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Mutagenicity and safety evaluation of Ashwagandha (*Withania somnifera*) root aqueous extract in different models

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ABSTRACT

Withania somnifera (Ashwagandha) also called as Indian ginseng, a revered herb from Indian traditional system of medicine is a rejuvenator and tonic (*Rasayana*) used for its varied benefits. The roots of ashwagandha exhibit properties like anti-inflammatory, aphrodisiac, anthelmintic, astringent, diuretic, stimulant and thermogenic. However, data of ashwagandha on its mutagenic effects are lacking. In the present study, in-vitro genotoxicity tests were used to evaluate the mutagenic potential of Ashwagandha Root Extract (ARE). Concentrations of 0.156 to 5.00 mg/plate ARE were used for conducting Bacterial reverse mutation test (BRMT). For chromosome aberration (CA) test ARE was used in concentrations of 0.25 to 2.00 mg/ml, and for micronucleus (MN) tests ARE concentrations of 500/1000/2000 mg/kg were used. Acute oral toxicity was conducted in Wistar rats (n = 25) as per the OECD guideline (#423) with doses of 500/1000/2000 mg/kg body weight in male Swiss albino mice for morbidity and mortality for 3 days. The BRMT and CA tests were conducted with and without metabolic activation (S9). The study was approved by the institutional ethics committee (IEC) and institutional animal ethics committee (IAEC). ARE failed to show any mutagenic effects up to a dose of 5 mg/plate in BRMT. Also, ARE did not show any clastogenic activity in doses up to 2 mg/ml in CA test and in micronucleus test up to 2000 mg/kg body weight. These results were observed with and without metabolic activation (S9) under the stated experimental conditions. No mortality, morbidity, or any clinical signs were observed up to 3 days following ARE administration. Ashwagandha root extract failed to show any mortality in doses up to 2000 mg/kg oral dosage and did not show any mutagenic (genotoxic) effects in high concentrations.

1. Introduction

Ashwagandha (*Withania somnifera*) commonly referred to as "Indian Ginseng" or "Winter Cherry," is a member of the Solanaceae family and holds significant medicinal value in both Ayurvedic and Unani medical systems [1]. Ashwagandha roots are used as adaptogenic, tonic, anti-inflammatory, aphrodisiac, anthelmintic, astringent, thermogenic and stimulant [2]. Other parts of the Ashwagandha plant have antioxidant and anti-bacterial actions [3]. Ashwagandha root extract contains over 200 primary and secondary metabolites, such as flavanol glycosides, alkaloids, glyco-withanolides, steroidal lactones (withanolides), and sterols [4]. One of the withanolides known as Withanone (win) is found in significant amounts in Ashwagandha extracts. Withanone (win) content is reported to be minimal in roots (3 mg/grams of extract) while high (19 mg/grams of extract) in leaves [4]. These are known to cause adverse drug reactions reported with the use of ashwagandha extract [5]. Although root extracts are mainly utilized in therapy, some of the over-the-counter formulations additionally employ leaf-based preparations. [6]. Indian government issued an advisory regarding the toxicity with *Withania somnifera* leaves extract [6]. The advisory emphasizes on an imminent need for generating substantial evidence and scientific literature pertaining to the efficacy and safety of various herbal formulations. Thus, there is a need for standardization of all plant-based products and stringent quality control.

Although many safety studies on animals and humans are reported with ashwagandha root extract (ARE), sufficient scientific data is not available on the safety of ARE with respect to the mutagenicity, genotoxicity, and single dose acute toxicity. This study evaluated the mutagenic potential of ARE in various OECD recommended in-vitro assay systems such as bacterial reverse mutation test (Ames II test), chromosomal aberration (CA) test, and micronucleus (MN) test). Although subacute and 90 days toxicity data are available for Ashwagandha root

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extract, literature of ashwagandha root extract (standardized) is not published. Hence, acute single dose oral toxicity of Ashwagandha root extract was performed with 500 to 2000 mg/kg. These tests were conducted as per the respective OECD guidelines [7–10].

2. Material and methodology

2.1. Ashwagandha (Withania somnifera): collection and Identification

Ashwagandha plant was collected from the cultivation farms with controlled conditions in the state of Rajasthan, India, and authenticated at NISCAIR (National Institute of Science Communication and Information Resources). A voucher specimen (identification number 653) was duly deposited in the herbarium.

2.2. Properties of Ashwagandha root extract (ARE) as test item

Dried roots were used for the preparation of extract as per the standardized method at Shri Kartikeya Pharma, Hyderabad, India. KSM-66 Ashwagandha is a root extract of ashwagandha manufactured using an aqueous-based extraction process. It is slightly hygroscopic and yellowish-brown in color. It is standardized to > 5 % of total withanolide content, consisting mainly of Withastramonolide A, Withanoside IV, Withanolide A, and Withanone. It also consists of less than 0.1 % of withaferin A. The standardized ARE powder contains 5.3 % withanolides and is confirmed by HPLC (supplementary material). The extract was assessed for microbials, heavy metals, pesticides and aflatoxin levels and their absence and the tests were conducted as per guidelines of United States Pharmacopeia [11]. The quality of ARE was compliant to the limits set by United States Pharmacopeia (USP) and British Pharmacopeia [11,12]. ARE stability was assessed as per protocols of Q1A(R2) ICH guidelines [13]. When stored under accelerated and real time conditions, ARE was found stable for 3 years and it is recommended to store ARE at room temperature 22 ± 3 °C in an airtight container.

2.3. ARE preparation as test Item

ARE being insoluble in water, a suspension of ARE was prepared using 0.1 % carboxy-methyl cellulose (CMC) as a suspending agent.

2.4. Source of chemicals used in the studies

Chemicals were procured from HIMEDIA, SRL, SIGMA ALDRICH, and MP Biomedicals. Test strains of *Salmonella typhimurium* as lyophilized Disc containing TA strains from Moltox, USA and Supplied by KRISHGEN Biosystems, Mumbai-400 018.

2.5. Bacterial strains

Salmonella typhimurium TA strains (TA 98, TA 100, TA 102, TA 1535, and TA 1537) were used for reverse mutation test for bacteria (Ame's assay) after confirming genotyping. The concentration of standard mutagen(s), S9 and incubation time were confirmed by keeping the previous publications as Refs. [14–18]. Cultures of Salmonella TA strains were grown overnight at 37 °C by using freshly thawed frozen strains spread on a growth medium. Environmental shaker was used and set at 120 RPM in the presence of 8 mg/ml of Ampicillin and 8 mg/ml of Tetracycline.

2.6. Human lymphocytes

Human peripheral blood lymphocytes were used for conducting chromosome Aberration Assay of ARE. Human blood was collected aseptically from three healthy male volunteers between 18–35 of age who reported no history of smoking, no recent illness record, or recent exposures to genotoxic agents (e.g., chemicals, ionizing radiations). Institutional Ethics Committee (IEC No. IEC-NI/21/APR/78/73) approval was received and informed written consent was obtained before collecting blood from healthy human volunteers. Lymphocytes were isolated from peripheral blood (0.5 ml) and were allowed to incubate in 9.5 ml growth medium (RPMI 1640 + presence of mitogen PHA-M (Phytohemagglutinin-M) for 48 h at 37 °C in a humidified atmosphere of 5 % CO_2 in air.

2.7. Mammalian Erythrocyte Cells from Swiss albino Mice

Mammalian Erythrocyte Cells were obtained from Swiss albino Mice to assess the clastogenic potential of ARE. Twenty-five healthy young 9-weeks aged male Swiss albino mice were used for the erythrocyte's extraction. Mice were housed in polypropylene cages and the temperature was maintained between 19.05 - 21.67 °C with 12 h each of dark and light cycle. The standard laboratory pellet was used for feeding the mice. The sampling of bone marrow was done within 23 – 24 h of last dosing time. Animals were euthanized by CO_2 asphyxiation, and bone marrow was collected from both the femurs by flushing 2 ml fetal bovine serum (FBS) into a centrifuge tube.

2.8. Bacterial reverse mutation test (Ame's assay)

Reverse mutation test for bacteria (Ame's assay) was used for identification of test substance to induce reverse mutation at histidine loci in Salmonella strains TA98, TA100, TA102, TA1535, TA1537. Standard procedure was followed to perform mutagenicity test [7,14–18]. *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535, TA1537 were exposed to ARE with and without S9 (metabolic activation). Dimethyl Sulfoxide (DMSO) was used to solubilize ARE and designated as vehicle control. The highest concentration of 5 mg/plate and subsequent concentrations of 5, 2.5, 1.25, 0.625, 0.3125 mg/plate for Trial-I and 5, 2, 0.8, 0.32, 0.128 mg/plate for Trial-II were selected to assess the mutagenic effect.

ARE was used in concentration range of 0.156 mg to 5.000 mg/plate. Salmonella tester strain TA 100 was used with S9 (metabolic activation) and without metabolic activation. For metabolic activation, S9 mix was prepared before start of experiment by adding 10 % v/v rat liver S9 fraction to cofactor mix. S9 mix is prepared by homogenization of liver in isotonic potassium chloride (0.15 M KCl) at a rate of 1 g wet tissue per 3 ml and then separated by centrifugation at 9000*g*. The mix was overlaid on basal agar plates and incubated for 48 h at 37 °C. All doses of the ARE, vehicle control (DMSO), and positive controls were plated in triplicates. Mixture of Mitomycin C (0.5 μg/plate) + Sodiumazide (1 μg/plate) + 2-Nitrofluorene (10 μg /plate) + 9-Aminoacridine (25 μg /plate) + Benzopyrane (10 μg /plate) were used as positive control in the absence and presence of S9 respectively. Following the incubation period, the number of revertant colonies were counted, and a positive result was determined based on a significant rise in the average number of revertant per plate in at least one of the tester strains compared to vehicle control. Revertant bacteria are those which are permanently converted to the prototrophic state, continue to grow and will become visible to the naked eye.

2.9. Chromosome aberration assay using human peripheral blood lymphocytes

Chromosome aberration test was performed as per OECD guideline 473 [7]. Four ARE concentrations (0.25, 0.5, 1 and 2 mg/ml) were selected for concentration range-finding study in presence and absence of the metabolic activation system to determine the cytotoxicity of ARE. The concentrations of 0.5, 1, and 2 mg/ml of ARE were selected for the different studies. Short term treatment (4 h) included both with and without metabolic activation (S9), whereas long term treatment (24 h) was without metabolic activation. Duplicate lymphocyte cultures were

used for both concentration range finding and main study. DMSO was used as vehicle control, whereas for positive control Mitomycin-C (0.5 μg/ml) as used for without metabolic activation, and cyclophosphamide monohydrate (3 μg/ml) was used for with metabolic activation.

No evidence of cytotoxicity to human lymphocytes was observed, hence the highest ARE concentration of 2 mg/ml was used for further study. After short-term exposure, the treatment medium was removed by centrifugation and cultures replenished medium was changed. The cells were reintroduced into the incubator to fulfil 1.5 cell cycle length. For long-term exposure, the cells were treated with test compound for the entire 1.5 cell cycle length. Cells were exposed to colchicine (0.1 μg/ml) for 3 h prior to harvest. After 3 h, cells were centrifuged, resuspended in hypotonic medium (75 mM KCl) and fixed (prechilled 3 parts of ethanol and 1 part of glacial acetic acid). Approximately, 3–4 drops of the fixed cell suspension were dropped from the height of 20–3 °Cm onto a clean microscope slide and stained with 10 % Giemsa solution for 10 min. Two hundred metaphase chromosome spreads per treatment were examined under the microscope for chromosome break, chromatid break, deletion, ring, dicentric and rearrangements as indicated by Savage et al. [19].

Only metaphases containing 46 ± 2 centromeres (chromosomes) were considered for analysis. Chromosomal aberrations in the form of total number of cells with aberrations and their number were recorded. Each treatment experiment was performed in duplicates. Mitotic index (MI) was used to for determining cytotoxicity and the following formula was used for its calculation.

- *MI = (No. of cells in metaphase/ Total number of cells) × 100*

The relative mitotic index (RMI) values were calculated as percentage with reference to the vehicle control (DMSO).

2.10. In-Vivo Micronucleus Test in Mammalian Erythrocyte Cells

The study assessed the clastogenic potential of ARE in Swiss Albino Mice. OECD guideline 474 was used for conducting micronucleus test [20]. Mammalian erythrocyte cells were obtained from Swiss albino mice. All the animals were dosed by oral gavage for two days separated by 24 h interval. The dose volume was 10 ml/kg body weight. Animals were observed for clinical signs, mortality, morbidity and body weight. The obtained bone marrow suspension was centrifuged, and smear was prepared on two glass microscope slides per animal. The slides were stained with 10 % Giemsa for 10 min after drying overnight in air. Experiments were performed in duplicates. Stained slides were air-dried and mounted using DPX mountant and examined under light microscopy with 100X oil objective. Slides were evaluated and 500 erythrocytes per animal were counted to determine the ratio of polychromatic erythrocytes (PCE) among the total erythrocytes. 4000 PCEs per animal were scored to evaluate the incidence of micronuclei in polychromatic erythrocytes (MNPCEs) in percentage.

The erythrocytes were incubated with ARE (500/kg, 1000/kg and 2000 mg/kg of bodyweight). The main study was conducted in 5 groups (G1- negative control vehicle- 0.1 % CMC - 0 mg/kg, G2, G3, and G4 - ARE with 500/kg, 1000/kg and 2000 mg/kg of body weight respectively) and G5 - Positive Control (Cyclophosphamide monohydrate 40 mg/kg body weight). CMC (0.1 %) solution was used to solubilize ARE.

Formula used for calculation:

- *Micronuclei Frequency (Total Number of MNPCEs) = (Total number of (MNPCEs)/ Total number of PCEs) X 100*
- *P/E ratio = No. of PCE / Total No. Erythrocytes*

2.11. Acute oral toxicity (LD_{50})

OECD test guideline 423 (Acute toxic class method adopted on 17th December 2001 and OECD series on Principles of Good Laboratory Practice and Compliance Monitoring, Number 1, ENY/MC/CHEM(98) 17 was used for estimation of acute toxicity of ARE in female Wistar rats.

The dose formulation of test item, ARE was freshly prepared in 0.1 % CMC sodium (vehicle) prior to administration. The dose was administered once orally to overnight fasted female Wistar rats at a dose of 2000 mg/kg body weight in two steps. The animal experiment was carried out in compliance with the guidelines of CCSEA (previously known as the Committee for the Purpose of Control and Supervision of Experiments on Animals) and after approval from the Institutional Animal Ethics Committee (IAEC). Animals were housed in polypropylene cages covered with stainless steel grid top. All surviving animals in both steps were euthanized for Gross pathology observations using CO_2 in the euthanasia chamber. For each step, animals were received single dose of test item by oral gavage route after being fasted for overnight prior to dosing with free access to water and after dosing, feed was withheld ~3 h. The dosing was performed based on their respective body weight and 1 0 ml/kg body weight was maintained as dose volume.

In first step, three female animals were administered with a single dose of 2000 mg/kg body weight of test item ARE, by oral route. As all the animals survived and no clinical signs of toxicity were observed up to 72 h in first step, next three animals were administered with same single dose of 2000 mg/kg body weight of test item (ARE) by oral route as second step. All the animals in both the steps were observed for clinical signs of toxicity, mortality, and morbidity for up to day 15. All animals were observed for mortality and morbidity twice daily from acclimatization till day 15. Clinical signs were observed approximately at 30 min, 1, 2 and 4 h on day 0 (after test item administration) and thereafter once daily till necropsy. All animals were euthanized for gross pathology on day 15.

2.12. Statistical methods

Data are expressed as mean ± standard deviation (SD). Various statistical test used for analysis were one way analysis of variance (ANOVA), Dunnett's test for multiple comparisons and GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) statistical software package was use for it. The significance level was chosen at $p < 0.05$ (95 % confidence).

3. Results

3.1. Bacterial reverse mutation test

All five tester strains exposed from 0.3125 to 5 mg/plate in Trial 1 and 0.128 to 5 mg/plate in Trial 2 showed comparable number of revertant colonies with respect to concurrent vehicle control both in the presence and absence of metabolic activation. No significant bacterial cell toxicity was observed after treatment with ARE, up to a maximum concentration of 5 mg/plate. The occurrence of spontaneous reversion for strains TA 98, TA 100, TA 102, TA 1535, and TA 1537 is in agreement with previous reports [14–18]. ANOVA followed by Dunnett's multiple comparison tests indicated that treatment of ARE at concentrations of 5.0, 2.5, 1.25, 0.3125, 0.625 mg/plate for Trial 1, and concentrations of 5.0, 2.0, 0.8, 0.32, 0.128 mg/plate for Trial 2 did not show any significant increase in the number of revertant colonies in Salmonella strains with and without metabolic activation. Positive controls, Mitomycin C (0.5 μg/plate) + Sodiumazide (1 μg/plate) + 2-Nitrofluorene (10 μg /plate) + 9-Aminoacridine (25 μg /plate) + Benzopyrane (10 μg /plate) demonstrated a significant ($p < 0.05$) increase in the number of revertant colonies in the absence and presence of S9 respectively (Tables 1 and 2). Ashwagandha root extract (ARE) did not induce any point mutations by base substitutions or frameshift in the genome of Salmonella typhimurium tester strains and was found to be non-mutagenic up to the tested concentration of 5.0 mg/plate with and without metabolic activation system under the tested experimental conditions.

Table 1
Mean revertant colonies per plate and fold increase (R) in Trial-1 of bacterial reverse mutation test.

Strain®	TA1537		TA1535		TA98		TA100		TA102	
Conc (mg/plate)	*Mean (SD)*	*R*	*Mean (SD)*	*R*	*Mean (SD)*	*R*	*Mean (SD)*	*R*	*Mean (SD)*	*R*
Without metabolic activation										
▪ DMSO (50 mg/ml)	9.33 (1.53)	1.00	19.00 (2.00)	1.00	30.67 (1.53)	1.00	114.67 (3.21)	1.00	249.00 (5.57)	1.00
▪ ARE 0.3125 mg/plate	8.33 (1.15)	0.89	17.00 (1.00)	0.89	28.00 (2.00)	0.91	104.33 (5.03)	0.91	230.67 (7.51)	0.93
▪ ARE 0.625 mg/plate	8.33 (0.58)	0.89	16.67 (2.52)	0.88	29.00 (2.00)	0.95	105.67 (7.09)	0.92	230.00 (4.58)	0.92
▪ ARE 1.25 mg/plate	8.67 (1.53)	0.93	16.33 (2.52)	0.86	25.67 (1.53)	0.84	102.00 (3.61)	0.89	227.00 (5.57)	0.91
▪ ARE 2.5 mg/plate	8.33 (1.53)	0.89	15.33 (2.08)	0.81	26.00 (1.73)	0.85	98.00 (4.58)	0.85	223.00 (3.61)	0.90
▪ ARE 5 mg/plate	8.00 (1.00)	0.86	16.00 (1.73)	0.84	24.67 (2.08)	0.80	95.67 (5.03)	0.83	216.33 (5.03)	0.87
▪ PC	119.00 (4.00)*	12.75	224.00 (6.24)*	11.79	325.33 (9.02)*	10.61	823.33 (19.86)*	718	967.00 (10.54)*	3.88*
With metabolic activation										
▪ DMSO (50 mg/ml)	10.33 (1.53)	1.00	21.67 (1.53)	1.00	31.67 (2.08)	1.00	120.67 (1.53)	1.00	262.67 (5.69)	1.00
▪ ARE 0.3125 mg/plate	9.67 (1.15)	0.94	19.00 (1.00)	0.88	29.67 (3.06)	0.94	113.67 (3.06)	0.94	247.00 (4.00)	0.94
▪ ARE 0.625 mg/plate	8.33 (0.58)	0.81	18.00 (2.65)	0.83	30.00 (2.00)	0.95	107.00 (5.57)	0.89	241.00 (3.00)	0.92
▪ ARE 1.25 mg/plate	8.67 (1.15)	0.84	17.33 (2.08)	0.80	29.00 (2.65)	0.92	108.00 (4.58)	0.90	249.00 (3.00)	0.95
▪ ARE 2.5 mg/plate	9.33 (0.58)	0.90	18.33 (2.08)	0.85	26.00 (2.65)	0.82	100.00 (4.58)	0.83	237.00 (5.57)	0.90
▪ ARE 5 mg/plate	8.67 (1.15)	0.84	17.33 (2.52)	0.80	25.67 (2.31)	0.81	94.00 (3.00)	0.78	232.00 (5.57)	0.88
▪ PC	120.07 (6.03)*	12.20	233.33 (6.66)*	10.77	344.67 (7.02)*	10.88	841.93 (19.20)*	6.97	983.93 (11.68)*	3.74

ARE: Ashwagandha root extract; DMSO: Dimethyl sulfoxide (vehicle control); PC: Positive control (Mitomycin C 0.5 ug/plate + Sodium azide 1 ug/plate + 2-Nitrofluorene 10 ug /plate + 9-Aminoacridine 25 ug /plate + Benzopyrane 10 ug /plate); R: Fold increase (ration of mean revertant compared to negative control); SD: Standard deviation; TA1537/TA1535/TA98/TA100/TA102 are strains of Salmonella typhimurium.
*Significant at 95 % confidence level (<0.05.)
Metabolic activation with S9 is a key determinant of the carcinogenic potential of compounds, was used in the study.
Revertant colony count and its fold increase was an indicator. Revertant bacteria are those which are permanently converted to the prototrophic state, continue to grow and will become visible to the naked eye. This was done with vehicle control, positive control and in various concentration of ARE with and without metabolic activation in trial 2 (This was done with increased concentration of ARE).

Table 2
Mean revertant colonies per plate and fold increase (R) in Trial-2 of bacterial reverse mutation test.

Strain®	TA1537		TA1535		TA98		TA100		TA102	
Conc. (mg/plate)	*Mean (SD)*	*R*	*Mean (SD)*	*R*	*Mean (SD)*	*R*	*Mean (SD)*	*R*	*Mean (SD)*	*R*
Without metabolic activation										
▪ **VC (50 mg/ml)**	8.67 (1.53)	1.00	19.67 (1.53)	1.00	32.33 (1.53)	1.00	119.67 (3.06)	1.00	252.00 (9.54)	1.00
▪ **ARE 0.128 mg/plate**	8.33 (0.58)	0.96	17.00 (1.00)	0.86	28.67 (1.53)	0.89	111.67 (3.06)	0.93	221.00 (4.00)	0.88
▪ **ARE 0.32 mg/plate**	7.00 (1.00)	0.81	19.33 (1.53)	0.98	30.67 (2.52)	0.95	103.67 (2.52)	0.87	236.33 (5.03)	0.94
▪ **ARE 0.8 mg/plate**	8.00 (1.00)	0.92	18.33 (1.53)	0.93	29.67 (2.52)	0.92	112.00 (5.00)	0.94	227.33 (3.06)	0.90
▪ **ARE 2 mg/plate**	7.00 (1.73)	0.81	18.00 (1.73)	0.92	28.67 (2.52)	0.89	102.67 (4.51)	0.86	224.00 (5.00)	0.89
▪ **ARE 5 mg/plate**	7.33 (0.58)	0.85	18.33 (1.53)	0.93	28.33 (1.53)	0.88	100.00 (5.57)	0.84	226.00 (5.00)	0.90
▪ **PC**	138.00 (8.19)*	15.92	223.00 (9.85)*	11.34	330. 33 (11.02)*	10.22	811.00 (20.30)*	6.78	963.33 (11.24)*	3.82
With metabolic activation										
▪ **VC (50 mg/ml)**	11.00 (1.00)	1.00	23.33 (1.53)	1.00	32.67 (2.52)	1.00	126.67 (5.13)	1.00	266.67 (6.03)	1.00
▪ **ARE 0.128 mg/plate**	9.33 (1.53)	0.85	21.67 (2.08)	0.93	29.00 (2.00)	0.89	116.33 (4.16)	0.92	251.00 (7.00)	0.94
▪ **ARE 0.32 mg/plate**	8.67 (0.58)	0.79	20.67 (1.53)	0.89	30.00 (2.00)	0.92	111.67 (4.04)	0.88	240.00 (6.56)	0.90
▪ **ARE 0.8 mg/plate**	9.00 (1.73)	0.82	19.33 (1.15)	0.83	26.67 (0.58)	0.82	103.33 (5.13)	0.82	234.67 (3.51)	0.88
▪ **ARE 2 mg/plate**	9.00 (1.00)	0.82	20.00 (1.73)	0.86	29.33 (1.53)	0.90	108.00 (4.58)	0.85	237.33 (4.51)	0.89
▪ **ARE 5 mg/plate**	8.67 (0.58)	0.79	19.00 (1.73)	0.81	27.00 (1.00)	0.83	109.33 (6.66)	0.86	230.00 (5.57)	0.86
▪ **PC**	163.67(10.21)*	14.88	235.33 (9.61)*	10.09	344.00 (13.75)*	10.53	843.33 (9.29)*	6.66	987.00 (14.53)*	3.70

ARE: Ashwagandha root extract; DMSO: Dimethyl sulfoxide; PC: Positive control (Mitomycin C 0.5 ug/plate + Sodium azide 1 ug/plate + 2-Nitrofluorene 10 ug /plate + 9-Aminoacridine 25 ug /plate + Benzopyrane 10 ug /plate); R: Fold increase (ration of mean revertant compared to negative control); SD: Standard deviation; TA1537/TA1535/TA98/TA100/TA102 are strains of Salmonella typhimurium.
*Significant at 95 % confidence level (<0.05.)
Metabolic activation with S9 is a key determinant of the carcinogenic potential of compounds, was used in the study.
Revertant colony count and its fold increase was an indicator. Revertant bacteria are those which are permanently converted to the prototrophic state, continue to grow and will become visible to the naked eye. This was done with vehicle control, positive control and in various concentration of ARE with and without metabolic activation in trial 2 (This was done with increased concentration of ARE).

3.2. Chromosome aberration analysis in human peripheral blood lymphocytes

Ashwagandha root extract (ARE) at 0.25, 0.5, 1.0 and 2.0 mg/ml was not found to be cytotoxic both in the absence and presence of metabolic activation system as compared to concurrent vehicle control. The mean percent relative mitotic index (RMI) values were 90.68, 88.14, 88.14, 77.12 at 0.25, 0.5, 1.0, 2.0 mg/ml concentrations of ARE respectively in the presence of metabolic activation, whereas the RMI (%) values were 90.44, 91.30, 86.08, 81.74 at 0.25, 0.5, 1.0, 2.0 mg/ml concentrations of ARE respectively in absence of metabolic activation. MI values indicate that ARE at dose level, 0.25, 0.5, 1.0 and 2.0 mg/ml, did not produce cell toxicity in both short-term (4 h) with and without S9, and without S9 on long-term exposure (24 h). Possible structural chromosomal aberrations were analyzed in treated cells arrested at metaphase stage. Exposure of ARE at the indicated concentrations (0.25, 0.5, 1 and 2 mg/ml) to human lymphocytes did not induce a statistically significant increase in the number of cells with chromosome aberrations in the in both short term (4 h) with and without S9, and without S9 on long term exposure (24 h) (Table 3).

ANOVA followed by Dunnett's multiple comparison tests revealed that only a small proportion of structural chromosomal aberrations in

Table 3
Aberration scoring in the chromosome aberration analysis in human peripheral blood lymphocytes.

	Total No. of aberrations					Total No. of aberrations without gaps	Aberrant cells without gaps Mean (SD)	% cells with aberrations Mean (SD)
	Chromatid type		Chromosome type		Others			
	Breaks	Gaps	Breaks	Gaps				
Short-term (With metabolic activation)								
▪ VC (DMSO 50 mg/ml)	2	0	1	0	0	3	1.50 (0.71)	1.00 (0.47)
▪ ARE 0.5 mg/ml	0	0	0	0	0	0	0.00 (0.00)	0.00 (0.00)
▪ ARE 1.0 mg/ml	1	0	0	0	0	1	0.50 (0.71)	0.33(0.47)
▪ ARE 2.0 mg/ml	0	0	0	0	1 (F)	1	0.50 (0.71)	0.33 (0.47)
▪ PC (CP 3 μg/ml)	7	1	7	0	9 (F), 5 (D), 1 (R)	29	14.50 (0.71)	9.67* (0.47)
Short-term (Without metabolic activation)								
▪ VC (DMSO 50 mg/ml)	1	0	0	0	1 (F)	2	1.00 (1.41)	0.67 (0.94)
▪ ARE 0.5 mg/ml	0	0	0	0	1 (F)	1	0.50 (0.71)	0.33 (0.47)
▪ ARE 1.0 mg/ml	0	0	0	0	0	0	-	-
▪ ARE 2.0 mg/ml	I	0	0	0	0	1	0.50 (0.71)	0.33 (0.47
▪ PC (MT 0.5 μg/ ml)	7	3	5	0	9 (F), 3 (E), 3 (D)	27	13.50 (0.71)	9.00 (0.47) *
Long-term (Without metabolic activation)								
▪ VC (DMSO 50 mg/ml)	1	0	1	0	1d	3	1.50 (0.71)	1.00 (0.47)
▪ ARE 0.5 mg/ml	0	0	0	0	If	1	0.50 (0.71)	0.33 (0.47)
▪ ARE 1.0 mg/ml	1	0	0	0	If	2	1.00 (0.00)	0.67 (0.00)
▪ ARE 2.0 mg/ml	0	0	0	0	1d	1*	0.50 (0.71)	0.33 (0.47)
▪ PC (MT 0.5 μg/ ml)	7	3	5	0	9 (F), 3 (D), 1 (E), 1 (R)	25	13.00 (1.41)	8.67* (0.94)

ARE: Ashwagandha root extract; CP: Cyclophosphamide monohydrate 3 μg/ml; PC: Positive control; MT: Mitomycin-C 0.5 μg/ml; F: Fragment; E: Exchange; D: Deletion; VC: Vehicle control;
*Significant at 95 % confidence level (<0.05.)
Increased level of chromosomal aberrations in peripheral blood lymphocytes is an early indicator of carcinogenic potential of compound. This was tested for ARE in presence and absence of metabolic activation system for short term period (4 h) and without metabolic activation for long term period (24hrs).

the control cultures of the vehicle were within the range of published data, indicating their insignificance [15,17,18]. The positive controls, Mitomycin-C without S9, and cyclophosphamide monohydrate with S9 were found to induce statistically significant ($p < 0.05$) number of structural chromosomal aberrations, namely, chromosome break, chromatid break, deletion in absence and presence of S9 respectively in human peripheral blood lymphocytes (Table 3). The results of this test conclude that ARE is non-clastogenic up to 2 mg/ml concentration.

3.3. Micronucleus test in mammalian erythrocyte cells: In-Vivo

PCE (Polychromatic Erythrocyte) ratio among the total erythrocytes, PCE: TE ratio and percentage of MNPCEs were calculated for all test item treated groups and positive control and compared with the negative control. No toxicity to bone marrow (decrease in P/E ratio) was observed in any of the treatment groups as compared to negative control. Positive control group yielded a statistically significant increase in micronucleated PCE as compared to negative control group thereby illustrating the validity of the experiment.

A statistically significant ($p < 0.05$) increase in MNPCE was observed in animals treated with cyclophosphamide monohydrate at 40 mg/kg, bodyweight which thereby illustrates the sensitivity of the test system, suitability of procedures and efficiency of test conditions employed in the study. Group-wise total PCE, MNPCE, percent MNPCE and mean P/E ratio in bone marrow cells are given (Table 4). From the above results, it is concluded that KSM-66 ashwagandha root extract under given experimental conditions, up to the guideline limit dose of 2000 mg/kg, body weight has been considered to be non-clastogenic and did not induce any cytogenetic damage to the chromosomes or mitotic apparatus of erythroblast in bone marrow of Swiss albino mice.

Table 4
Micronucleus test results in mammalian erythrocyte cells in Swiss albino mice.

	MNPC Mean (SD)	MNPC % Mean (SD)	P/E ratio Mean (SD)	Mean % Reduction in P/E ratio
▪ VC (CMC)	7.6 (4.278)	0.188 (0.106)	0.494 (0.006)	0.000
▪ ARE 500 mg/kg	6.6 (1.949)	0.164 (0.047)	0.489 (0.01)	1.012
▪ ARE 1000 mg/ kg	2.0 (1.225)	0.048 (0.033)	0.479 (0.005)	3.036
▪ ARE 2000 mg/ kg	7.2 (0.837)	0.178 (0.022)	0.445 (0.008)	9.919
▪ PC (CP 40 mg/ kg)	57.6 (9.42)*	1.440 (0.2361)*	0.474 (0.013)	4.049

ARE: Ashwagandha root extract; CMC: Carboxy-methyl cellulose; CP: Cyclophosphamide monohydrate; MNPC: Micro nucleated polychromatic erythrocytes; PC: Positive control; P/E: Ratio of polychromatic erythrocytes; SD: Standard deviation; VC: Vehicle control.
*Significant at 95 % confidence level (<0.05.)
Micronucleus test is the most successful and reliable assays for evaluation of genotoxic carcinogens. Micro nucleated polychromatic erythrocytes were measured in presence of various concentrations of ARE. This was compared with vehicle and positive control.

3.4. Acute oral toxicity (LD_{50})

All the experimental animals showed gain in body weight on day 8 and 15 in comparison to their day 1 body weight. No mortality and morbidity were observed throughout the experiment period. All animals (n = 25) appeared normal and did not exhibit any clinical signs of toxicity throughout the observation period. No gross lesions were observed in any of the organs of all the animals.

In sighting and main studies, treatment with ARE does not reveal any adverse effects on the body weight gain at 1 day, 2 day and 3 days of

Table 5
Body weight (grams) of female Wistar rats.

	N	Body weight (g) Mean SD		
		Day 1	Day 2	Day 3
VC	5	33.626 (3.709)	33.710 (3.789)	33.722 (3.757)
ARE 500 mg/kg	5	33.836 (3.677)	33.910 (3.666)	33.874 (3.711)
ARE 1000 mg/kg	5	33.458 (4.085)	33.560 (4.126)	33.466 (4.052)
ARE 2000 mg/kg	5	33.172 (4.113)	33.250 (4.187)	33.330 (4.180)
PC	5	32.868 (4.131)	32.992 (4.153)	32.880 (4.164)

ARE: Ashwagandha root extract; CMC: Carboxy-methyl cellulose; CP: Cyclophosphamide monohydrate; PC: Positive control; SD: Standard deviation.
* Body weight variation is one of the indicators used to evaluate the acute toxic effect of compound. This was done in various concentrations of ARE and compared with vehicle and positive control.

observation. Overall, it was observed that the percentage increase in body weight over the entire three-day monitoring period was within the expected range for all the treated animals. On necropsy, no major gross pathological changes were observed in ARE treated Wistar rats (Table 5). Based on the above observations, the LD_{50} cut-off value of Ashwagandha root extract (ARE) was found to be greater than 5000 mg/kg body weight and classified as Category-5 or unclassified, as per Globally Harmonized Classification System (OHS) for Chemical Substances and Mixtures.

4. Discussion

In this study, mutagenic potential, and acute single oral dose toxicity of standardized ashwagandha root extract (ARE) was estimated in a variety of tests. These tests are an integral part of pre-clinical studies aimed to establish the balance of benefits and risks for use in humans for their health benefits in a range of clinical conditions. Genotoxic potential of ARE was evaluated using a bacterial reverse mutation assay, chromosomal aberration, and micronucleus assays.

Bacterial reverse mutation test is an adequate primary test for the detection of potential mutagens and carcinogens, is well recognized, and has been widely used since 1970 s to screen and test potential carcinogens. [21] ARE did not show any increase in the number of reverted colonies at concentrations from 0.128 to 5.0 mg/plate, with or without metabolic activation. In Salmonella strains such as TA 98, TA 100, TA 102, TA 1535 and TA 1537, these results confirmed the absence of mutagenic activity. These findings are consistent with those reported by Rani G et al. (2005) [22]. Apart from lack of mutagenicity, antimutagenic properties have been reported in AREs with respect to a range of classic mutagenic agents [3].

An important part of a genotoxicity screening is assessment of DNA damage and micronucleus formation at chromosome level [8,9]. Chromosomal aberrations and micronuclei formation with ARE was tested in the human peripheral blood lymphocytes and mammalian (Swill albino mice) red blood cells. The effects of AREs on cell division were studied by examining the mitotic index of peripheral blood lymphocytes and polychromatic erythrocytes, micronucleated polychromatic erythrocytes, MNPCE percentages, and the average P/E ratio of mammalian erythroid cells. MI is used to quantify differences in cell division when environmental parameters are changed [8]. Micronucleated polychromatic erythrocytes indicate chromosomal breakage (clastogenicity) or aneuploidy [23]. ARE failed to show any effects on MI and MNPCE, and there were no differences in cell proliferation with ARE compared to vehicle controls. Also, in mammalian cells, ARE was not capable of inducing many chromosome anomalies or micronuclei formation. The results are consistent with the prior testing and reported lack of genotoxic activity in ashwagandha extracts [24]. Furthermore, the study performed by Jayaprakasam et al. (2013) shows that, Ashwagandha extract was significantly more effective than the well-known anticancer drug doxorubicin in inhibiting the growth of breast and colon cancer cell lines [25]. The authors report the potential role of ashwagandha extract in chemotherapy of malignancy.

We tried to establish the median lethal dose (LD_{50}) of single dose oral administration of ARE in female Wistar rats to meet the hazard classification and labeling requirements for human health and environmental risk assessment [2]. ARE failed to cause any mortality, abnormal clinical symptoms, or significant pathological changes in doses up to 2000 mg/kg body weight. Furthermore, overall weight gain was found to be normal in all dosed mice and ARE did not fall into the internationally harmonized system risk category. [11]. The current results of acute oral toxicity studies are consistent with previous study by Antony et al. [1]. ARE used in the study complies with the as pharmacopeial acceptable limits for contaminants like microorganisms, pesticides, heavy metals, and aflatoxins. This further assures the quality of this extract. Genotoxicity studies are designed to determine whether AREs affect genetic material or cause cancer. As a result, no genotoxic effects of ARE were confirmed in a series of genotoxicity studies [10].

Ethical approval

The study was approved by the Institutional Animal Ethics Committee (IAEC) registered under CCSEA, India.

Institutional Ethics Committee (IEC No. IEC-NI/21/APR/78/73) approval was received and informed written consent was obtained from human volunteers.

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CRediT authorship contribution statement

Dr. Deepak Langade: Writing – original draft, Writing – review & editing. **Dr. V. Gayathri:** Project administration, Visualization. **Dr. R. Siva:** Formal analysis, Methodology. **Dr. P. Kalaivani:** Conceptualization, Investigation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential conflict of interests. Gayathri V, Centre For Toxicology and Developmental Research (CEFTE), Sri Ramachandra University, Chennai, Tamil Nadu, India) reports financial support, administrative support, article publishing charges, equipment, drugs, or supplies, and statistical analysis were provided by Shri Kartikeya Pharma, Hyderabad, India.

Data availability

Ashwagandha root extract (ARE) characteristics are provided as supplementary material [26].

Data will be made available on request.

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