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# Safety Assessment of a Novel Valerian Powder 2% (Sleeproot®): Acute and Sub-Chronic Oral Toxicity, Mutagenicity, and Genotoxicity Studies

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## Abstract

Valeriana officinalis is a perennial herb traditionally used for the management of sleep disorders. Here we report the results from the battery of toxicology tests that were conducted to evaluate the safety of Valerian extract powder 2% (VE), commercially known as Sleeproot®. The present investigation was designed to establish the safety of VE by conducting a comprehensive list of toxicity experiments, including acute and sub-chronic oral toxicity, mutagenicity and genotoxicity studies. These studies were conducted under Good Laboratory Practice (GLP) in accordance with OECD guidelines. Based on acute oral toxicity study, no lethality was observed in Wistar rats at dose as high as 2000 mg/kg body weight (b.w.) of VE with LD50 cut-off value of 5000 mg/kg b.w. Further, based on the 90-day repeat dose oral toxicity study followed by 28-day recovery period in Wistar rats, No Observed Adverse Effect Level (NOAEL) was noted to be 1000 mg/kg b.w./day. The VE sample did not induce gene mutations and was considered non-mutagenic up to concentration of 2.5 mg/plate in Salmonella typhimurium reverse mutation assay (AMES). Further, no genotoxicity was noted in the micronucleus assay conducted using polychromatic erythrocytes from Swiss albino mice and, chromosomal aberrations test in human peripheral blood lymphocytes. Overall, VE was found to be safe in rodents, non-mutagenic, non-genotoxic, and had no observed adverse effects under experimental conditions tested.

**Keywords:** Valerian; Toxicity; Safety; Mutagenicity; Genotoxicity; Sleep Disorder

**Abbreviations:** : ALP: Alkaline Phosphatase, ALT: Alanine Aminotransferase, ANOVA: Analysis of Variance, APTT: Activated Partial Thromboplastin Time, AST: Aspartate Aminotransferase, BUN: Blood Urea Nitrogen, $CO_2$: Carbon Dioxide, CPCSEA: Committee for the Purpose of Control and Supervision on Experiments on Animals, Cl: Chloride, DC: Differential Count, DNA: Deoxyribonucleic Acid, DRDE: Defense Research and Development Establishment, DRDO: Defense Research and Development Organization, FDA: Food and Drug Administration, GHS: Globally Harmonized System (of Classification and Labelling of Chemicals), GI: Gastrointestinal, GLP: Good Laboratory Practice, HCT: Hematocrit, HDL: High Density Lipoprotein, HGB: Hemoglobin, HPLC: High Performance Liquid Chromatography, K: Potassium, LD: Lethal Dose, LDL: Low Density Lipoprotein, MCH: Mean Corpuscular Hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration, MCV: Mean Corpuscular Volume, N: Number of observations/animals, NOAEL: No Observed Adverse Effect Level, Na: Sodium, OECD: Organization for Economic Co-operation and Development, PCE: Polychromatic Erythrocyte, PLT: Platelet, PT: Prothrombin Time, RBC: Red Blood Cell, RPMI: Roswell Park Memorial Institute (medium), SD: Standard Deviation, T3: Triiodothyronine, T4: Thyroxine, TA100: Tester Strain 100 of Salmonella typhimurium, TA102: Tester Strain 102 of S. typhimurium, TA1535: Tester Strain 1535 of S. typhimurium, TA1537: Tester Strain 1537 of S. typhimurium, TA98: Tester Strain 98 of S. typhimurium, TCHO: Total Cholesterol, TSH: Thyroid Stimulating Hormone, VE: Valerian Extract, WBC: White Blood Cell

## Introduction

Valeriana officinalis is a perennial herb used for the management of sleep disorders across the world, including the Americas and Europe [1,2]. Root extract of Valerian has also been used as a sedative [3,4]. V. officinalis extract possess antioxidant, anti-inflammatory, sedative, anxiolytic, anticonvulsant, cytoprotective and neuroprotective activities [5]. Valerian extract reduces sleep latency and improves sleep architecture and perception of insomnia in healthy volunteers as well as patients suffering from sleep disorders [6,7]. Volatile oils and valepotriates present in the root extract are considered responsible for its sedative activity [2,4,8]. Several preclinical and clinical studies showed valerian as an effective alternate treatment for insomnia

and overall improvement in multiple sleep-related parameters [6,7] but also have established the safety [9,10]. Valerenic acid from valerian acts as a sedative by modulating GABA (gamma-aminobutyric acid) receptor function [2] as well as inhibition of enzymatic breakdown of GABA [11] and partial agonists of 5-HT5a (5-Hydroxytryptamine (serotonin) receptor 5A) receptor activities [8]. Valerian extract contains a class of iridoid compounds called valepotriates which are known to possess anxiolytic effect. Valerian extract significantly ($p<0.05$) increased the sleep duration with shorter sleep latency that was comparable to melatonin through modulation of GABA and 5-HT5a receptor expression in brain tissues in a pentobarbital-induced sleep model in mice [12]. Previously we have developed a novel and improved root extract from Valeriana officinalis (VE) containing 2% total valerenic acid content as compared to the 0.5-0.8% used in most of the published preparations explored in the past.

We have conducted comprehensive human clinical study that demonstrated significant improvement of both subjective and objective parameters of sleep, such as the Pittsburgh Sleep Quality Index, wrist actigraphy, and polysomnography in young subjects with mild insomnia symptoms. We demonstrated not only significant improvement in actual sleep with a single dose of VE but also improved overall sleep quality, sleep latency, sleep efficiency, and total sleep time but also decreased anxiety and daytime sleepiness, and improved feelings of being refreshed after waking up with VE supplementation. Further, we found VE to be safe and well tolerated throughout the study [9]. Our mechanistic studies conducted in a pentobarbital-induced sleep model in mice indicated that VE enhanced both the quantity and quality of sleep through the GABAergic pathway and effectively increased sleep duration while reducing the time to fall asleep [12]. The objective of the current study was to evaluate adverse events and the safety of VE through a battery of toxicology tests. A single-dose acute and sub-chronic (90-day) repeated dose-response oral toxicity studies in Wistar rats were conducted for assessing toxicity. The genotoxic potential of VE was conducted through reverse mutation (AMES) test using Salmonella typhimurium strains and in vivo micronucleus assay to induce the formation of micronuclei in polychromatic erythrocytes in the bone marrow of Swiss albino mice. The chromosomal aberration (in vitro genotoxicity) test was conducted using human peripheral blood lymphocytes.

## Materials and Methods

### Acute studies

The acute oral toxicity study was performed at Vipragen Biosciences Private Limited, Mysuru, India, according to the protocol as prescribed by the Organization for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals (No. 423, Section 4, Health Effects). Female Wistar rats from Hylasco Biotechnology (India) Pvt. Ltd., Hyderabad, were used in the studies. In two separate experiments, three female rats (8-10 weeks of age; body weight range: 197.72 to 221.06 g), were administered a single dose of VE, using oral gavage at a dose of 300 mg/kg body weight for Step I and Step II experiments. As all the animals dosed in Step I & II survived, three females were treated at a dose of 2000 mg/kg body weight (Step III & IV). In both the experiments, animals were observed once daily for 14 days for any clinical signs of morbidity or mortality. On Day 15, all the animals were euthanized, and gross pathological examinations were done. Mean and standard deviation were calculated on the basis of the results using GraphPad Prism (Version 10.0.1).

### Sub-chronic studies

#### Study design

The sub-chronic oral toxicity study was performed at Vipragen Biosciences Private Limited, Mysuru, India according to a well-designed protocol based on OECD Guidelines for Testing Chemicals, Health Effects Test Guidelines, for Repeated Dose 90-Day Oral Toxicity Study in Rodents, Section 408 and US FDA OECD 2018. The study was conducted in compliance with the OECD principles and Good Laboratory Practices in accordance with the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) approved laboratory (registration number 1683/PORcBiBt/S/13/CPCSEA) following all ethical practices as laid down in the guidelines for animal care and institutional animal ethics committee approval.

#### Test item

A dark greenish-brown colored homogenous powder of Valerian extract, prepared from roots of V. officinalis, containing total valerenic acid content of minimum 2%, commercially known as Sleeproot® manufactured by OmniActive Health Technologies Limited, India, was used in the study.

#### Animals

A total of 100 Wistar rats (50 males and 50 females) 6-8 weeks old, from Hylasco Biotechnology Private Limited (Hyderabad, India) were selected after physical examination for the study. Female rats used in the study were nulliparous and non-pregnant. All animals were maintained according to standard guidelines. The animals were housed in groups of three in standard polycarbonate cages with autoclaved clean corn cob bedding. The animal room was air-conditioned with adequate air changes per hour and a 12 h light/dark cycle. The experimental room was continuously monitored for temperature and relative humidity. The room temperature and relative humidity were 22.0±3°C and 30 to 70%, respectively. The animals were allowed to acclimatize for a minimum of five days before the initiation of experiments. Purina Lab Diet 5L79 Rat and Mouse 18% pellet feed (PMI Nutrition International, USA) and drinking water were provided ad libitum throughout the study period.

#### Treatment

For the sub-chronic study, Wistar rats (10/sex/group) were divided into six groups based on stratified randomization by


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using body weights taken before treatment. At randomization, the animals were approximately 6-7 weeks old, and their body weight was within ±20% of the overall mean of each sex (male- 202.73-252.86 g; female- 157.05-193.78 g). Rats were given VE preparation by mouth once a day for 90 days at different doses: 0 mg/kg (Group I - control), 250 mg/kg (Group II - low dose), 500 mg/kg (Group III - mid dose), or 1000 mg/kg (Group IV - high dose), using a volume of 10 mL/kg. Two more groups of animals in the recovery study were given either 0 (Group V-R) or 1000 (Group VIR) mg/kg bw/day of VE for 90 days, and then they had no treatment for 28 days. The analytical verification of VE was carried out using the HPLC (High-Performance Liquid Chromatography) method. The dosage formulations were prepared by thoroughly mixing appropriate amounts of the test material, VE, with Milli-Q water for administration to rats shortly before dosing. The homogeneity of VE in the vehicle was maintained during the daily administration period using a magnetic stirrer. During the course of the sub-chronic study, all animals were provided ad libitum feed until the day prior to the scheduled euthanasia. At the end of the 90-day treatment period, all the animals in the main group were euthanized. In the recovery group, after completion of the 90-day treatment period, the animals were kept under post-treatment observations for 28 days and then euthanized.

**Parameters investigated**

**Clinical signs, body weight, and feed consumption**: All animals were observed daily for mortality and morbidity. Clinical signs were recorded once daily during the treatment period. The cage-side observations included changes in handling response, skin, fur, eyes and mucous membranes, breathing pattern, occurrence of secretions and excretions, ears, eyes, oral cavity, ventral and posterior side of the body, checking injury, wound or swelling, posture, gait, and behavior. An ophthalmological examination was performed using a Welch Allyn direct ophthalmoscope. The weight of each rat was recorded on Day 1 and at weekly intervals throughout the study period. For the corresponding intervals, mean body weights and mean body weight changes were computed. The amount of feed consumed by each cage of animals was recorded weekly. Feed intake was calculated as g/animal/day for the corresponding body weight intervals.

Clinical laboratory investigations: Urine and blood samples were collected for clinical evaluations (urinalysis, hematology, and serum chemistry) from all animals in the 90-day study and the recovery groups during the last week of the treatment period, prior to the scheduled necropsy. For urine collection, each animal was kept in a metabolic cage and fasted for 12-14 hours, and the urine sample was collected in a specimen vial. Urinalysis included volume, appearance, specific gravity, color, pH, blood cells, bilirubin, urobilinogen, leukocytes, ketone bodies, proteins, glucose, and nitrite. Additionally, microscopic examination of urine was also carried out. Urine analysis was performed using a Cobas u411 urine analyzer, Roche, USA. Blood samples were collected from all animals under a light isoflurane anesthesia. Before blood collection, the animals were fasted overnight. Animals were given unlimited access to water while they were fasting. Blood samples were taken from a vein behind the eyes using a small glass tube that contains heparin. Blood samples were centrifuged, and the plasma was separated for clinical biochemistry analysis. Hematology parameters analyzed included erythrocyte count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (thrombocyte) count (PLT), total leukocyte count (WBC), differential leukocyte count (DC), reticulocyte count, and coagulation efficiency. Clinical biochemistry parameters analyzed included glucose, urea, creatinine, cholesterol, total triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, sodium (Na), potassium (K), chloride (Cl), total protein, albumin, and globulin.

Necropsy, organ weight and histopathology: On the completion of the treatment period, complete necropsy was performed on all animals. Animals were euthanized under carbon dioxide inhalation anesthesia. At the scheduled necropsies (on Day 91 for main groups I-IV and Day 121 for the recovery group), the following organs were weighed from all animals: brain, thymus, spleen, ovaries, heart, kidneys, lungs, prostate plus seminal vesicles with coagulating glands, thyroid gland with parathyroid gland, pituitary gland, testes, liver, adrenals, uterus, and epididymides. Over 40 tissues and organs were collected and placed in 10% neutral-buffered formalin for microscopic examinations. For histopathology, tissues were processed, embedded in paraffin blocks, sectioned at 4 to 5 micrometers, and stained with hematoxylin and eosin.

**Statistical analysis**

Statistical analysis was performed using "GraphPad Prism (Version 10.0.1)". Analyses were conducted using two-tailed tests for a minimum significance level of 5%, comparing each test item treated group to the control group by sex. Each group mean was presented with the standard deviation (SD) and the number of animals/observations (N) used to calculate the mean. The "p" value ≤ 0.05 was considered statistically significant. Males and females were considered separately for analysis. The data was visually examined for outliers and subjected to Grubb's test at a 1% significance level. The significant values in Grubb's test were excluded from statistical analysis. The numerical data, including but not necessarily limited to body weight, body weight change, food consumption, grip strength, hind limb foot splay, motor activity count, body temperature, hematology, clinical chemistry, thyroid hormone analysis, urinalysis (pH and specific gravity), and absolute and relative organ weights, were subjected to statistical analysis. Initially, data were subjected to the Shapiro-Wilk normality test to check the normal distribution of data and


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Bartlett's test for homogeneity of variance. When Bartlett's test or Shapiro-Wilk normality tests were non-significant, Dunnett's post hoc test was used to compare the control group with test item-treated groups. When Bartlett's test or the Shapiro-Wilk normality test was significant, data were subjected to Kruskal-Wallis ANOVA followed by Dunn's post hoc test.

**Mutagenicity study**

The mutagenic activity of VE was investigated by means of the AMES test conducted at Ross Lifescience Limited, Pune, India, in compliance with Good Laboratory Practices (GLP) requirements as per OECD Guidelines 471. The mutagenicity study was conducted at Ross Lifesciences Limited, Pune, India. The tester strains used in this study were the histidine-requiring auxotrophic strains of S. typhimurium (TA98, TA100, TA102, TA1535, and TA1537). The cultures were maintained at Ross Lifesciences Limited, which were originally obtained from Dr. G. P. Meshram, DRDO-DRDE, Ambernath, India. Strains TA100, TA102, and TA1535 are designed to find changes in base pairs, while strains TA98 and TA1537 are used to identify mutagens that create frameshift mutations. Metabolic activation was performed using a cofactor-supplemented post-mitochondrial fraction (S9 fraction). Based on range-finding test results, five concentrations of test items (0.156, 0.313, 0.625, 1.25, and 2.5 mg/plate) were selected for vehicle and reference controls in three replicates for the first definitive study in the presence of 5% S9 and without S9. Spacing factor 2 was used to elect the test item concentration in the above definitive study. In the second definitive study, again five test item concentrations of test items 0.025, 0.079, 0.250, 0.791, and 2.5 mg/plate were selected for with (10% S9 mix) and without metabolic activation along with solvent and reference controls with three replicates. In both definitive studies, all five S. typhimurium tester strains were tested with and without metabolic activation (S9). The inoculation of the Salmonella tester strains for each study was done using master vial copies. The culture was mixed in a shaker water bath at 37 ± 2 °C at 110-120 rpm to get a cell density of 1-2 x 109 viable cells/mL. The pH was maintained at 7.2±0.2. The following mixture was added on to the minimal glucose agar plates: 100 µL of test solution at each dose/vehicle/positive control; 500 µL of S9 mix (for test metabolic activation) or sodium phosphate buffer (for test without metabolic activation); 100 µL bacterial suspension; and 2000 µL top agar preheated at 46±1°C. After solidification, the plates were incubated upside down for 48-72 hours (approx.) at 37±2°C. The colonies were counted manually. The mean values from the plates for each concentration, together with standard deviations, were compared to the spontaneous reversion rates.

**Micronucleus study**

The in vivo micronucleus test was conducted to evaluate the genotoxic potential of VE to induce the formation of micronuclei in polychromatic erythrocytes in the bone marrow of Swiss albino mice. The study was conducted at Ross Lifescience Limited, Pune, India, in compliance with OECD 474 (2016) [24] and GLP C (97)186/Final. Initially, a dose range finding study (DRF) was conducted at the dose levels of 2000, 1000, 500, and 250 mg/kg bw per day to identify study-limiting toxicity and select the doses for the main study. Based on the results, a 2000 mg/kg dose was considered for the main study and 50 mg/kg of cyclophosphamide monohydrate was used as a positive control. Milli-Q water was used as a vehicle for the test item formulations. The dose volume used was 10 mL/kg bw. Eight-week-old male and female Swiss albino mice were utilized for this study. The mice were acclimatized, observed, and examined for a period of 5 days to confirm that the animals were in good health. Animals were housed in individually sterilized polycarbonate cages with autoclaved clean corn cob bedding material; the room temperature was 20.1-23.8°C with 48.0-69.0% relative humidity and 12-hour light-dark cycles. All animals had access to rodent feed (Purina Lab Diet 5L79 Rat and Mouse 18%) and water ad libitum. Animals were randomized into groups of 5 animals/sex/group at a dose level of 2000 mg/kg bw/day, negative (0) and positive control groups (cyclophosphamide monohydrate - 50 mg/kg bw). The VE formulations were administered through the oral route by gavage. The positive control was administered through the intraperitoneal route.

All the animals were observed for clinical signs of toxicity approximately at 1 hr, 2 hr, and 4 hr on the first day of test item administration, twice on the second day, and once on the day of sacrifice. The test article VE and vehicle control were administered twice at an interval of 24 h by oral gavage using a 1 mL disposable syringe. The positive control was administered on Day 2. The dose-volume, route, and frequency of administration for the vehicle were consistent with those of the test item. The volume of the dose administered to each animal was calculated based on its most recent body weight. Animals were observed for clinical signs at pre-dose and at 1, 2, 3, 4, and 24 hr post-dosing. As the positive control animals were dosed once on day 2, the observations were performed accordingly, and they were observed twice daily for mortality or moribund condition. Body weights were recorded on the day of receipt, before randomization, on treatment days, and on the day of sacrifice for both phases of the study. Bone marrow samples were collected from both exposed femurs of each animal 18-24 hours after the last dose. Slides were prepared, blind coded, and examined for incidence of micronucleated cells. At least 4000 polychromatic erythrocytes (PCEs) were looked at for each animal, and the number of micronucleated polychromatic erythrocytes was given as a percentage of the total micronucleated polychromatic erythrocytes. The proportion of polychromatic erythrocytes of total erythrocytes was determined for each animal by counting a total of at least 500 erythrocytes.

**Chromosomal aberration study**

The chromosomal aberration test is an in vitro genotoxicity test that is conducted using a mammalian cell line of human peripheral blood lymphocytes. The study was conducted in two phases: pre-experiment for cytotoxicity and main study with two exposure regimes: short (for 3-6 hours; with or without metabolic activation system) and long (18-24 hours exposure;


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without metabolic activation system) term exposure to test items. The study was conducted at Ross Lifescience Limited, Pune, India, as per OECD guidelines TG 473(4) 2016: "In Vitro Mammalian Chromosomal Aberration Test" and GLP (1997). Human peripheral blood lymphocyte cells were obtained from healthy donors of ages 27 and 30 years with non-smoking behavior and without any specific medical history of illness. The age of the culture used was 64-65 hours old, with cells derived from human peripheral blood. RPMI (Roswell Park Memorial Institute) 1640 media with L-glutamine supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 units and 100 µg/mL), and phytohemagglutinin-M was used as a culture medium for culture initiation aseptically in the biosafety cabinet. The pH was maintained up to 7.4±0.2. To evaluate the toxicity of the test item, a cytotoxicity assay was performed both in the presence and absence of a metabolic activation system. At least 3 concentrations based on the solubility, precipitation, and pH of the test item were tested. Cytotoxicity was determined by the difference in the mitotic index. For the cytotoxic test item, the highest concentration chosen for the main study was the dose that caused about a 45±5% decrease in the mitotic index.

To assess cytotoxicity, researchers prepared 83 mL of RPMI-1640, 1.3 mL of phytohemagglutinin, 0.4 mL of antibiotic solution (penicillin/streptomycin), 9.3 mL of fetal bovine serum, and 7.1 mL of heparinized blood. The culture media was equally distributed into 3 tissue culture flasks measuring 75 $cm^2$. Then after 68 hours of incubation, the same medium was distributed in 20 sterile Falcon tubes (5 mL/tube) for test item exposure. At the same time, an additional 50 mL of media was prepared for pH control. For the definitive study, a quantity of 124.5 mL of RPMI-1640, 1.95 mL of phytohemagglutinin, 0.6 mL of antibiotic solution (penicillin/streptomycin), 13.95 mL of fetal bovine serum, and 10.56 mL of heparinized blood was prepared. The culture media was equally distributed into 5 x 75 $cm^2$ tissue culture flasks. Then, after 68 hours of incubation, the same medium was distributed in 30 sterile Falcon tubes (5 mL/tube) for test item and reference item exposure. The culture tubes were incubated at 37°C and 5% $CO_2$ for 68 hours in both phases. After incubation of 68 hours, cultures were exposed to test items and vehicle controls for 4 hours in the presence (10% v/v S9) and absence of a metabolic activation system. In the definitive study, cultures were exposed to the test item, vehicle, and positive controls for 21 hours in the absence of a metabolic activation system and an additional 4 hours in the presence (10% v/v S9) or absence of a metabolic activation system. During treatment, contamination and precipitation were observed visually in all the test concentrations, including the control. Approximately 2 to 2.5 hours into the cytotoxicity/definitive study, metaphase arresting agent (Colemid 0.2 µg/mL) was added to all culture tubes and incubated at 37°C and 5% $CO_2$.

The culture was harvested by centrifugation at 1000 rpm for 10 minutes, followed by the addition of 5 mL of prewarmed hypotonic KCl (0.56%) to each culture tube. After hypotonic KCl treatment for 20 minutes, culture tubes were centrifuged at 1000 rpm for 10 minutes. Supernatants were discarded, and 5 mL of freshly prepared chilled Carnoy's fixative were added in each tube and centrifuged at 1000 rpm for 10 minutes. The same procedure was repeated three times in both the cytotoxicity study and the definitive study. During the final wash, 0.5 mL of solution was left at the bottom of the test tube, and the pellet was mixed by air bubbling using a Pasteur pipette. Slides were prepared for each concentration by dropping cell suspension on clean, chilled, wet slides, which were then fixed by exposing slides immediately to vapor for about 2-3 seconds. They were air-dried after being stained with 5% Giemsa solution and mounted using DPX (Distyrene, Plasticizer, Xylene) mounting medium. A minimum number of 1000 consecutive cells were counted in different fields of slides for each concentration to determine the mitotic index. A minimum of 300 consecutive metaphases were scored under a 100x oil immersion objective in a microscope. Both chromatid and chromosome-type aberrations were scored, and the mitotic index was calculated. The statistical analysis was carried out by using the one-way ANOVA (Analysis of Variance) test, and Dunnett's multiple comparison test was applied for comparing the vehicle control, treatment groups, and positive control.

## Results

### Acute toxicity studies

No mortality or abnormal clinical signs were observed for Wistar rats after VE administration at 2000 mg/kg bw up to day 15 during detailed clinical examination. No additional body weight gain was observed in all animals on days 7 and 14 after the administration of VE as compared to day 1. No abnormality was detected in any of the animals during gross pathological observation.

### Sub-chronic study

#### Survival, clinical observations, and body weight

No test item-related mortality or clinical signs were observed in any of the treated groups during the experiment period. During detailed clinical examination, no test item-related clinical signs were observed across all the groups in both sexes. No ocular abnormalities were observed in any of the animals before the start of the treatment or on the last day of treatment. There were no statistically significant differences in the mean body weights of the treated groups as compared to their respective control groups in both sexes during the treatment and recovery periods (Figures 1 & 2). No statistically significant changes in body weight were observed in males. However, females in the mid-dose test item (500 mg/kg bw) showed a statistically significant increase in body weight gain from Day 71 to Day 78 as compared to the control group. In the recovery group males, there was a statistically significant increase in body weight gain from Day 36 to Day 43 as compared to the control group. In the recovery group females, there was a statistically significant decrease in body weight gain from Day 71 to Day 78 and an increase from Day 85 to Day 92 in 1000 mg/kg bw/day (high dose of test item recovery) when compared to the vehicle control recovery group (mg/kg bw/day).


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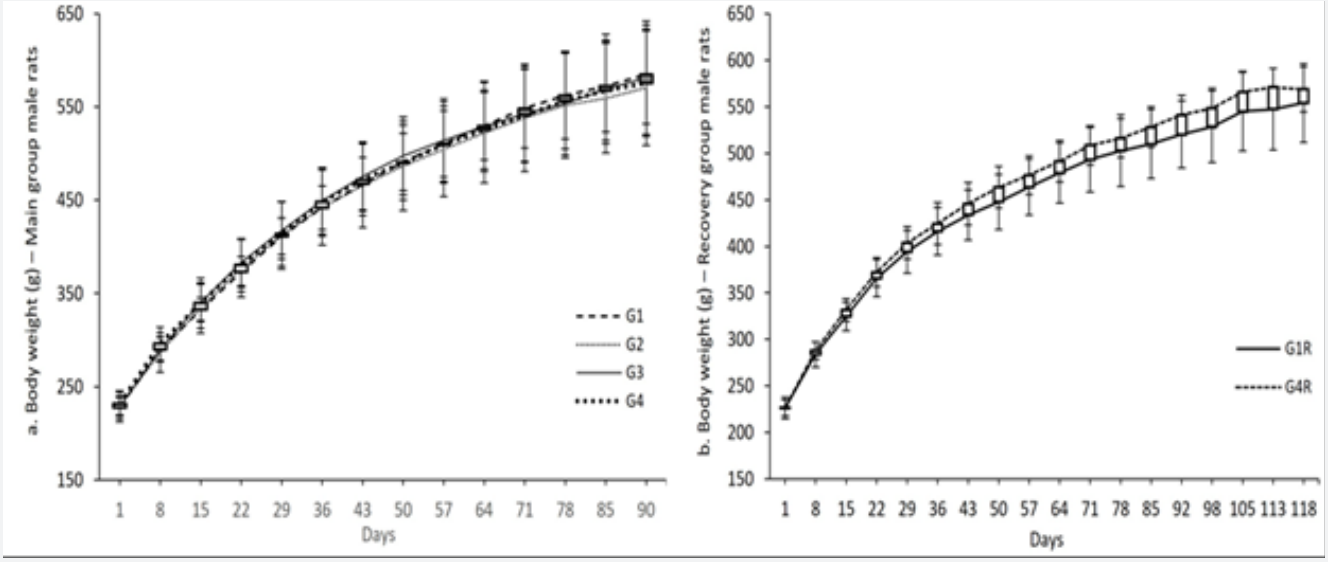


**Figure 1:** Effect of VE on body weights in male rats. Mean body weights for male rats during a 90-day oral (gavage) toxicity study and 28-day recovery study with VE. The values are presented as means ± standard deviation [10 rats/sex/group for test (T) groups and 5 rats/sex/group for recovery (R) group]. (a) G1 - 0 mg/kg bw/day; G2 - 250 mg/kg bw/day; G3 - 500 mg/kg bw/day; G4 - 1000 mg/kg bw/day; (b) G1R - 0 mg/kg bw/day and G4R - 1000 mg/kg bw/day.

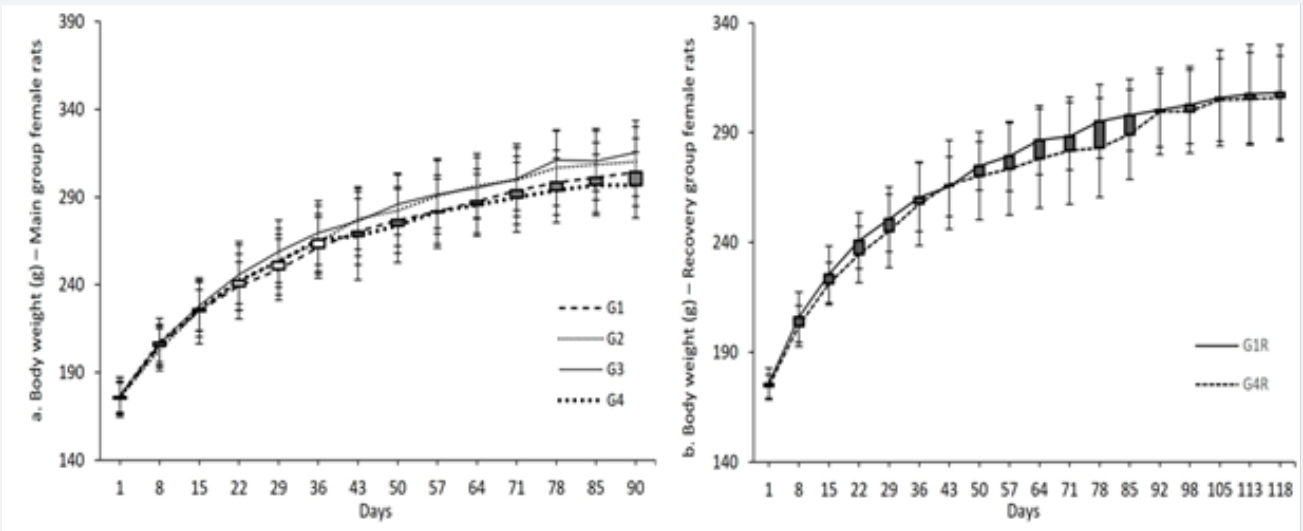


**Figure 2:** Effect of VE on body weights in female rats. Mean body weights for male rats during a 90-day oral (gavage) toxicity study and 28-day recovery study with VE. The values are presented as means ± standard deviation [10 rats/sex/group for test (T) groups and 5 rats/sex/group for recovery (R) group]. (a) G1 - 0 mg/kg bw/day; G2 - 250 mg/kg bw/day; G3 - 500 mg/kg bw/day; G4 - 1000 mg/kg bw/day; (b) G1R - 0 mg/kg bw/day and G4R - 1000 mg/kg bw/day.

The observed changes/variation in the body weight gain, though statistically significant, were minimal as compared to the corresponding control group. These slight differences are considered incidental and not toxicologically relevant, as body weights were within the historical control ranges, with the magnitude of the body weight variation being small. In this case, the changes were not linked to the dose of the test item, suggesting they were not related to the toxicity of VE. The absence of other adverse effects further supports the safety of VE. No significant changes were observed in food consumption, organ weight, or clinical signs of toxicity, which are critical markers when assessing the biological impact of a test item. When body weight changes occur without these accompanying symptoms, they are often regarded as incidental, possibly due to natural variation rather


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than a direct toxicological effect. Thus, despite being statistically significant, the changes in body weight gain in this study do not suggest any toxicological risk and are viewed as incidental variations.

### Feed consumption

No statistically significant changes in food intake were observed at any dose level during the treatment and recovery period.

### Serum Hormone Estimation

There were no statistically significant differences in T4, TSH, or T3 levels in any of the treatment groups compared to the vehicle control groups for either sex.

### Necropsy and Gross Pathology

There were no gross changes observed in the main groups as well as in the recovery group animals.

### Urinalysis

No test item-related changes were observed in urinalysis for either sex. In males, there were no statistically significant changes in any urinalysis parameters compared to the vehicle control group. In females, statistically significant increases in urine pH were observed in 500 mg/kg bw/day and 1000 mg/kg bw/day compared to the vehicle control group (0 mg/kg bw/day). There were no statistically significant changes in the recovery group males. Conversely, in females, a statistically significant decrease in urine pH was observed in 1000 mg/kg bw/day compared to the recovery group receiving the vehicle control (0 mg/kg bw/day). The changes in urine pH observed in the main and recovery groups could not be correlated with other related parameters, such as ketone levels or gross and histopathological observation of kidneys, bladder, pancreas, and gastrointestinal tract. Therefore, pH changes observed in urinalysis were considered not related to the test item and toxicologically irrelevant.

### Hematology

No test item-related alterations were detected in any hematological parameters in either of the sexes (Tables 1 & 2). In the main group of males, no statistically significant changes were observed for any treatment groups as compared to the control group. In the main group of females, there was a statistically significant increase in the percentage of eosinophils observed in the group - 250 mg/kg/bw/day. Additionally, in group 1000 mg/kg bw/day, an increase in HCT and MCV and a decrease in the absolute and percentage of neutrophils and MCHC were observed compared to the vehicle control group (0 mg/kg bw/day). However, these changes were deemed unrelated to the test item because similar shifts were not observed in male animals and in the females of the recovery high-dose group of females. No changes were observed during histopathological examinations of key organs, including the hematopoietic and immune-related organs, such as spleen, bone marrow, and thymus. The observed increase in parameters like HCT and MCV may also be attributed to normal biological variation or physiological responses that are not associated with any pathological condition. Since there were no clinical signs, changes in organ weight, or tissue damage, these small changes in blood measurements are seen as unimportant. Therefore, based on the lack of dose-response, the sex-specific nature of the findings, and the absence of corroborating histopathological changes in relevant organs, the observed hematological changes in the female groups were considered non-adverse and incidental, with no toxicological significance related to the test item.

**Table-1:** Effect of VE on hematological parameters in male rats.

| Group | | | G1 (n=10) | G2 (n=10) | G3 (n=10) | G4 (n=10) | G1R (n=5) | G4R (n=5) |
|---|---|---|---|---|---|---|---|---|
| Dose (mg/kg bw/day) | | | 0 | 250 | 500 | 1000 | 0 | 1000 |
| | | Units | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Parameters | WBC | $x10^3$ cells/µL | 8.58±1.57 | 7.9±1.6 | 9.07±1.98 | 8.01±1.95 | 7.93±3.28 | 7.76±2.59 |
| | RBC | $x10^6$ cells/µL | 8.85±0.39 | 8.82±0.14 | 8.79±0.27 | 8.92±0.31 | 7.49±3.86 | 8.68±0.54 |
| | HGB | g/dL | 15.76±0.55 | 15.67±0.43 | 15.49±0.67 | 15.93±0.37 | 15.52±0.24 | 14.68±0.91 |
| | HCT | % | 45.36±1.45 | 44.88±0.86 | 44.73±1.73 | 45.93±1.08 | 46.5±0.63 | 44.36±2.13 |
| | MCV | fL | 51.34±2.27 | 50.88±0.95 | 50.88±1.33 | 51.54±1.78 | 51.22±1.86 | 51.18±2.84 |
| | MCH | pg. | 17.84±0.82 | 17.78±0.42 | 17.62±0.59 | 17.88±0.66 | 17.08±0.54 | 16.94±1.05 |
| | MCHC | g/dL | 34.74±0.36 | 34.91±0.4 | 34.63±0.48 | 34.71±0.42 | 33.36±0.32 | 33.08±0.53 |
| | RDW | % | 13.82±0.73 | 13.84±0.39 | 13.85±0.51 | 13.78±0.57 | 14.1±0.53 | 13.98±0.95 |
| | PLT | $x10^3$ cells/µL | 997.8±86.79 | 1072±133.2 | 985.6±173.49 | 1032.3±135.67 | 1046.4±69.89 | 1025±68.16 |
| | MPV | fL | 9.56±0.53 | 9.39±0.4 | 9.39±0.28 | 9.29±0.36 | 9.64±0.36 | 9.52±0.23 |
| | RRC | % | 2.33±0.25 | 2.47±0.16 | 2.38±0.29 | 2.42±0.26 | 2.52±0.16 | 2.5±0.07 |
| | PT | Sec | 11.51±1.26 | 12.15±2.88 | 12.12±1.13 | 10.37±1.56 | 11.58±1.54 | 11.38±1.4 |
| | APTT | Sec | 17.35±1.35 | 16.64±1.79 | 17.44±2.47 | 19.19±4.38 | 18.76±1.9 | 16.26*±1.45 |



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| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Relative Differential Leukocyte Count | Neut | % | 27.32±5.71 | 25.86±4.93 | 26.8±5.82 | 28.34±10.99 | 30.32±11.48 | 30.9±9.07 |
| | Lymph | % | 63.39±6.17 | 64.62±5.34 | 64.09±6.75 | 62.29±12.07 | 61.7±13 | 59.64±8.97 |
| | Mono | % | 6.48±1.24 | 6.75±1.29 | 6.67±1.42 | 6.99±1.65 | 5.98±1.55 | 7.64±1.32 |
| | Eos | % | 2.81±0.84 | 2.77±0.64 | 2.44±0.6 | 2.38±0.94 | 2±0.45 | 1.82±0.75 |
| | Baso | % | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |
| Absolute Differential Leucocyte Count | Neut | $x10^3$ cells/µL | 2.3±0.45 | 2.06±0.64 | 2.4±0.59 | 2.16±0.74 | 2.37±1.34 | 2.54±1.55 |
| | Lymph | $x10^3$ cells/µL | 5.5±1.55 | 5.1±1.04 | 5.87±1.73 | 5.13±1.85 | 4.91±2.34 | 4.49±1.16 |
| | Mono | $x10^3$ cells/µL | 0.55±0.1 | 0.53±0.17 | 0.6±0.14 | 0.54±0.14 | 0.49±0.26 | 0.61±0.27 |
| | Eos | $x10^3$ cells/µL | 0.24±0.07 | 0.21±0.04 | 0.21±0.04 | 0.19±0.07 | 0.16±0.08 | 0.12±0.04 |
| | Baso | $x10^3$ cells/µL | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |

G1 - Vehicle control; G2 - Low dose; G3 - Mid dose; G4 - High dose; G1R - Vehicle control recovery and G4R - High dose recovery.
* - indicates statistically significant changes at p<0.05 when compared with Vehicle control.

**Table-2:** Effect of VE on hematological parameters in female rats.

| Group | | | G1 (n=10) | G2 (n=10) | G3 (n=10) | G4 (n=10) | G1R (n=5) | G4R (n=5) |
|---|---|---|---|---|---|---|---|---|
| Dose (mg/kg bw/day) | | | 0 | 250 | 500 | 1000 | 0 | 1000 |
| | | Units | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Parameters | WBC | $x10^3$ cells/µL | 5.5±1.17 | 4.73±0.53 | 5.55±1.77 | 5.32±1.16 | 7.38±3.23 | 7.97±3.12 |
| | RBC | $x10^6$ cells/µL | 7.76±0.37 | 7.83±0.42 | 7.56±0.62 | 7.94±0.47 | 8.33±0.47 | 8.52±0.56 |
| | HGB | g/dL | 14.96±0.55 | 14.75±0.62 | 14.64±1.02 | 15.31±0.74 | 15.16±0.31 | 15.08±1.14 |
| | HCT | % | 44.99±4.22 | 42.53±1.39 | 46.07±4.92 | 51.26*±2.53 | 45.48±1.08 | 45.52±3.01 |
| | MCV | fL | 57.99±4.41 | 54.39±2.07 | 60.94±4.96 | 64.60*±1.67 | 54.72±2.48 | 53.52±3.64 |
| | MCH | pg. | 19.3±0.61 | 18.85±0.79 | 19.37±0.47 | 19.28±0.49 | 18.22±0.9 | 17.78±1.42 |
| | MCHC | g/dL | 33.42±2.58 | 34.67±0.5 | 31.95±2.5 | 29.87*±0.45 | 33.3±0.61 | 33.16±0.62 |
| | RDW | % | 16.21±3.25 | 13.63±0.39 | 16.25±2.81 | 18.87±0.87 | 13.28±0.43 | 13.36±1.05 |
| | PLT | $x10^3$ cells/µL | 984.3±73.14 | 999.9±191.19 | 1075.3±147.45 | 1008.8±115.43 | 943.2±69.43 | 1026±127.76 |
| | MPV | fL | 9.97±0.89 | 9.28±0.47 | 10.17±0.96 | 11±0.38 | 9.62±0.24 | 9.64±0.25 |
| | RRC | % | 2.37±0.21 | 2.49±0.15 | 2.26±0.2 | 2.44±0.15 | 2.38±0.29 | 2.32±0.11 |
| | PT | Sec | 11.27±2.14 | 11.56±1.09 | 11.07±1.93 | 10.89±2.03 | 11.32±0.7 | 14.66*±1.47 |
| | APTT | Sec | 20.31±1.62 | 18.6±2.33 | 21.47±2.62 | 21.02±4.43 | 17.42±0.95 | 17.24±1.71 |
| Relative Differential Leukocyte Count | Neut | % | 17.49±9.84 | 24.43±8.01 | 14.63±6.38 | 8.36*±2.6 | 27.06±7.68 | 22.12±5.55 |
| | Lymph | % | 71.94±7.52 | 66.41±7.04 | 71.87±5.43 | 75.92±7.38 | 67.2±7.75 | 70.32±7.95 |
| | Mono | % | 12.45±13.24 | 6.18±3.87 | 11.18±6.05 | 13.29±4.99 | 3.82±1.27 | 5.42±3.18 |
| | Eos | % | 2.1±0.52 | 2.98*±0.52 | 2.32±0.57 | 2.33±0.63 | 1.92±0.51 | 2.14±0.34 |
| | Baso | % | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |
| Absolute Differential Leucocyte Count | Neut | $x10^3$ cells/µL | 0.96±0.56 | 1.16±0.39 | 0.79±0.37 | 0.43*±0.1 | 2±1.2 | 1.85±1.04 |
| | Lymph | $x10^3$ cells/µL | 3.99±1.1 | 3.15±0.49 | 4.06±1.5 | 4.08±1.05 | 4.94±2.09 | 5.52±2.02 |
| | Mono | $x10^3$ cells/µL | 0.44±0.25 | 0.29±0.18 | 0.58±0.31 | 0.7±0.29 | 0.3±0.2 | 0.43±0.25 |
| | Eos | $x10^3$ cells/µL | 0.11±0.03 | 0.14±0.02 | 0.13±0.06 | 0.12±0.03 | 0.14±0.07 | 0.17±0.09 |
| | Baso | $x10^3$ cells/µL | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |

G1 - Vehicle control; G2 - Low dose; G3 - Mid dose; G4 - High dose; G1R - Vehicle control recovery and G4R - High dose recovery.
* - indicates statistically significant changes at p<0.05 when compared with Vehicle control.


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### Coagulation Parameters

No test item-related alterations were noted in any coagulation parameters for either sex. In the recovery group males, a statistically significant decrease in APTT was observed, and in the recovery group females, a statistically significant decrease in PT was observed compared to their respective control group (0 mg/kg bw/day). However, the changes seen in the recovery groups were very small, with no related changes in the main groups and no associated changes in other important tests, like complete blood count and platelet count, or any significant issues in the organs like the bone marrow, spleen, and liver. In addition, the values were within the historical control range of the test facility. Hence, these changes were considered to be of no toxicological significance.

### Clinical biochemistry

No test item-related changes were observed in clinical chemistry parameters for either sex (Tables 3 & 4). In the main group of males, a statistically significant decrease in ALT in 250 mg/kg bw/day and 500 mg/kg bw/day, an increase in HDL and A/G ratio in 500 mg/kg bw/day, an increase in LDL in 250 mg/kg bw/day, an increase in albumin in 1000 mg/kg bw/day, and an increase in calcium in 500 mg/kg bw/day and 1000 mg/kg bw/day were observed when compared to the control. In the main group of females, statistically significant increases in TCHO and HDL were observed at 500 mg/kg bw/day, and sodium and chloride at 1000 mg/kg bw/day. Additionally, there was a statistically significant decrease in creatinine in 1000 mg/kg bw/day compared to the vehicle control group (1 mg/kg bw/day). The variations observed in the main group of males were of lower magnitude and also fell within the historical control range of the test facility; in some cases, these variations were limited to the mid-dose group, indicating a lack of dose dependency. Moreover, no correlating changes were observed in other related parameters, gross pathology, or histopathology. In the recovery group males, statistically significant decreases in urea, LDL, and BUN levels were observed in 1000 mg/kg bw/day compared to 0 mg/kg bw/day. These variations were observed in a single sex and were within the historical control range of the test facility and lacked corresponding changes in supporting parameters (creatinine, calcium, chloride, and inorganic phosphorus), gross pathology (kidneys, heart, and liver), and histopathology (kidneys, heart, and liver). Therefore, these changes are considered toxicologically irrelevant. In the recovery group females, a statistically significant increase in total protein was observed in 1000 mg/kg bw/day compared to 0 mg/kg bw/day. These changes only happened in females; similar changes were not seen in the main dose groups and did not relate to changes in creatinine, sodium, chloride, or blood urea nitrogen levels, and the values were within the normal range for the test facility. Consequently, these changes were deemed incidental and toxicologically insignificant. Overall, all observed variations in clinical chemistry parameters were not considered adverse or toxicologically relevant.

**Table-3:** Effect of VE on blood biochemistry parameters in male rats.

| Group | | | G1 (n=10) | G2 (n=10) | G3 (n=10) | G4 (n=10) | G1R (n=5) | G4R (n=5) |
|---|---|---|---|---|---|---|---|---|
| Dose (mg/kg bw/day) | | | 0 | 250 | 500 | 1000 | 0 | 1000 |
| | | Units | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Parameters | ALB | g/L | 36.49±1.08 | 37.70±0.93 | 37.62±1.33 | 37.69*±2.24 | 34.32±1.53 | 35.08±1.05 |
| | ALPI | U/L | 78.99±20.1 | 73.82±18.96 | 85.02±29.42 | 89.03±19.75 | 85.12±10.76 | 67.08±19.48 |
| | ALTI | U/L | 69.67±19.52 | 52.40*±13.26 | 51.96*±4.11 | 53.23±8.25 | 105.59±39.22 | 66.14±22.22 |
| | AST | U/L | 154.97±37.33 | 137.14±26.89 | 133.35±30.56 | 134.41±35.67 | 242.14±19.21 | 201.03±40.74 |
| | BUN | mg/dL | 15.62±1.36 | 15.97±1.36 | 15.89±3.44 | 16.18±2.94 | 19.95±3.03 | 14.55*±2.81 |
| | CA | mg/dL | 9.92±0.23 | 9.83±0.28 | 10.45*±0.27 | 10.38*±0.44 | 10.63±0.37 | 10.45±0.06 |
| | TBI | mg/dL | 0.13±0.01 | 0.13±0.02 | 0.11±0.03 | 0.11±0.04 | 0.23±0.02 | 0.22±0.02 |
| | TGL | mg/dL | 126.79±65.89 | 139±36.29 | 110.64±35.81 | 130.1±58.76 | 43.41±13.73 | 42.3±29.61 |
| | CHOL | mg/dL | 65.82±16.91 | 65.44±10.25 | 75.48±12.42 | 63.07±16.43 | 64.69±8.82 | 63.26±4.76 |
| | ALDL | mg/dL | 2.44±0.9 | 5.53*±3.84 | 3.33±2.31 | 2.32±0.54 | 9.18±1.83 | 6.81*±0.66 |
| | AHDL | mg/dL | 17.48±3.81 | 17.2±1.94 | 21.51*±3.07 | 15.61±3.17 | 19.57±2.03 | 18.43±2.15 |
| | GLUC | mg/dL | 109.48±16.35 | 108.81±8.13 | 116.65±22.24 | 129.26±21.07 | 106.1±7.43 | 120.35±36.49 |
| | TP | g/L | 61.66±1.78 | 63.63±1.78 | 62.32±2.08 | 62.64±4.31 | 58.19±3.74 | 58.63±3.41 |
| | GLOB | g/L | 25.18±1.13 | 25.94±1.36 | 24.7±0.95 | 24.95±2.41 | 23.87±2.39 | 23.55±2.53 |
| | A/G Ratio | - | 1.45±0.07 | 1.46±0.08 | 1.52*±0.05 | 1.52±0.12 | 1.44±0.1 | 1.5±0.12 |
| | CRE2 | mg/dL | 0.63±0.04 | 0.67±0.18 | 0.63±0.04 | 0.64±0.03 | 0.57±0.02 | 0.59±0.03 |
| | PHOS | mg/dL | 6.24±1.23 | 5.69±0.42 | 6.37±0.41 | 5.85±0.37 | 5.86±0.25 | 5.46±0.32 |



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| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Na+ | mmol/L | 141.3±1.42 | 141.2±1.4 | 141.4±0.97 | 141.3±1.42 | 142.4±1.52 | 141.6±1.67 |
| | K+ | mmol/L | 4.41±0.18 | 4.5±0.24 | 4.47±0.21 | 4.47±0.18 | 4.5±0.5 | 3.98±0.54 |
| | $Cl^-$ | mmol/L | 101.9±1.91 | 102.4±1.35 | 102.2±1.48 | 102.8±1.69 | 101.6±2.51 | 104±1.87 |
| | Urea | mg/dL | 33.43±2.9 | 34.18±2.92 | 34.01±7.36 | 34.63±6.28 | 42.7±6.48 | 31.13*±6.01 |

G1 - Vehicle control; G2 - Low dose; G3 - Mid dose; G4 - High dose; G1R - Vehicle control recovery and G4R - High dose recovery.
* - indicates statistically significant changes at p<0.05 when compared with Vehicle control.

**Table-4:** Effect of VE on blood biochemistry parameters in female rats.

| Group | | | G1 (n=10) | G2 (n=10) | G3 (n=10) | G4 (n=10) | G1R (n=5) | G4R (n=5) |
|---|---|---|---|---|---|---|---|---|
| Dose (mg/kg bw/day) | | | 0 | 250 | 500 | 1000 | 0 | 1000 |
| | | Units | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Parameters | ALB | g/L | 38.87±1.70 | 38.20±3.21 | 38.82±1.08 | 38.76±1.82 | 35.51±1.18 | 36.08±0.81 |
| | ALPI | U/L | 0.35±0.08 | 0.33±0.04 | 0.34±0.07 | 0.37±0.08 | 0.23±0.02 | 0.22±0.04 |
| | ALTI | U/L | 38.91±6.45 | 39.23±12.47 | 37.94±7.23 | 39.79±8.16 | 44.31±10.8 | 52.38±19.52 |
| | AST | U/L | 137.55±29.59 | 131.97±60.66 | 112.33±22.16 | 128.82±20.45 | 134.03±45.01 | 111.23±46.32 |
| | BUN | mg/dL | 19.76±2.08 | 17.85±2.64 | 17.28±3.15 | 17.53±1.94 | 17.01±0.87 | 19.72±4.19 |
| | CA | mg/dL | 4.85±0.53 | 4.67±0.62 | 5.39±1.07 | 5.43±1.48 | 4.35±1.14 | 4.94±1.46 |
| | TBI | mg/dL | 10.22±0.3 | 9.97±0.44 | 10.27±0.35 | 10.29±0.26 | 10.49±0.28 | 10.41±0.2 |
| | TGL | mg/dL | 73.51±16.07 | 67.63±18.17 | 74.36±22.51 | 67.7±20.27 | 48.2±8.18 | 55±15.89 |
| | CHOL | mg/dL | 64.31±10.43 | 73.56±20.68 | 82.56*±11.47 | 66.4±11.07 | 52.17±7.43 | 68.37±15.57 |
| | ALDL | mg/dL | 2.27±1.17 | 3.62±2.75 | 1.9±1.39 | 1.98±1.19 | 5.83±2.76 | 4.95±1.72 |
| | AHDL | mg/dL | 17.54±2.45 | 19.29±4.6 | 21.17*±2.51 | 19.21±2.7 | 19.2±2.6 | 21.74±3.18 |
| | GLUC | mg/dL | 102.46±16.01 | 97.41±10.16 | 106.73±10.42 | 99.52±8.45 | 114.7±20.41 | 90.65±11.93 |
| | TP | g/L | 64.3±3.1 | 64.18±4.35 | 65.46±1.91 | 63.86±2.66 | 58.38±0.91 | 60.81*±1.58 |
| | GLOB | g/L | 39.44±8.55 | 41.97±11.42 | 42.72±6.54 | 45.91±17.85 | 49.36±18.14 | 38.48±4.46 |
| | A/G Ratio | - | 1.02±0.19 | 0.97±0.28 | 0.93±0.17 | 0.92±0.24 | 0.79±0.25 | 0.95±0.12 |
| | CRE2 | mg/dL | 0.64±0.03 | 0.62±0.02 | 0.62±0.03 | 0.59*±0.06 | 0.64±0.05 | 0.63±0.04 |
| | PHOS | mg/dL | 25.42±2.7 | 25.98±1.35 | 26.63±1.79 | 25.1±1.34 | 22.87±0.47 | 24.74±1.6 |
| | Na+ | mmol/L | 139.6±1.78 | 139.6±2.37 | 140.5±0.85 | 142.00*±1.25 | 140.8±0.84 | 141.4±2.07 |
| | K+ | mmol/L | 4.3±0.31 | 4.22±0.33 | 4.07±0.17 | 4.12±0.19 | 4.5±0.55 | 4.5±0.46 |
| | $Cl^-$ | mmol/L | 100.9±1.45 | 100.5±1.27 | 101.8±1.03 | 104.10*±0.99 | 101±1.22 | 102.4±1.67 |
| | Urea | mg/dL | 42.28±4.46 | 38.21±5.64 | 36.99±6.73 | 37.52±4.16 | 36.4±1.86 | 42.21±8.97 |

G1 - Vehicle control; G2 - Low dose; G3 - Mid dose; G4 - High dose; G1R - Vehicle control recovery and G4R - High dose recovery.
* - indicates statistically significant changes at p<0.05 when compared with Vehicle control.

### Organ weight

In male animals, a statistically significant increase in kidney weight at 500 mg/kg bw/day and a decrease in absolute adrenal weight at 500 mg/kg bw/day and 1000 mg/kg bw/day and a decrease in absolute thymus weight at 250 mg/kg bw/day and 1000 mg/kg bw/day were observed as compared to the vehicle control group. We also observed a statistically significant increase in absolute thyroid weight at 500 mg/kg bw/day and 1000 mg/kg bw/day and a decrease in lung weight at 1000 mg/kg bw/day in female animals as compared to the vehicle control group. Similarly, male animals showed a statistically significant increase in relative kidney weight in 500 mg/kg bw/day and 1000 mg/kg bw/day and a decrease in relative thymus weight in 1000 mg/kg bw/day when compared to the vehicle control group. A statistically significant increase in relative thyroid gland weight was noted at 1000 mg/kg bw/day in female animals as compared to the vehicle control group. However, the observed organ weight changes in the main group could not be correlated with any gross or histopathological findings of these organs. Additionally, the changes observed in the recovery group were present in only


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one sex, and similar changes were not observed in the main group. The increase in relative thyroid gland weight in females was considered incidental in the absence of any changes in the gross pathology, histopathology, and thyroid hormone levels. Therefore, these changes were considered incidental and not adverse or toxicologically relevant. In the recovery group males, a statistically significant decrease in absolute thymus weight was observed. Conversely, there were no statistically significant changes in absolute organ weight in females. Additionally, there were no statistically significant changes in relative organ weight in either males or females as compared to the vehicle control group. These isolated changes observed in the recovery group were considered incidental and not related to the test item, as these were individual animal variations in organ weight and the changes were minimal.

### Histopathology

Histopathological examination of the animals treated with the test item (1000 mg/kg bw/day) did not show any lesion(s) of pathological significance when compared to vehicle control groups (0 mg/kg bw/day). Histopathological findings were observed as follows: hepatocellular vacuolation (minimal and multifocal) in the liver in one male rat each in the 0 mg/kg bw/day and 1000 mg/kg bw/day groups and in one female rat of the 0 mg/kg bw/day group; focal and minimal osseous metaplasia in the lungs of one 0 mg/kg bw/day male rat; chronic inflammatory foci (minimal in severity) in the lungs in one male and one female rat of the 1000 mg/kg bw/day group; focal and minimal mononuclear cell infiltration in the myocardium of the heart in one 1000 mg/kg bw/day female; and perivascular mononuclear cell infiltration (minimal and focal) in the epididymides of one 0 mg/kg bw/day male animal. The lesions observed in the liver and osseous metaplasia in the lungs were considered incidental findings, as these lesions were observed in the control group also. The other lesions observed in the lungs, heart, and epididymides are common background findings in rats. Hence all these histopathology lesions were considered incidental and not toxicologically relevant. No treatment-related findings were observed during the qualitative assessment of spermatogenesis stages or during the morphological examination of interstitial testicular cell structures in high-dose (1000 mg/kg bw/day) male animals when compared to the vehicle control group (0 mg/kg bw/day). The stage of the estrous cycle in each female rat in 0 mg/kg bw/day and 1000 mg/kg bw/day at the terminal sacrifice was in correlation with the histology of the respective female reproductive organs.

### Mutagenicity study

VE did not induce any gene mutation by base pair changes or frame shift in the genome of the S. typhimurium strains used for testing. Hence, the test product is considered non-mutagenic up to a concentration of 2.5 mg/plate for the S. typhimurium reverse mutation assay.

### Micronucleus study

Based on the results, it was concluded that oral administration of VE once a day each for two consecutive days did not induce the formation of micronucleated polychromatic erythrocytes in the bone marrow of Swiss albino mice at the dose of 2000 mg/kg bw.

### Chromosomal aberration study

There was no statistically significant increase in the percentage of aberrant cells observed both in the absence and in the presence (10% v/v S9 mix) of metabolic activation systems in any of the experiments carried out with VE when compared to concurrent vehicle control. Positive controls showed a statistically and biologically significant increase in the percentage of aberrant cells both in the absence and in the presence (10%, v/v S9 mix) of the metabolic system. In conclusion, the test product did not induce chromosomal aberrations in the human peripheral blood lymphocytes assay up to the concentration of 0.25 mg/mL.

## Discussion

V. officinalis is a medicinal plant used for the management of sleep disorders [4]. The root extract of V. officinalis exhibits anxiolytic, antioxidant, anti-inflammatory, sedative, anxiolytic, spasmolytic, anticonvulsant, cytoprotective, and neuroprotective activities [5]. Valerian extracts reduced sleep latency and improved sleep in patients with sleep disorders [1,3,4]. Valerian as an herb is well tolerated and found to be safe across multiple animal and human clinical studies [13,14]. Valerenic acid is used as the chemical marker for qualitative and quantitative evaluation of valerian extract and to measure sleep-inducing properties [2,4,8]. Valerenic acid and valepotriates are believed to mediate sedative and hypnotic effects through modulation of the A1 adenosine receptor [15], benzodiazepine receptor [11], enhanced GABA release and inhibition of GABA reuptake [16]. In mice sleep models, VE that contain 2% valerenic acid effectively reduced sleep latency and extended sleep duration by modulating neurotransmitter levels in the brain and increased expression of GABA receptors as well as blocking the adenosine receptors at doses of 100 mg/kg and 300 mg/kg bw [12]. Further VE significantly improved overall sleep quality, latency, efficiency, and total sleep time and reduced daytime sleepiness in young and healthy subjects with mild insomnia symptoms. Also, valerian extract significantly ($p<0.05$) increased the sleep duration with shorter sleep latency, which was comparable to melatonin through modulation of GABA and 5-HT5a receptor expression in brain tissues in a pentobarbital-induced sleep model in mice [12]. The VE is a low-dose valerian root extract that contains 2% valerenic acid and is formulated for longer stability and reduced odor associated with valerian extracts.

The formulation was tested for its safety through acute and sub-chronic studies, bacterial revertant assays, in vivo mammalian erythrocyte micronucleus tests, and in vitro mammalian chromosomal aberration tests. The acute oral


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toxicity study results following a single oral administration in female Wistar rats suggest VE is non-toxic. Based on the results, VE is classified as Category 5/Unclassified as per GHS (Globally Harmonized System), with an LD50 range greater than 2000 mg/kg body weight and an LD50 (cut-off value) of 5000 mg/kg body weight. Additionally, results from a 90-day study showed that the highest dose of VE that did not cause any harmful effects in Wistar rats was 1000 mg per kg of body weight per day. The bacterial reverse mutation test results concluded that VE did not induce any gene mutations, including base pair changes or frame shifts, in the genome of S. typhimurium tester strains and hence was considered to be non-mutagenic up to a concentration of 2.5 mg/plate in the S. typhimurium reverse mutation assay. The in vivo mammalian erythrocyte micronucleus test in Swiss albino mice revealed VE as non-genotoxic with no significant micronuclei formation in mice bone marrow cells up to a 2000 mg/kg bw dose level. Similarly, in vitro chromosomal aberration tests showed no significant structural aberrations in human peripheral blood lymphocytes up to a concentration of 0.25 mg/mL, and thus VE can be considered non-clastogenic. Bao et al. 2024 [17] evaluated toxicology of aqueous extract of valerian root through acute oral toxicity, sub-chronic toxicity, bacterial reverse mutation, chromosomal aberration, and teratogenicity test.

The study results established that the aqueous extract of valerian root has a non-genotoxicity profile, with an LD50 greater than 96 g/kg body weight in mouse models, according to an acute oral toxicity study. Sub-chronic toxicity and teratogenicity tests showed that the NOAEL of aqueous extract of valerian root was no less than 14 g/kg body weight. Furthermore, a systemic review and meta-analysis suggests that valerian root extracts improve sleep quality without any side effects [6]. Our cell culture studies also revealed no significant mutagenic or structural chromosomal aberrations or micronuclei formation with VE. Finally, our results for VE from acute and sub-chronic studies indicated no signs of any adverse effects, which is in alignment with previously published studies for V. pavonii in Wistar rats with a NOAEL of 2000 mg/kg in the case of acute toxicity and 1000 mg/kg weight through sub-chronic toxicity studies [18]. Similarly, hydroethanolic extract from another valerian species, V. wallichii, was found to be safe in Swiss albino mice studies at an oral dose of up to 2000 mg/kg, with no signs of abnormality, morbidity, or mortality [19]. This study reports that VE is non-toxic when administered orally with an LD50 cut-off value of 5000 mg/kg b.w. and a NOAEL of 1000 mg/kg b.w. per day. The test product did not cause any toxicity, as evidenced by the results of biochemical testing, body weight changes, and histopathology. In addition, the data showed no mutagenicity or genotoxicity. Hence, valerian extract used in the current study is considered safe and suitable for oral consumption.

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## Author Contributions

Satendra Pratap Singh, Aleem Sarangi Muneer, Samit Kadam and Rishi Kumar Mishra contributed to the study conception and design. Abhijeet Morde and Paras Patni provided the study material, reviewed and approved the study plans. Material preparation, data collection and analysis were performed by Satendra Pratap Singh, Aleem Sarangi Muneer, Samit Kadam and Rishi Kumar Mishra. The first draft of the manuscript was written by Satendra Pratap Singh, Aleem Sarangi Muneer, Samit Kadam, Rishi Kumar Mishra, and all authors commented on previous versions of the manuscript. All authors read and approved of the final manuscript.

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## Data Availability Statement

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

## Declarations

### Conflict of Interest

Satendra Pratap Singh and Rishi Kumar Mishra are employees of Ross Lifesciences Limited, Pune, India, where the study was performed. Aleem Sarangi Muneer and Samit Kadam are employees of Vipragen Biosciences Private Limited, Mysuru, India. Abhijeet Morde and Paras Patni are employed by Omni Active Health Technologies Ltd.

### Ethics Statement

The studies were conducted as per applicable OECD guidelines.

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