275.44 ± 22.35	295.92 ± 22.57	330.25 ± 1.11	400.84 ± 3.60	320.97 ± 29.97	328.53 ± 32.41	338.98 ± 32.30	348.13 ± 34.99
Mid dose—500 mg/kg b.w.	281.14 ± 29.78	303.84 ± 30.28	346.60 ± 31.02	426.41 ± 39.77	325.7 ± 42.11	333.27 ± 44.34	340.92 ± 44.83	356.06 ± 44.33
High dose—1000 mg/kg b.w.	265.33 ± 28.29	284.67 ± 31.65	321.29 ± 32.01	399.38 ± 41.99	302.46 ± 29.28	309.26 ± 28.81	317.56 ± 27.85	331.52 ± 26.41

Note: Values are expressed as mean ± SD.

TABLE 12 | Effect of absolute organ weights (g) of animals in the reproductive/developmental toxicity study of ARE.

	Male						Female		
Treatment groups	**Testes**	**Epididymidis**	**Glans penis**	**Thyroid with parathyroid**	**LABC**	**Cowper's glands**	**Ovaries**	**Uterus with cervix**	**Thyroid and parathyroid**
Vehicle control	3.4350 ± 0.3812	1.3501 ± 0.1961	0.1326 ± 0.0350	0.0198 ± 0.0047	1.3919 ± 0.1811	0.1178 ± 0.0180	0.2077 ± 0.2686	0.4983 ± 0.1113	0.0211 ± 0.0054
Low dose—100 mg/kg b.w.	4.2609 ± 0.8309	1.3732 ± 0.0661	0.1274 ± 0.0146	0.0206 ± 0.0045	1.4627 ± 0.2053	0.1333 ± 0.0214	0.1181 ± 0.0186	0.4700 ± 0.1450	0.0171 ± 0.0033
Mid dose—500 mg/kg b.w.	3.9676 ± 0.5388	1.4080 ± 0.0617	0.1340 ± 0.0139	0.0200 ± 0.0017	1.4124 ± 0.1175	0.1449 ± 0.0216	0.1068 ± 0.0180	0.4445 ± 0.1252	0.0188 ± 0.0037
High dose—1000 mg/kg b.w.	3.3118 ± 1.2789	1.2608 ± 0.3236	0.1184 ± 0.0192	0.0239 ± 0.0031	1.3241 ± 0.1359	0.1342 ± 0.0212	0.1109 ± 0.0268	0.4223 ± 0.0918	0.0189 ± 0.0051

Note: Values are expressed as mean ± SD.
Abbreviation: LABC, levator ani plus bulbocavernosus muscle complex.

TABLE 13A | Summary data of average P/E ratio and % average reduction (main study) in mammalian erythrocyte micronucleus test of ARE in female mice.

Group	Dose (mg/kg b.w.)	PCE	NCE	TE	% P/E ratio	% P/E ratio average	% PCE compared to VC	% reduction
Vehicle control (water) G1	0	309	191	500	61.80	60.96	NA	NA
		312	188	500	62.40			
		304	196	500	60.80			
		301	199	500	60.20			
		298	202	500	59.60			
ARE low dose (G2)	500	294	206	500	58.80	58.04	95.21	4.79
		285	215	500	57.00			
		291	209	500	58.20			
		288	212	500	57.60			
		293	207	500	58.60			
ARE mid dose (G3)	1000	275	225	500	55.00	55.40	90.88	9.12
		272	228	500	54.40			
		279	221	500	55.80			
		276	224	500	55.20			
		283	217	500	56.60			
ARE high dose (G4)	2000	257	243	500	51.40	52.20	85.63	14.37
		263	237	500	52.60			
		265	235	500	53.00			
		259	241	500	51.80			
		261	239	500	52.20			
Cyclophosphamide monohydrate (G5)	50	240	260	500	48.00	47.20	77.43	22.57
		238	262	500	47.60			
		236	264	500	47.20			
		235	265	500	47.00			
		231	269	500	46.20			

Abbreviations: NA, not applicable; NCE, normochromatic erythrocytes; P/E, PCE/total erythrocyte ratio; P/E, ratio of polychromatic erythrocytes to total number of erythrocyte; PCE, polychromatic erythrocytes; TE, total erythrocytes.

TABLE 13B | Summary data of total MNPCE and % MNPCE (main study) in mammalian erythrocyte micronucleus test of ARE in female mice.

Group	Dose (mg/kg b.w.)	Total PCE considered for % MNPCE	Total MNPCE	Average MNPCE	Average % MNPCE	±SD
Vehicle control (water G1)	0	20,000	24	4.8	0.120	0.01
ARE low dose (G2)	500	20,000	23	4.6	0.115	0.02
ARE mid dose (G3)	1000	20,000	22	4.4	0.110	0.02
ARE high dose (G4)	2000	20,000	21	4.2	0.105	0.02
Cyclophosphamide monohydrate (G5)	50	20,000	256	51.2	1.280[a]	0.08

Abbreviations: MNPCE, micronucleated polychromatic erythrocytes; SD, standard deviation.
[a]Statistically significant increase in mean %MNPCE was observed compared to the concurrent vehicle control.

and not treatment-related due to the lack of dose-dependency and histopathological correlation. From the results obtained, the NOAEL of ARE was found to be 1000 mg/kg body weight for the reproductive and developmental toxicity study.

In a bacterial reverse mutation study, ARE was found to be non-mutagenic, as it does not induce (point) gene mutations by base-pair changes or frameshift in the histidine operon in any of the bacterial strains of *Salmonella typhimurium* (either with or without metabolic activation). Likewise, in a mammalian erythrocyte micronucleus study, ARE did not induce micronuclei formation in female animals treated with dose levels up to 2000 mg/kg b.w. justifying it as non-mutagenic.

6 | Conclusion

In summary, the findings indicate that administration of ARE at the tested doses to Wistar rats showed no adverse effects or mortality in acute (at a single dose of 2000 mg/kg body weight), subacute, subchronic, and reproductive/developmental toxicity studies. Based on these results, the NOAEL was determined to be up to 2000 mg/kg body weight for subacute and subchronic studies, and up to 1000 mg/kg body weight for reproductive/developmental studies in both male and female animals. The NOAEL for subchronic studies was established at up to 2000 mg/kg body weight, which is the highest dose tested. However, the actual NOAEL value for ARE may be higher than the highest dose tested. Additionally, the findings suggest that ARE is unlikely to cause any genotoxic effects based on mammalian erythrocyte micronucleus and bacterial reverse mutation tests.

Author Contributions

Anju Majeed and **Shaheen Majeed:** conceptualization, resources, writing – review and editing. **Anjali Pandey** and **Sarang Bani:** methodology, investigation, supervision, project administration, data curation, writing – review and editing. **Smitha Thazhathidath:** writing – original draft, writing – review and editing.

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Disclosure

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Histopathological findings in liver section were examined in vehicle and high dose groups in (A) Subacuteand (B) Subchronic toxicity.