Research Article ISSN 2639-944X

Journal of Medical - Clinical Research & Reviews

Toxicity and Clastogenic Potential of Ageratum Conyzoides

Narendra S Deshmukh[1], Prahanth Nalge[1], Sedatrul Muntha[2], Silma Subah[2] and Paul R Clayton[2*]

[1]*Intox Private Ltd, 375 Urawade, Tal. Mulshi, Dist. Pune 412 115. Maharashtra, India.*

[2]*Gencor Pacific Ltd, Unit 3, 1st Floor, Discovery Bay North Plaza, Discovery Bay, Hong Kong.*

***Correspondence:**
Paul Clayton, Gencor Pacific Ltd, Unit 3, 1st Floor, Discovery Bay North Plaza, Discovery Bay, Hong Kong.

Received: 11 Mar 2024; **Accepted:** 17 Apr 2024; **Published:** 25 Apr 2024

Citation: Deshmukh NS, Jaideep K, Muntha S, et al. Toxicity and Clastogenic Potential of Ageratum Conyzoides. J Med - Clin Res & Rev. 2024; 8(4): 1-22.

Introduction

Billygoat Weed *(Ageratum conyzoides Linn*.,family Asteraceae) is an annual invasive herb which grows in tropical and subtropical regions. Its ethnobotanical profile includes use in wound dressing, and as an anti-microbial, anti-inflammatory, analgesic and gastro-protective agent [1-7]. It has been characterized as broadly beneficial in humans [8].

Secondary metabolites including flavonoids, alkaloids, chromenes, sterols and coumarins give *A. conyzoides* its distinct biological, pharmacological and insecticidal properties [9]. It also contains essential oils, including benzofurans and eugenols, which have anticonvulsant and antimicrobial properties [10]. Extracts of *A. conyzoides* exert anti-infective properties via microbial inhibition and killing [11,12]. These and other activities are implicated in studies of wound healing, where methanolic extracts of *A. conyzoides* reduced site-related inflammation and enhanced local restoration of ECM [13]. Anti-insecticidal and anti-malalarial properties have been shown in pre-clinical models [14], and anti-cancer effects in several human cancer cell lines [2].

The Asteraceae family are known to contain hepatotoxic compounds [15]. These include the pyrrolidine alkaloids 1,2-desifropirrolizidinic and lycopsamine, and also hexamethoxyflavone and 1-2 benzopyrone. In rodent models, however, *A conyzoides* has demonstrated an absence of hepato-toxicity [5,16], and positive hepato-protective effects [17]. These findings are not entirely consistent.

A recent series of studies which positioned *A conyzoides* as a novel treatment for alopecia [18,19] has increased public interest in the plant, accelerating the need for a robust safety evidence base.

Our investigation of the hepatotoxicity, global toxicity and possible genotoxicity of *A. conyzoides* includes three studies: a chronic 180-day oral toxicity study in Wistar rats, a Salmonella typhimurium reverse mutation assay and a mammalian cell micronucleus test.

Materials and Methods

Repeated Dose Oral Toxicity Study (6 Months) with 28 Days Recovery Period in Rat

Groups of 40 Wistar rats, 20 female and 20 male, were administered with *A conyzoides* extract in analytical grade water, daily by oral gavage for 180 consecutive days, at dose of 500 mg/kg, 1000 mg/kg and 2000 mg/kg b.wt.

Table 1: Study Design of Repeated Dose Oral Toxicity Study.

Dose Group		Dose (mg/kg/day) of Ageratum conyzoides Extract	No. of Animals per Group			
			Treatment Period (180 Days)		Treatment period + Recovery Period (28 Days)	
			Male	Female	Male	Female
G1 and G1 (R)	Vehicle Control	0	20	20	10	10
G2	Low Dose	500	20	20	-	-
G3	Mid Dose	1000	20	20	-	-
G4 and G4 (R)	High Dose	2000	20	20	10	10

M – Males, F – Females, R – Recovery group (following 180-Day treatment)

The doses of *A conyzoides* extract were derived from published findings of a 90-day oral toxicity study in rats [20]. A control group of rats was treated with vehicle alone. Animals were observed during the treatment period for signs of toxicity. After cessation of treatment, additional groups of 10 rats of each sex at control and high dose levels were observed for reversal of toxicity or delayed toxicity during a post-treatment period of 28 days.

Throughout the study, rats were examined daily for signs of toxicity am and pm. Signs of ill health and behavioral changes were recorded for each individual animal. Animals were subjected to general and detailed clinical examinations prior to the first exposure and weekly thereafter during the treatment and recovery periods. Changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity such as lacrimation, piloerection, pupil size, unusual respiratory pattern were observed. Changes in gait, posture and response to handling were recorded, as were clonic or tonic movements, stereotypies or bizarre behaviors.

Ophthalmoscopic examination was carried out on all animals on Day 0. On day 180, before termination of the treatment period, ophthalmological examination was performed on rats from control (G1) and high dose groups (G4).

Eye examinations utilized a direct ophthalmoscope (Heine Mini 3000). After initial examination of eyes for pupillary reflexes, pupils were dilated using a 1% Tropicamide ophthalmic solution (Tropicamet®, manufactured by Sun ways (India) Pvt. Ltd., Mumbai) to facilitate examination of fundus.

During the 25th and 26th week of treatment all animals were assessed for sensory reactivity, grip strength and motor activity. Neurological examinations include in home-cage assessment, open field assessment, manipulative examinations and assessment of responses to stimuli. Posture, movement and respiration were examined, together with palpebral closure, lacrimation, salivation and skin and hair quality.

Urination, defecation and rearing frequency and gait were examined by placing animals in a standard open arena. Manipulative examinations / evaluation of responses to stimuli included tactile response, tail pinch, pupil response to light, proprioception–righting reflex, auditory response, head shaking and landing foot splay.

Locomotor activity was assayed during the 26th week of treatment. Rats were placed in a multiple unit open field enclosure, with four chambers, one animal in each chamber. An overhead camera sent signal to a validated software system - Anymaze® (Stoelting Co., 620 Wheat Lane, Wood Dale, IL 60191, USA). Movement was tracked for 600 seconds per rat, while the Anymaze® software analysed specified parameters and generated graphical and numeric reports for each animal. Locomotor assessment included total distance travelled, average speed, rotations and absolute turn angle.

Body weights were recorded on the day of grouping, day 1 of treatment, weekly thereafter, on day 180 and on day 181, the day of necropsy. Body weights of all animals in recovery groups were recorded weekly during the recovery period, on day 208 and on day 209, the day of necropsy. The quantity of food consumed by rats in each cage was recorded weekly. Food intake per rat per day was averaged and back-calculated.

Blood and plasma samples were subjected to clinical haematology and clinical chemistry evaluations at day 98, 181/182 (termination of the treatment), or at end of the 28-day recovery period (day 209). Animals were fasted overnight prior to sampling. Sampling of blood was conducted, under light isoflurane anesthesia, through the orbital sinus and collected separately in tubes containing EDTA (dipotassium salt) for haematology, Sodium citrate for determination of coagulation parameters and Heparin, for clinical chemistry, as anticoagulants. For hormone estimations, sera samples were separated from blood collected in tube devoid of anticoagulant for analysis of T3, T4, TSH, FSH, LH, OE and TST.

After completion of 180 days of treatment and at termination of the 28-day recovery period, the Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) were determined using 'Semi Automated Coagulation Analyser STart® Max, (Diagnostica Stago, France). General Blood Picture: Blood smear, stained as above, were assessed for abnormal and immature cells by microscopy. Plasma samples were analysed individually for determination of clinical chemistry parameters using fully automated Clinical Chemistry System, Dimension Xpand Plus (Siemens Healthcare Diagnostics Inc. Newark, U.S.A.).

Urinalysis was performed in the last week of the treatment period on rats from control and high dose groups. Urine samples were summed over a period of 2-3 hours. Samples were centrifuged before being subjected to qualitative / semi-quantitative and microscopic evaluation. Multistix® 10 SG multiple reagent diagnostic strips were used for the tests and results were read using 'Clinitek Status' Urine Analyser'(Siemens Medical Solutions Diagnostics). Colour, Glucose, Nitrite, Appearance, Ketone, Leucocyte, Specific gravity, pH, Protein, Volume (timed), Bilirubin, Urobilinogen and Occult blood were assessed.

Endocrine and reproductive functions were assessed by determining levels of T3, T4, TSH, FSH, LH, OE and TST, along with other parameters. Male rats were assessed for cauda epididymis sperm reserves, sperm motility and sperm morphology.

After sacrifice all animals were subjected to detailed necropsy, and organ weights were recorded. Histopathological evaluation was performed on all study plan-listed tissues in all rats from the vehicle control and high dose groups. Assessment of immune toxicity was based on the primary indicators of immune toxicity derived from routine measurements and additional examinations performed during toxicity studies.

AMES test

The Ames Test measured *A conyzoides* extract's ability to induce reverse mutation at selected histidine loci in five tester strains of Salmonella typhimurium viz. TA1535, TA97a, TA98, TA100 and TA102 in the presence and absence of metabolic activation system (S9). The pre-incubation method was employed.

Suspensions of bacterial cells were incubated with the test item in a reaction mixture, at 37 °C for 20 minutes in an orbital shaker,

in presence and absence of an exogenous metabolic activation system (S9). The reaction mixture was then mixed with top agar and plated immediately onto minimal medium. After about 65 to 67 hours of incubation at 37 °C, revertant colonies were counted and compared to the number of spontaneous revertant colonies on solvent control plates. The entire study was carried out in duplicate. Based on solubility, precipitation and preliminary cytotoxicity testing, ethanol was chosen as the vehicle and six doses were tested: 15, 50, 150, 500, 1500 and 5000 µg of extract/plate.

Liver S9 fraction induced in rats with combination of sodium phenobarbitone and 13-naphthoflavone was used for metabolic activation. The exposed bacteria were plated onto minimal glucose agar medium supplemented with L-histidine D-biotin solution. The plates were incubated at 37 °C for 65 to 67 hours after which the histidine revertant colonies were counted and their frequency was compared with vehicle controls. Concurrent vehicle control and positive control groups were included in all the experiments as required by test guidelines.

In Vitro Mammalian Cell Micronucleus Test using Human Lymphoblastoid Cell Line (TK6)

In Vitro Mammalian Cell Micronucleus Test using Human Lymphoblastoid Cell Line - TK6 was used to evaluate the potential of *A conyzoides* extract to induce formation of small membrane-bound DNA fragments (micronuclei) in the cytoplasm of interphase cells. The formation of micronuclei may come from acentric fragments or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of the cell division. The test therefore detects both clastogens and aneugens.

Prior to the main study, solubility, precipitation test and preliminary cytotoxicity tests were carried out to determine the exposure concentrations. TK6 cells were exposed to the test item at concentrations of 312.5, 156.25 and 78.125 µg/ml with and without metabolic activation for 3 hours and 24 hours exposure. Liver S9, induced in Wistar rats with sodium phenobarbitone and 13 - naphthoflavone, was used as the metabolic activator. Triplicate cultures were used at each concentration.

In experiment no. 1, cells were exposed to the test item for 3 hours in presence of the metabolic activation system, cell harvesting was done after 1.5-2 cell cycle length after the beginning of treatment.

In experiments 2 and 3, the exponentially proliferating cells were exposed to the test item in absence of a metabolic activation system for 3 hours and 24 hours respectively. Cell harvesting was done after 1.5-2 cell cycle length after the beginning of treatment.

Positive and vehicle controls with and without metabolic activation were tested concurrently with the test item. Analytical grade water was used as a vehicle control, aneugenic and clastogenic agents were employed as positive controls. Mitomycin C and Vinblastine were used at 160 ng/ml and 3.5 ng/ml respectively, for experiments without metabolic activation system, Cyclophosphamide was employed at the concentration of 6.25 µg/m for experiment with metabolic activation system.

At the end of treatment/recovery period, each culture was harvested and processed separately. Cells were lysed, stained and approximately 10,000 healthy nuclei per group were analyzed by micronuclei scoring using flow cytometric method (Litron-MicroFlow).

Litron- MicroFlow uses sequential staining that differentiatesion MN and the chromatin of apoptotic and necrotic cells. Reliable MN measurements are obtained even when appreciable numbers of dead cells are present.

The cells collected were washed and the cytoplasmic membranes digested with detergent to liberate nuclei and MN. During the lysis step, Nucleic Acid Dye B (i.e., SYTOX Green) is introduced which labels all chromatin. In this way, differential staining of healthy chromatin versus that of dead/dying cells is achieved. Stained cells were acquired on BD FACSVerse flow cytometer (BD Biosciences, USA) equipped with 488 nm laser and analyzed using BD FACSuite (version 1.0.6) software for MN scoring.

Micronucleus values were expressed as percent by dividing the number of events that fall within the "MN" region by the number of events that fall within the "Nucleated" region and multiplying by 100 according to the formula:

$$\%MN = \frac{\text{Number of events in the MN region}}{\text{Number of events in the Nucleated region}} \times 100$$

STUDY DESIGN

Expt. Sr. No.	Metabolic Activation (S9)	Treatment Period (hours)	Treatment Groups : Dose Levels (µg/mL)			Control Groups	
			High	Mid	Low	Vehicle Control	Positive Control
1	Present	3	312.5	156.25	78.125	Analytical grade water	CPM
2	Absent	3	312.5	156.25	78.125		MMC
3	Absent	24	312.5	156.25	78.125		VBL

MMC- Mitomycin C, CPM – Cyclophosphamide monohydrate, VBL – Vinblastine

Table 2: Study Design of *In Vitro* Mammalian Cell Micronucleus Test.

Results

Chronic Oral Toxicity Study (6 Months) with 28 Days Recovery Period

During the treatment and post-treatment periods, daily oral administration of *A conyzoides* extract at doses of up to 2000 mg/kg/day had no effect on survival rates. No ophthalmological abnormalities were seen at any dose level.

Qualitative / quantitative parameters including sensory activity, grip strength and motor activity measured during weeks 25 and 26 of the study showed no changes at any time point. Responses to touch and tail-pinch, pupil constriction in response to light, righting reflex, auditory response and head-shaking response remained within normal variations for all animals. Mean frequencies of urination, defecation and rearing of the treatment group rats did not differ significantly (P>0.05) from those of the vehicle control group.

The test item did not affect locomotor activity assessed in open field analysis. Total distance travelled, average speed, body rotations and absolute turn angle (°), for the treatment group did not differ significantly (P>0.05) from those of the vehicle control group. *A conyzoides* Extract therefore demonstrated no neurotoxic potential in this study.

Daily oral administration of *A conyzoides* extract at up to the maximum dose of 2000 mg/kg did not affect body weight and body weight gain in female rats. Statistically significant (P<0.05) decreases in mean body weight were observed from day 49 to day 180 in male rats given 2000 mg/kg, but the degree of reduction was marginal and was maintained during the recovery period. Daily oral administration of *A conyzoides* extract at doses of up to 2000 mg/kg/day did not affect average daily food consumption during the treatment and recovery periods.

Haematological evaluations were performed at day 98, 181/182 (termination of the 180-day treatment) or the 28-day recovery period (day 209). Treatment with *A conyzoides* extract at up to 2000 mg/kg/day induced no significant changes in haemoglobin, haematocrit (PCV), total red cell (RBC) counts, erythrocyte indices (MCV, MCH, MCHC), total and differential white cell (WBC) counts, reticulocyte counts, platelet counts and coagulation parameters (prothrombin time and activated partial thromboplastin time) and in the morphology of their blood cells. The evaluation of stained blood smear made by microscopic examination did not reveal any abnormal cells.

Clinical Chemistry evaluations were performed at the end of the treatment and the recovery periods. *A conyzoides* extract at doses of up to 2000 mg/kg/day did not induce any changes i.

Table 3: Summary of overall food consuption**.**

Sex	Group	G1 (Vehicle Control)	G2 500 mg/kg	G3 1000 mg/kg	G4 2000 mg/kg
Male Rats	Average*	29.88	29.71	29.86	29.63
	As % of Control	-	99	100	99
Female Rats	Average*	19.15	19.19	19.24	19.04
	As % of Control	-	100	100	99

Table 4: Statistically Significant (P<0.05) Haematological Alterations.

Sr. No.	Parameter (Unit)	Sex	Group and Change	**Mean ± SD Control group value is presented in parenthesis**	Remark	Conclusion
	End of Treatment Period (Day 98)					
1	Total WBC (x 103 cells/µ L)	Male	G4 ↑	11.07 ± 1.14 (8.60 ± 1.46)	Although G2 (8.77 ± 2.11) & G3 (9.65 ± 1.14) value not statistically significant, increase in WBC count is dose dependent; Slight decrease (28%), within HCR of INTOX 5.14 to 11.76.	Within Biological Limits, Toxicologically Insignificant
2	Neutrophil Count (x 103 cells/µ L)	Males	G4 ↑	2.96 ± 0.62 (2.39 ± 0.33)	No dose dependency; Increase (24%) within HCR of INTOX 1.08 to 4.88.	Within Biological Limits, Toxicologically Insignificant
3	Lymphocyte Count (x 103 cells/µ L)	Males	G4 ↑	7.46 ± 0.88 (5.74 ± 1.67)	Although G2 (5.99 ± 1.60) & G3 (6.25 ± 0.89) value not statistically significant increase (30%) in lymphocyte count is dose dependent However, values are within HCR of INTOX 3.98 to 13.55.	Within Biological Limits, Toxicologically Insignificant
4	Monocyte Count (x 103 cells/µ L)	Males	G4 ↑	0.39 ± 0.08 (0.26 ± 0.07)	Although G2 (0.27 ± 0.09) & G3 (0.34 ± 0.05) value not statistically significant increase (50%) in monocyte count is dose dependent However, values are within HCR of INTOX 0.00 to 0.47.	Within Biological Limits, Toxicologically Insignificant

↓ and ↑ : values of the parameter are lower (↓) or higher (↑) than the control group value at P<0.05. # Control group value is presented in parenthesis HCR - Historical control range in the test facility for this strain of rat.

Table 5: Statistically Significant (P<0.05) Clinical Chemistry Alterations.

Sr. No.	Parameter (Unit)	Sex	**Group & Change**	**Mean ± SD Control group value is presented in parenthesis**	Remark	Conclusion
	End of Treatment Period (Day 98)					
1	Total protein (g/dL)	Male	G3 ↑ G4 ↑	7.27 ± 0.25 7.79 ± 0.54 (6.76 ± 0.25)	Dose dependent Increase (8% to 15%) Within HCR of INTOX 5.55 to 8.10 Due to increase in albumin levels	Within Biological Limits, Incidental
2	Albumin (g/dL)	Male	G3 ↑ G4 ↑	1.26 ± 0.12 1.60 ± 0.19 (1.10 ± 0.08)	Dose dependent Increase (15% to 45%), G3 group value within the HCR while G4 value slightly higher than HCR of INTOX 0.87 to 1.59	Toxicologically Insignificant
3	ALT (U/L)	Male	G4 ↓	27.40 ± 6.90 (46.70 ± 19.90)	Not Dose dependent, Within HCR of INTOX 31.08 to 68.04, Decrease in ALT value do not have any significance	Incidental
		Female	G3 ↑ G4 ↑	41.60 ± 9.10 41.90 ± 14.00 (30.00 ± 6.20)	Dose Dependent Increase (39% to 40%) Within HCR of INTOX 21.04 to 58.26.	Associated with microsomal enzyme induction; Induced but non adverse
4	ALP (U/L)	Male	G4 ↓	38.50 ± 19.10 (63.00 ± 13.50)	Not Dose dependent, Within HCR of INTOX 50.66 to 92.53. Decrease in ALP value do not have any significance	Incidental
5	Na (mmol/L)	Male	G2 ↓ G3 ↓ G4 ↓	146.40 ± 4.70 140.80 ± 1.20 142.40 ± 0.80 (152.00 ± 7.30)	Not Dose dependent, Within HCR of INTOX 133.03 to 162.00.	Incidental
6	Calcium (mg/dL)	Male	G3 ↑ G4 ↑	10.66 ± 0.38 10.74 ± 0.31 (10.26 ± 0.28)	Although G2 (10.46 ± 0.22) value not statistically significant increase (4% to 5%) in calcium levels is dose dependent. However, values are within HCR of INTOX 9.20 to 12.75	Within Biological Limits, Incidental
		Female	G2 ↑ G3 ↑	11.68 ± 0.92 11.92 ± 0.56 (10.95 ± 0.37)	Not Dose Dependent Within HCR of INTOX 9.32 to 12.58.	Incidental
7	Phosphorus (mg/dL)	Male	G3 ↑	6.01 ± 0.50 (5.28 ± 0.31)	Not Dose Dependent, Within HCR of INTOX 4.07 to 10.07.	Incidental
8	HDL (mg/dL)	Male	G2 ↑ G4 ↑	85.70 ± 15.40 86.60 ± 14.40 (70.70 ± 9.30)	Not Dose Dependent, G5 value slightly higher than HCR of INTOX 35.00 to 86.32	Within Biological Limits, Incidental
9	Globulin (g/dL)	Male	G3 ↑ G4 ↑	6.01 ± 0.28 6.19 ± 0.35 (5.66 ± 0.23)	Although G2 (5.68 ± 0.23) value not statistically significant increase (6% to 9%) in Globulin levels is dose dependent. However, values are within HCR of INTOX 4.47 to 6.72.	Within Biological Limits, Incidental
		Female	G2 ↑	6.83 ± 0.39 (6.38 ± 0.31)	Not Dose Dependent, Within HCR of INTOX 4.47 to 7.17.	Incidental
10	A/G Ratio	Male	G4 ↑	0.26 ± 0.02 (0.20 ± 0.02)	Not Dose Dependent, Within HCR of INTOX 0.15 to 0.29.	Incidental
11	AST (U/L)	Female	G3 ↑ G4 ↑	113.50 ± 12.90 96.10 ± 16.20 (73.00 ± 7.10)	Not Dose Dependent, Within HCR of INTOX 59.47 to 124.37	Incidental
12	Glucose (mg/dL)	Female	G3 ↓ G4 ↓	69.70 ± 10.70 69.60 ± 10.10 (107.20 ± 10.30)	Not Dose Dependent, Within HCR of INTOX 49.81 to 119.38.	Incidental
13	Urea Nitrogen (mg/dL)	Female	G3 ↑ G4 ↑	26.60 ± 4.40 26.40 ± 3.70 (16.70 ± 2.50)	Not Dose Dependent Higher (58% to 59%) than control; However, values are within HCR of INTOX 12.83 to 28.38. No change in creatinine levels.	Incidental/ Toxicologically insignificant
14	Potassium (mmol/L)	Female	G3 ↑	4.27 ± 0.34 (3.68 ± 0.30)	Not Dose Dependent Within HCR of INTOX 3.52 to 6.29	Incidental

End of Treatment Period (Day 181/182)						
15	Total Protein (g/dL)	Male	G2 ↑ G3 ↑	7.22 ± 0.29 7.43 ± 0.27 (6.94 ± 0.36)	Not Dose Dependent Increase within HCR of INTOX 5.55 to 8.10 Due to increase in albumin levels	Within Biological Limits, Incidental
16	Albumin (g/dL)	Male	G3 ↑	1.15 ± 0.11 (1.05 ± 0.10)	Not Dose Dependent Increase within HCR of INTOX 0.87 to 1.59	Incidental
17	Glucose (mg/dL)	Male	G3 ↓	79.25 ± 17.20 (99.25 ± 16.22)	Not Dose Dependent Decrease within HCR of INTOX 56.27 to 128.11	Incidental
		Female	G3 ↑ G4 ↑	105.70 ± 21.28 107.70 ± 17.62 (90.20 ± 9.37)	Although G2 (102.0 ± 13.3) value not statistically significant increase (17% to 19%) in Glucose levels is dose dependent. However, values are within HCR of INTOX 49.81 to 119.38.	Incidental/ Toxicologically insignificant
18	Globulin (g/dL)	Male	G3 ↑	6.28 ± 0.26 (5.90 ± 0.36)	Not Dose Dependent Increase within HCR of INTOX 4.47 to 6.72	Incidental
19	GGT (U/L)	Male	G4 ↑	10.60 ± 1.27 (9.50 ± 0.95)	Although G2 (9.85 ± 1.60) & G3 (9.85 ± 1.09) value not statistically significant, increase (12%) in GGT levels is dose dependent; Slightly higher than HCR of INTOX 5.42 to 6.50. Even control value is higher than HCR.	Within Biological Limits, Toxicologically Insignificant
		Female	G4 ↓	8.75 ± 1.16 (9.65 ± 1.50)	Not Dose Dependent Within HCR of INTOX 5.44 to 10.48	Incidental
20	Calcium (mg/dL)	Male	G2 ↑ G3 ↑	11.47 ± 0.30 11.59 ± 0.31 (11.22 ± 0.43)	Not Dose Dependent Within HCR of INTOX 9.20 to 12.75	Incidental
21	Phosphorus (mg/dL)	Male	G4 ↑	4.84 ± 0.41 (4.47 ± 0.31)	Not Dose Dependent Within HCR of INTOX 4.07 to 10.07	Incidental
22	Creatinine (mg/dL)	Male	G2 ↑ G3 ↑	0.46 ± 0.06 0.49 ± 0.06 (0.41 ± 0.06)	Not Dose Dependent Within HCR of INTOX 0.40 to 0.79.	Within Biological Limits, Incidental
23	HDL (mg/dL)	Female	G2 ↑	101.20 ± 10.43 (88.70 ± 16.46)	Not Dose Dependent Slightly higher than HCR of INTOX 34.77 to 92.91.	Incidental
End of Recovery Period (Day 209)						
23	ALP (IU/L)	Male	G4 ↑	90.00 ± 18.00 (71.10 ± 12.20)	Within HCR of INTOX 50.66 to 92.53.	Within Biological Limits, Incidental
		Female	G4 ↑	60.70 ± 25.70 (19.30 ± 6.90)	Within HCR of INTOX 20.06 to 60.79.	
24	Total Protein (g/dL)	Female	G4 ↓	7.09 ± 0.51 (7.88 ± 0.24)	Within HCR of INTOX 5.81 to 8.71.	Within Biological Limits, Incidental
25	Albumin (g/dL)	Female	G4 ↓	1.05 ± 0.21 (1.52 ± 0.19)	Within HCR of INTOX 0.99 to 1.88.	Incidental
26	Total Bilirubin(mg/dL)	Female	G4 ↓	0.10 ± 0.07 (0.21 ± 0.03)	Within HCR of INTOX 0.06 to 0.18.	Incidental
27	Na (mmol/L)	Female	G4 ↑	136.90 ± 2.10 (135.00 ± 1.60)	Within HCR of INTOX 134.45 to 155.94.	Incidental
28	Phosphorus (mg/dL)	Female	G4 ↑	4.71 ± 0.67 (3.98 ± 0.34)	Not Dose Dependent Within HCR of INTOX 3.30 to 9.17	Incidental
29	HDL (mg/dL)	Female	G4 ↓	78.10 ± 11.60 (97.70 ± 13.20)	Within HCR of INTOX 34.77 to 92.91.	Incidental
30	Globulin (g/dL)	Female	G4 ↓	6.04 ± 0.38 (6.36 ± 0.18)	Within HCR of INTOX 4.47 to 7.17.	Incidental
31	A/G Ratio	Male	G4 ↓	0.17 ± 0.03 (0.24 ± 0.03)	Within HCR of INTOX 0.16 to 0.34.	Incidental

↓ and ↑ : Mean value is lower (↓) or higher (↑) than the control group mean at $P<0.05$.
Control group mean is presented in parenthesis;
HCR - Historical control range in the test facility for this strain of rat.

Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Bilirubin (total), Glucose, Total Cholesterol, Triglycerides, Creatinine, Calcium, Urea nitrogen, Total Bile acid, Urea (calculated), Phosphorus, Protein (total), Albumin, Sodium, Potassium, Low -density Lipoprotein Cholesterol (LDL), High -density Lipoprotein Cholesterol (HDL), Globulin (calculated), gamma glutamyl transpeptidase and Albumin/Globulin (A/G) Ratio (calculated). Mean values of all clinical chemistry parameters at days 98, 181/182 and 209 remained within normal biological ranges.

Oral administration of *A conyzoides* extract to male and female rats for 180 days at doses up to 2000 mg/kg/day, did not affect serum levels of Triiodothyronine (T3), Thyroxine (T4) and Thyroid Stimulating hormone (TSH). At necropsy there were no gross pathological findings in the thyroid, and no changes in the absolute or relative weights of this gland. Histopathological evaluations did not reveal any abnormalities.

Oral administration of *A conyzoides* extract to male and female rats for 180 days, at up to 2000 mg/kg/day, did not induce any alterations in Follicle Stimulating hormone (FSH), testosterone (TST), Luteinizing hormone (LH) and Oestradiol (OE). No abnormalities were seen in the reproductive organs and in the biochemistry panel.

After treatment of male and female rats with *A conyzoides* extract at up to 2000 mg/kg/day, no changes were observed in any of the urinalysis parameters. Urine colour, appearance, specific gravity, pH and analytes protein, glucose, ketones, occult blood, urobilinogen, nitrite, leucocytes and bilirubin were unaffected, as was microscopic appearance of the centrifuged deposits.

Oral administration of *A conyzoides* extract for 180 days up to the dose of 2000 mg/kg/day did not affect the absolute and relative weights of liver, kidneys, adrenals, testes / ovaries, epididymides, prostate + seminal vesicles with coagulating gland, uterus with cervix, brain, spleen, thymus, heart, pituitary gland and thyroid gland (fixed).

Table 6: Statistically Significant (P<0.05) Circulating Thyroid Hormones.

Sr. No.	Parameter (Unit)	Sex	Group and Change	**Mean ± SD Control group value is presented in parenthesis**	Remark	Conclusion
End of Treatment Period (Day 98)						
1	TSH (mIU/L)	Male	G4 ↑	6.61 ± 1.05 (5.47 ± 0.22)	Although G2 (5.36 ± 0.29) & G3 (5.74 ± 0.35) value not statistically significant, increase in TSH levels is dose dependent; Slightly higher (21%) than control. Higher than HCR of INTOX 2.80 to 4.80. Even control group values are higher than HCR.	Inconsistent with co-relating findings hence considered as incidental/ Toxicologically insignificant
2	T4 (pmol/L)	Male	G2 ↑ G3 ↓ G4 ↓	548 ± 99 256 ± 37 297 ± 42 (471 ± 72)	Not Dose dependent, Within HCR of INTOX 159.69 to 374.71; Control and G2 rats values are higher than HCR.	Incidental
		Female	G4 ↑	384 ± 71 (328 ±49)	Although G2 (340 ± 39) & G3 (359 ± 70) value not statistically significant, increase in T4 levels is dose dependent; Slightly higher (17%) than control.G2 & G3 values within HCR, while G4 value is slightly higher than HCR of INTOX 177.90 to 363.44.	Inconsistent with co-relating findings, no changes in absolute and/ or relative organ weight or no histopathological or no clinical pathology findings hence considered as toxicologically insignificant
3	T3 (pmol/L)	Male	G3 ↑ G4 ↑	9.20 ± 0.61 9.30 ± 0.58 (8.02 ± 0.48)	Although G2 (8.46 ± 0.64) value not statistically significant, increase in T3 levels is dose dependent; Slightly higher (15%-16%) than control. G2 values within HCR, while G3 & G4 value is slightly higher than HCR of INTOX 4.22 to 8.87.	
		Female	G3 ↓ G4 ↓	7.40 ± 0.71 7.70 ± 1.11 (10.35 ± 1.78)	Not dose dependent; Within HCR of INTOX 5.14 to 8.54.	Incidental
4	TSH (mIU/L)	Male	G2 ↑ G4 ↑	4.64 ± 0.92 4.48 ± 0.28 (4.05 ± 0.29)	Not dose dependent; Within HCR of INTOX 2.06 to 4.80	Incidental
		Female	G2 ↑ G4 ↑	6.86 ± 1.54 7.06 ± 2.48 (4.68 ± 1.08)	Not dose dependent; Higher than HCR of INTOX 2.04 to 4.69.	Incidental
End of Recovery Period (Day 209)						
5	T3 (pmol/mL)	Female	G4 ↑	9.80 ± 1.68 (8.00 ± 1.86)	Slightly higher than HCR of INTOX 5.14 to 8.54. Decrease in T3 levels was observed in rats of main group which is inconsistent with recovery group rats	Incidental
6	TSH (mIU/L)	Female	G4 ↑	8.43 ± 1.57 (5.92 ± 1.33)	Higher than HCR of INTOX 2.04 to 4.69. Even control values are higher than HCR of INTOX.	Incidental

Table 7: Statistically Significant (P<0.05) Reproductive Hormone Alteration.

Sr. No.	Parameter (Unit)	Sex	**Group and Change**	**Mean ± SD Control group value is presented in parenthesis**	Remark	Conclusion
End of Treatment Period (Day 98)						
1	TST (ng/mL)	Female	G4 ↓	18.3 ± 8.4 (33.9 ± 7.8)	Not Dose dependent, Within HCR of INTOX 10.76 to 21.82.	Incidental
2	OE (pg/mL)	Female	G4 ↓	1363 ± 664 (1999 ± 205)	Not Dose dependent, Higher than HCR of INTOX 491.57 to 1179.53. Even control group value is higher than HCR.	Incidental
3	LH (ng/mL)	Female	G4 ↑	23.9 ± 2.9 (15.8 ± 3.0)	Although G2 (18.8 ± 7.8) & G3 (19.4 ± 2.4) value not statistically significant, increase in LH levels is dose dependent; Higher (51%) than control. Not Dose dependent, Within HCR of INTOX 7.31 to 32.63.	Inconsistent with co-relating findings, no histopathological or sperm parameters hence considered as toxicologically insignificant
End of Treatment Period (Day 181/182)						
4	TST (ng/mL)	Male	G2 ↓ G3 ↓ G4 ↓	41.9 ± 15.3 41.4 ± 19.5 33.9 ± 13.0 (56.3 ± 19.6)	Not Dose dependent, Within HCR of INTOX 19.09 to 47.82. Control group values is higher than HCR.	Incidental
5	FSH (mIU/mL)	Male	G4 ↓	40.9 ± 8.9 (60.5 ± 15.3)	Although G2 (57.061 ± 10.200) & G3 (54.373 ± 15.590) value not statistically significant, decrease in LH levels is dose dependent; Lower (32%) than control. Not Dose dependent, Within HCR of INTOX 7.31 to 32.63.	Inconsistent with co-relating findings, hence considered as toxicologically insignificant
		Female	G4 ↓	66.8 ± 10.1 (57.9 ± 12.2)	Not Dose dependent, Within HCR of INTOX 56.12 to 131.58.	Incidental
6	LH (ng/mL)	Male	G2 ↓	7.6 ± 4.3 (10.9 ± 4.0)	Not Dose dependent, Lower than HCR of INTOX 10.86 to 27.16.	Incidental
End of Recovery Period (Day 209)						
7	OE (pg/mL)	Female	G4 ↓	1592 ± 111 (1864 ± 261)	Not Dose dependent, Higher than HCR of INTOX 491.57 to 1179.53. Even control group value is higher than HCR.	Incidental

A summary of stages of oestrus cycle of all females were determined at necropsy by taking vaginal smears as tabulated below. These observations aided interpretation of the histological evaluation of oestrogen-sensitive tissues. No adverse effects were observed at the end of the treatment and recovery periods.

Oral administration of *A conyzoides* extract to male rats at dose of up to 2000 mg/kg/day for 180 days, did not affect sperm concentration, motility and morphology. Sperm motility parameters including motile sperms, immotile sperms, total sperms, motility rate (%), straight line velocity (VSL-µm/sec), average path velocity (VAP- µm/sec), curvilinear velocity (VCL- µm/sec), linearity, straightness, amplitude of lateral head displacement (ALH- µm) and beat cross frequency (BCF) did not differ significantly from the vehicle control group values (P>0.05). Statistically significant (p<0.05) changes were observed in several parameters (sperm concentrations, motile sperms, total sperms, motility rate (%), curvilinear velocity (VCL- µm/sec), linearity, straightness and amplitude of lateral head displacement (ALH- µm)) but only at the end of the recovery period. These changes were considered to be incidental.

Necropsy examinations conducted at termination of the study, and the microscopic examination of all tissues /organs of all vehicle control group rats and all rats treated with *A conyzoides* extract at the dose level of 2000 mg/kg/day(G4), showed no gross pathological and histopathological alterations suggestive of systemic toxicity.

Table 8: Statistically Significant (P<0.05) Organ weight Alterations.

Sr. No.	Parameter (Unit)	Sex	Group and Change	**Mean ± SD Control group value is presented in parenthesis**	Remark	Conclusion
End of Treatment Period (Day 181/182)						
1	Spleen Absolute Weight (g)	Male	G4 ↑	1.24 ± 0.14 (1.10 ± 0.14)	Although G2 (1.11 ± 0.15) & G3 (1.16 ± 0.13) values are not statistically significant increase in spleen weight (0% to 12%) is dose dependent. Within HCR of INTOX 0.46 to 1.27.	Within Biological Limits, Toxicologically Insignificant
2	Thyroid Absolute Weight (g)	Male	G3 ↓ G4 ↓	0.032 ± 0.006 0.032 ± 0.007 (0.038 ±0.007)	Although G2 (0.036 ± 0.004) value is not statistically significant decrease (5% to 16%) in thyroid weight is dose dependent. Within HCR of INTOX 0.023 to 0.039.	Within Biological Limits, Toxicologically Insignificant
		Female	G2 ↓ G3 ↓	0.031 ± 0.004 0.031 ± 0.006 (0.036 ±0.008)	No dose dependency. Within HCR of INTOX 0.018 to 0.042.	Incidental
3	Testes Relative Weight (%)	Male	G4 ↑	0.76 ± 0.08 (0.68 ± 0.06)	Although G2 (0.70 ± 0.10) & G3 (0.71 ± 0.08) values are not statistically significant increase in testes weight (3% to 12%) is dose dependent. Within HCR of INTOX 0.54 to 1.17.	Within Biological Limits, Toxicologically Insignificant
4	Kidney Relative Weight (%)	Male	G4 ↑	0.70 ± 0.07 (0.63 ± 0.06)	Although G2 (0.64 ± 0.07) & G3 (0.66 ± 0.08) values are not statistically significant increase in kidney weight (2% to 11%) is dose dependent. Within HCR of INTOX 0.53 to 0.78.	Within Biological Limits, Toxicologically Insignificant
5	Liver Relative Weight (%)	Male	G3 ↑ G4 ↑	3.04 ± 0.23 3.05 ± 0.22 (2.77 ± 0.23)	Although G2 (2.88 ± 0.24) value is not statistically significant, increase (5% to 16%) in liver weight is dose dependent. Within HCR of INTOX 2.15 to 3.38.	Induced but Non-Averse. Attributed as an adaptive response of liver for the metabolism of test item.
		Female	G4 ↑	3.40 ± 0.32 (3.05 ± 0.42)	Although G2 (3.13 ± 0.41) & G3 (3.16 ± 0.42) value is not statistically significant, increase (3% to 11%) in liver weight is dose dependent. Within HCR of INTOX 2.14 to 3.53. Correlated with hepatocyte hypertrophy observed in females of high dose group.	
6	Spleen Relative Weight (%)	Male	G4 ↑	0.21 ± 0.02 (0.18 ± 0.02)	Although G2 (0.18 ± 0.03) & G3 (0.19 ± 0.03) value is not statistically significant, increase (0% to 17%) in spleen weight is dose dependent. Within HCR of INTOX 0.13 to 0.26.	Within Biological Limits, Toxicologically Insignificant
7	Thyroid Relative Weight (%)	Female	G2 ↓ G3 ↓	0.010 ± 0.001 0.009 ± 0.002 (0.012 ± 0.003)	No dose dependency. Within HCR of INTOX 0.006 to 0.016.	Incidental
End of Recovery Period (Day 209)						
8	Spleen Absolute weight	Female	G4 ↑	0.78 ± 0.15 (0.64 ± 0.13)	No changes seen in rats of main group. Within HCR of INTOX 0.42 to 0.89.	Incidental
9	Liver Relative Weight (%)	Male	G4 ↑	2.90 ± 0.27 (2.57 ± 0.33)	Within HCR of INTOX 2.15 to 3.38.	Recovery in adaptive response Toxicologically Insignificant
10	Heart Relative Weight%	Male	G4 ↑	0.34 ± 0.04 (0.30 ± 0.04)	No changes seen in rats of main group. Within HCR of INTOX 0.42 to 0.89	Incidental
11	Epididymides Relative Weight(%)	Male	G4 ↑	0.31 ± 0.03 (0.28 ± 0.02)	No changes seen in rats of main group. Within HCR of INTOX 0.22 to 0.47.	Incidental
12	Spleen Relative Weight(%)	Female	G4 ↑	0.24 ± 0.03 (0.20 ± 0.03)	No changes seen in rats of main group. Within HCR of INTOX 0.13 to 0.26.	Incidental

↑ : Mean value is higher (↑) than the control group mean at P<0.05. # Control group mean is presented in parenthesis;
HCR - Historical control range in the test facility for this strain and source of rat.

Table 9: Terminal Vaginal Cytology.

Stage of Oestrus Cycle	Number of Females in Respective Stage of the Oestrus Cycle					
	On day-181/182 (End of Treatment)				On day-209 (End of Recovery Period)	
	G1 Vehicle Control	G2	G3	G4	G1(R)	G4(R)
No of Females	20	20	20	20	10	10
Proestrus	4	6	4	3	1	3
Oestrus	9	3	8	6	3	1
Metestrus	3	4	3	4	3	3
Diestrus	4	7	5	7	3	3

Male Rats

Day of Termination: 181/182

Group	Dose mg/kg/day		Motile (Count)	Immotaile (Count)	Total (Count)	Conentration (M/mL)	Motile (%)	VSL (M/m/s)	VCL (M/m/s)	VAP (M/m/s)	LIN	STR	ALH (M/m)	BCF (Hz)
Vehicle Control - Analytical grade water														
G1	0	Mean	2288.70	223.20	2511.90	209.48	86.67	11.85	50.10	26.45	0.26	0.45	1.89	9.16
		S.D.	1619.04	229.17	1601.28	133.54	11.69	3.00	20.66	9.00	0.10	0.13	1.30	0.39
		n	10	10	10	10	10	10	10	10	10	10	10	10
Test Item: *Ageratum conyzoides* Extract														
G2	500	Mean	1724.10	221.00	1945.10	162.21	81.25	9.94	47.95	24.11	0.21	0.39	1.96	9.37
		S.D.	1555.23	179.20	1601.09	133.52	16.24	3.50	17.41	10.69	0.06	0.11	1.03	0.22
		n	10	10	10	10	10	10	10	10	10	10	10	10
G3	1000	Mean	1637.40	396.10	2033.50	169.59	85.76	10.79	51.63	28.12	0.19	0.35	2.05	8.99
		S.D.	1131.51	751.44	1595.35	133.05	14.02	4.94	19.87	12.22	0.05	0.03	1.01	1.32
		n	10	10	10	10	10	10	10	10	10	10	10	10
G4	2000	Mean	1505.20	183.40	1688.60	140.82	87.08	12.71	60.82	32.74	0.20	0.36	2.57	9.29
		S.D.	1181.68	213.28	1213.74	101.22	9.67	4.15	14.07	7.82	0.05	0.05	0.90	0.34
		n	10	10	10	10	10	10	10	10	10	10	10	10

Mean values for treated groups do not differ significantly ($p > 0.05$) from those of vehicle control group

Table 10: Summary of Assessment of Sperm Parameters.

Male Rats

Day of Termination: 209

Group	Dose mg/kg/day		Motile (Count)	Immotaile (Count)	Total (Count)	Conentration (M/mL)	Motile Rate (%)	VSL (M/m/s)	VCL (M/m/s)	VAP (M/m/s)	LIN	STR	ALH (M/m)	BCF (Hz)
Vehicle Control - Analytical grade water														
G1	0	Mean	3203.20	90.60	3293.80	274.69	93.22	12.88	41.72	25.14	0.32	0.50	1.17	9.34
		S.D.	1396.78	174.18	1231.92	102.73	14.39	2.17	8.53	3.80	0.10	0.13	0.84	0.21
		n	5	5	5	5	5	5	5	5	5	5	5	5
Test Item: Ageratum conyzoides Extract														
G4	2000	Mean	860.80	322.40	**1183.20**$^{S-}$	**98.68**$^{S-}$	72.37	10.42	54.98	28.41	**0.18**$^{S-}$	**0.33**$^{S-}$	2.66	9.27
		S.D.	215.77	140.46	191.16	15.94	13.03	1.01	3.41	1.38	0.02	0.02	0.27	0.46
		n	5	5	5	5	5	5	5	5	5	5	5	5

S-: Mean values of treated groups significantly lower ($P < 0.05$) from those of the vehicle control group.

Table 10: Summary of Assessment of Sperm Parameters (Contd.)

Male Rats

Day of Termination: 181/182

Group	G1			G2			G3			G4		
Dose (mg/kg)	0			500			1000			2000		
No. of Males Examined	10			10			9			10		
No. of Sperms Examined	2000			2000			1800			2000		
Observations	Incidence			Incidence			Incidence			Incidence		
	Absolute	% of abnormality	No. of males affected	Absolute	% of abnormality	No. of males affected	Absolute	% of abnormality	No. of males affected	Absolute	% of abnormality	No. of males affected
Normal	1966	-	-	1975	-	-	1771	-	-	1976	-	-
Head												
Blunt head	1	0.05	1	1	0.05	1	2	0.11	2	-	-	-
Fused head	-	-	-	-	-	-	-	-	-	-	-	-
Short/Narrow head	-	-	-	-	-	-	-	-	-	-	-	-
Round head	-	-	-	-	-	-	-	-	-	-	-	-
Double head	-	-	-	-	-	-	-	-	-	-	-	-
Straight head	-	-	-	-	-	-	-	-	-	-	-	-
Misshapen head	-	-	-	-	-	-	-	-	-	-	-	-
Mid piece	-	-	-	-	-	-	-	-	-			
Thick neck	-	-	-	-	-	-	-	-	-	-	-	-
Fused neck	-	-	-	-	-	-	-	-	-	-	-	-
Coild neck	-	-	-	-	-	-	-	-	-	-	-	-
Fused head and neck	-	-	-	-	-	-	-	-	-	-	-	-
Flagella												
Coiled tail/ flagellum	12	0.61	9	19	0.96	10	18	1.02	9	16	0.81	9
Double flagellum / tail	-	-	-	-	-	-	-	-	-	-	-	-
Bent neck or tail	20	1.02	10	5	0.25	4	9	0.51	6	8	0.40	5
Bifurcated tail	1	0.05	1	-	-	-	-	-	-	-	-	-
Misshapen head	-	-	-	-	-	-	-	-	-	-	-	-

Note: Sperm morphology was not performed in rat Rm1169 due to aspermia attributed to degenerative changes in testes.

Table 11: Summary of Sperm Morphology Findings.

Male Rats

Day of Termination: 209

Group	G1			G4		
Dose (mg/kg)	0			2000		
No. of Males Examined	5			5		
No. of Sperms Examined	1000			1000		
Observations	Incidence			Incidence		
	Absolute	% of abnormality	No. of males affected	Absolute	% of abnormality	No. of males affected
Normal	988	-	-	986	-	-
Head						
Blunt head	-	-	-	-	-	-
Fused head	-	-	-	-	-	-
Short/Narrow head	-	-	-	-	-	-
Round head	-	-	-	-	-	-
Double head	-	-	-	-	-	-
Straight head	-	-	-	-	-	-
Misshapen head	-	-	-	-	-	-
Mid piece				-	-	-
Thick neck	-	-	-	-	-	-
Fused neck	-	-	-	-	-	-
Coild neck	1	0.10	1	-	-	-
Fused head and neck	-	-	-	-	-	-
Flagella						
Coiled tail/ flagellum	5	0.51	4	9	0.91	5
Double flagellum / tail	-	-	-	-	-	-
Bent neck or tail	6	0.61	4	5	0.51	5
Bifurcated tail	-	-	-	-	-	-
Misshapen head	-	-	-	-	-	-

Table 11: Summary of Sperm Morphology Findings(Contd.).

Fate: Terminal Sacrifice

Time: Termination of Treatment Period (Day 181 & 182)

Group	G1	G2	G3	G4
	Vehicle control	*Ageratum conyzoides* Extract		
Dose (mg/kg b. wt./day)	0	500	1000	2000
Findings	Incidence (No. of animals with findings)			
MALE RATS				
Number of Rats examined	20	20	20	20
No abnormality detected	18	20	19	20
Testes				
Small, soft, bilateral	-	-	1	-
Epididymides				
Nodule, yellow, right	-	-	1	-
Eyes				
Discoloration, lens, grey, bilateral, diffuse, minimal	1	-	-	-
Thorax- Right				
Mass, soft, lateral	1	-	-	-
FEMALE RATS				
Number of Rats examined	20	20	20	20
No abnormality detected	19	19	18	19
Kidneys				
Cyst, cortex, unilateral	-	-	1	-
Ovaries				
Enlarged, yellow, right, diffuse, moderate	-	1	-	1
Uterus				
Enlarged, yellow, bilateral, diffuse, minimal / moderate	1	1	2	1
Mammary glands				
Enlarged, inguinal, soft, right, single, moderate	1	-	-	-

Table 12: Summary of Necropsy Findings.

Fate: Terminal Sacrifice

Time: Termination of Recovery Period (Day 209)

Group	G1-R	G4-R
	Vehicle control	***Ageratum conyzoides* Extract**
Dose (mg/kg b. wt./day)	0	2000
Findings	Incidence (No. of animals with findings)	
MALE RATS		
Number of Rats examined	10	10
No abnormality detected	10	10
FEMALE RATS		
Number of Rats examined	10	10
No abnormality detected	10	9
Ovary		
Mass, Para ovarian, yellow	-	1

Table 12: Summary of Sperm Morphology Findings(Contd.)

Primary indicators of immune toxicity and other data from the study indicated that A conyzoides extract, at the doses used, did not affect the immune system of rats during the treatment and recovery periods.

Primary indicators of immune toxicity are basic Type 1 Tests, and were derived from routine measurements and examinations performed during the study including haematology and serum chemistry profiles, routine histopathology examinations, and organ and body weight measurements.

Haematology Indicators

As determined at termination of the treatment and recovery periods, the treatment of male and female rats with *A conyzoides* extract at doses up to 2000 mg/kg did not affect white blood cell (WBC) counts, activated partial thromboplastin time, differential WBC counts, neutrophils, monocytes, basophils, RBC indices, mean corpuscular haemoglobin and mean corpuscular volume.

Clinical Chemistry Indicators

As determined at termination of the treatment and recovery periods, treatment of male and female rats with *A conyzoides* extract at doses up to 2000 mg/kg did not affect total serum protein, albumin-to-globulin (A/G) ratio, globulin, and the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Histopathology Indicators

At termination of the 180-day treatment period, no abnormalities were found during gross and routine histological evaluation of the lymphoid tissues: spleen, lymph nodes, thymus, bone marrow, GALT and Peyer's patches. *A conyzoides* extract did not stimulate cell proliferation or cause atrophy and cell depletion in any lymphoid organ.

Organ and Body Weight Indicators

At termination of the treatment and the recovery periods, there were no treatment-related changes in spleen and thymus weights and elevated or depressed organ-to-body-weight ratios for these tissues. There were no changes in body weight, an indicator of endocrine function which may also indicate indirect immunotoxic effects.

Indicators From Other Toxicity Data

The absence of clinical and behavioural abnormalities provided further evidence of a lack of immunological damage.

AMES test

A conyzoides extract, when exposed to the tester strains, did not induce cytotoxicity in any of the tester strains TA1535, TA97a, TA98, TA100 and TA102 when tested at and up to the highest dose of 5000 µg/plate, both in the presence and absence of Liver S9.

TABLE 1

EXPERIMENT NO.: 1 SUMMARY DATA ON HISTIDINE REVERTANT COLONIES

Treatment	Concentration		TA1535		TA97a		TA98		TA100		TA102	
	(µg/plate)	S9*	Mean	± S.D.	Mean	± S.D.	Mean	± S.D.	Mean	± S.D.	Mean	± S.D.
Ageratum conyzoides extract powder	5000	–	12.00	1.00	106.67	10.07	44.00	4.58	142.67	24.19	266.67	12.22
		+	11.00	2.00	101.33	8.33	49.00	10.58	99.33	1.15	258.67	14.05
	1500	–	14.00	3.61	107.33	12.06	45.67	4.16	118.00	23.58	257.33	14.05
		+	11.67	1.53	104.67	8.08	44.33	3.21	113.33	12.70	256.00	8.00
	500	–	13.67	7.57	101.33	10.26	45.00	4.58	119.33	21.94	268.00	12.00
		+	11.33	0.58	117.33	11.55	41.33	13.32	118.00	9.17	249.33	8.33
	150	–	13.33	4.51	102.67	5.77	46.33	2.52	110.67	10.26	284.00	10.58
		+	13.67	0.58	118.00	5.29	42.00	8.54	122.67	12.70	249.33	9.24
	50	–	13.00	3.61	102.67	17.01	40.33	2.31	96.67	5.03	257.33	8.33
		+	14.67	3.51	116.00	5.29	41.67	2.52	115.33	13.01	253.33	8.33
	15	–	14.00	1.73	91.33	2.31	38.67	2.08	102.67	12.86	257.33	14.05
		+	9.67	8.62	121.33	10.26	40.00	11.79	96.00	2.00	268.00	6.93
Vehicle Control												
Ethanol	100 µL	–	18.00	3.00	120.00	20.78	47.33	7.09	164.00	34.87	300.00	12.00
		+	16.67	1.53	117.33	11.02	45.00	5.29	118.00	25.53	282.67	4.62
Positive Controls												
Sodium azide	2	–	498.67	41.05	-	-	-	-	-	-	-	-
ICR 191	1	–	-	-	1077.33	36.07	-	-	-	-	-	-
4-Nitroquinoline-N-oxide	0.5	–	-	-	-	-	954.67	53.27	-	-	-	-
3-Methylmethane Sulphonate	1 µL	–	-	-	-	-	-	-	781.33	274.15	1984.00	60.40
2-Aminoanthracene	10	+	504.00	83.52	-	-	-	-	-	-	-	-
2-Aminofluorene	20	+	-	-	901.33	40.27	570.67	149.74	1008.00	84.66	-	-
Danthron	30	+	-	-	-	-	-	-	-	-	1528.00	236.37

Table 12: Experiment No.1 Summary Data On Histidine Revertant Colonies.

Treatment	Concentration		TA1535		TA97a		TA98		TA100		TA102	
	(µg/plate)	S9*	Mean	± S.D.	Mean	± S.D.	Mean	± S.D.	Mean	± S.D.	Mean	± S.D.
Ageratum conyzoides extract powder	5000	–	14.67	4.51	109.33	9.87	38.33	3.51	136.67	8.08	284.00	6.93
		+	11.33	2.08	120.67	17.24	42.33	6.66	122.67	4.16	270.67	12.86
	1500	–	11.00	1.73	124.00	16.37	39.33	8.50	136.67	14.47	286.67	18.04
		+	14.67	3.06	115.33	15.01	39.33	3.06	150.00	9.17	258.67	26.63
	500	–	13.33	2.52	122.67	11.02	42.33	6.03	142.00	10.58	288.00	27.71
		+	9.33	4.16	126.67	14.47	37.33	4.04	132.67	5.03	266.67	6.11
	150	–	12.00	2.00	136.67	3.06	40.67	3.21	132.67	22.03	258.67	12.22
		+	14.67	3.51	124.00	5.29	46.67	3.06	146.67	17.01	280.00	13.86
	50	–	11.67	3.79	136.00	8.00	43.00	3.61	146.67	8.08	256.00	22.27
		+	13.67	2.08	113.33	31.77	41.00	2.65	137.33	20.53	274.67	18.04
	15	–	12.33	5.51	134.00	13.11	41.00	5.20	146.00	12.49	258.67	18.90
		+	9.67	2.52	136.00	8.00	42.67	3.06	137.33	13.32	270.67	32.08
Vehicle Control												
Ethanol	100 µL	–	18.00	2.00	142.00	8.72	38.67	3.51	146.00	8.00	286.67	30.02
		+	17.00	2.65	144.67	7.02	39.33	3.06	146.00	19.08	286.67	33.31
Positive Controls												
Sodium azide	2	–	640.00	90.86	-	-	-	-	-	-	-	-
ICR 191	1	–	-	-	944.00	44.54	-	-	-	-	-	-
4-Nitroquinoline-N-oxide	0.5	–	-	-	-	-	717.33	40.27	-	-	-	-
3-Methylmethane Sulphonate	1 µL	–	-	-	-	-	-	-	994.67	132.99	1552.00	209.69
2-Aminoanthracene	10	+	621.33	100.03	-	-	-	-	-	-	-	-
2-Aminofluorene	20	+	-	-	1192.00	346.78	738.67	28.10	997.33	93.75	-	-
Danthron	30	+	-	-	-	-	-	-	-	-	1394.67	56.76

Table 13: Experiment No.2 Summary Data On Histidine Revertant Colonies.

INDIVIDUAL PLATE COUNT OF HISTIDINE REVERTANT COLONIES - (PRE INCUBATION METHOD)

Strain : TA1535 Experiment No. : 1

Treatment	(-) Without Metabolic Activation										(+) With Metabolic Activation									
	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control
Ageratum conyzoides extract powder	5000	11	12	13	4+	4+	4+	12.00	1.00	0.67	5000	9	13	11	4+	4+	4+	11.00	2.00	0.66
	1500	11	13	18	4+	4+	4+	14.00	3.61	0.78	1500	12	10	13	4+	4+	4+	11.67	1.53	0.70
	500	19	5	17	4+	4+	4+	13.67	7.57	0.76	500	11	12	11	4+	4+	4+	11.33	0.58	0.68
	150	9	13	18	4+	4+	4+	13.33	4.51	0.74	150	13	14	14	4+	4+	4+	13.67	0.58	0.82
	50	17	12	10	4+	4+	4+	13.00	3.61	0.72	50	11	15	18	4+	4+	4+	14.67	3.51	0.88
	15	12	15	15	4+	4+	4+	14.00	1.73	0.78	15	19	2	8	4+	4+	4+	9.67	8.62	0.58
Vehicle Control																				
Ethanol	100 µL	21	15	18	4+	4+	4+	18.00	3.00	-	100 µL	18	17	15	4+	4+	4+	16.67	1.53	-
Positive Control (PC)*																				
PC	2	464	488	544	4+	4+	4+	498.67	41.05	27.70	10	544	408	560	4+	4+	4+	504.00	83.52	30.24

*Positive Controls - Sodium Azide (-), 2- Aminoanthracene (+) # Bacterial Background Lawn Evaluation Codes: 4+: Thick lawn (Normal)

Multiples of vehicle control = Mean number of colonies at the specific concentration/ Mean number of colonies of vehicle control

For 5000µg/plate, without S9 mix

Multiples of vehicle control = 12.00/ 18.00 = 0.67

Table 14: Experiment No.1 Individual Plate Count of Histidine Revertant Colonies (Pre-incubation method) [TA1535].

Strain : TA97a Experiment No. : 1

Treatment	(-) Without Metabolic Activation										(+) With Metabolic Activation									
	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control
Ageratum conyzoides extract powder	5000	96	116	108	4+	4+	4+	106.67	10.07	0.89	5000	108	92	104	4+	4+	4+	101.33	8.33	0.86
	1500	120	106	96	4+	4+	4+	107.33	12.06	0.89	1500	114	100	100	4+	4+	4+	104.67	8.08	0.89
	500	110	90	104	4+	4+	4+	101.33	10.26	0.84	500	124	124	104	4+	4+	4+	117.33	11.55	1.00
	150	106	96	106	4+	4+	4+	102.67	5.77	0.86	150	122	112	120	4+	4+	4+	118.00	5.29	1.01
	50	122	90	96	4+	4+	4+	102.67	17.01	0.86	50	120	110	118	4+	4+	4+	116.00	5.29	0.99
	15	90	94	90	4+	4+	4+	91.33	2.31	0.76	15	130	124	110	4+	4+	4+	121.33	10.26	1.03
Vehicle Control																				
Ethanol	100 µL	132	96	132	4+	4+	4+	120.00	20.78	-	100 µL	112	110	130	4+	4+	4+	117.33	11.02	-
Positive Control (PC)*																				
PC	1	1112	1080	1040	4+	4+	4+	1077.33	36.07	8.98	20	944	896	864	4+	4+	4+	901.33	40.27	7.68

*Positive Controls - ICR-191 (-), 2-Aminofluorene (+)

Bacterial Background Lawn Evaluation Codes: 4+: Thick lawn (Normal)

Table 15: Experiment No.1 Individual Plate Count of Histidine Revertant Colonies (Pre-incubation method) [TA97a].

INDIVIDUAL PLATE COUNT OF HISTIDINE REVERTANT COLONIES - (PRE INCUBATION METHOD)

Strain : TA98 Experiment No. : 1

Treatment	(-) Without Metabolic Activation										(+) With Metabolic Activation									
	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control
Ageratum conyzoides extract powder	5000	48	45	39	4+	4+	4+	44.00	4.58	0.93	5000	45	41	61	4+	4+	4+	49.00	10.58	1.09
	1500	41	49	47	4+	4+	4+	45.67	4.16	0.96	1500	48	42	43	4+	4+	4+	44.33	3.21	0.99
	500	49	40	46	4+	4+	4+	45.00	4.58	0.95	500	26	48	50	4+	4+	4+	41.33	13.32	0.92
	150	44	49	46	4+	4+	4+	46.33	2.52	0.98	150	51	41	34	4+	4+	4+	42.00	8.54	0.93
	50	39	43	39	4+	4+	4+	40.33	2.31	0.85	50	39	44	42	4+	4+	4+	41.67	2.52	0.93
	15	37	41	38	4+	4+	4+	38.67	2.08	0.82	15	53	37	30	4+	4+	4+	40.00	11.79	0.89
Vehicle Control																				
Ethanol	100 µL	55	46	41	4+	4+	4+	47.33	7.09	-	100 µL	39	49	47	4+	4+	4+	45.00	5.29	-
Positive Control (PC)*																				
PC	0.5	1000	968	896	4+	4+	4+	954.67	53.27	20.17	20	400	680	632	4+	4+	4+	570.67	149.74	12.68

*Positive Controls – 4-Nitroquinoline-N-Oxide (-), 2-Aminofluorene (+)
Bacterial Background Lawn Evaluation Codes: 4+: Thick lawn (Normal)

Table 16: Experiment No.1 Individual Plate Count of Histidine Revertant Colonies (Pre-incubation method) [TA98].

INDIVIDUAL PLATE COUNT OF HISTIDINE REVERTANT COLONIES - (PRE INCUBATION METHOD)

Strain : TA100 Experiment No. : 1

Treatment	(-) Without Metabolic Activation										(+) With Metabolic Activation									
	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control
Ageratum conyzoides extract powder	5000	124	170	134	4+	4+	4+	142.67	24.19	0.87	5000	98	100	100	4+	4+	4+	99.33	1.15	0.84
	1500	92	138	124	4+	4+	4+	118.00	23.58	0.72	1500	128	106	106	4+	4+	4+	113.33	12.70	0.96
	500	94	132	132	4+	4+	4+	119.33	21.94	0.73	500	108	120	126	4+	4+	4+	118.00	9.17	1.00
	150	102	122	108	4+	4+	4+	110.67	10.26	0.67	150	130	108	130	4+	4+	4+	122.67	12.70	1.04
	50	92	102	96	4+	4+	4+	96.67	5.03	0.59	50	128	116	102	4+	4+	4+	115.33	13.01	0.98
	15	112	108	88	4+	4+	4+	102.67	12.86	0.63	15	98	96	94	4+	4+	4+	96.00	2.00	0.81
Vehicle Control																				
Ethanol	100 µL	188	180	124	4+	4+	4+	164.00	34.87	-	100 µL	146	96	112	4+	4+	4+	118.00	25.53	-
Positive Control (PC)*																				
PC	1 µL	696	560	1088	4+	4+	4+	781.33	274.15	4.76	20	976	1104	944	4+	4+	4+	1008.00	84.66	8.54

*Positive Controls – 3- Methyl Methane Sulphonate (-), 2-Aminofluorene (+)
Bacterial Background Lawn Evaluation Codes: 4+: Thick lawn (Normal)

Table 17: Experiment No.1 Individual Plate Count of Histidine Revertant Colonies (Pre-incubation method) [TA100].

INDIVIDUAL PLATE COUNT OF HISTIDINE REVERTANT COLONIES - (PRE INCUBATION METHOD)

Strain : TA102 Experiment No. : 1

Treatment	(-) Without Metabolic Activation										(+) With Metabolic Activation									
	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control
Ageratum conyzoides extract powder	5000	256	280	264	4+	4+	4+	266.67	12.22	0.89	5000	272	244	260	4+	4+	4+	258.67	14.05	0.92
	1500	256	272	244	4+	4+	4+	257.33	14.05	0.86	1500	264	256	248	4+	4+	4+	256.00	8.00	0.91
	500	280	256	268	4+	4+	4+	268.00	12.00	0.89	500	252	256	240	4+	4+	4+	249.33	8.33	0.88
	150	276	280	296	4+	4+	4+	284.00	10.58	0.95	150	244	260	244	4+	4+	4+	249.33	9.24	0.88
	50	248	264	260	4+	4+	4+	257.33	8.33	0.86	50	244	256	260	4+	4+	4+	253.33	8.33	0.90
	15	244	256	272	4+	4+	4+	257.33	14.05	0.86	15	272	260	272	4+	4+	4+	268.00	6.93	0.95
Vehicle Control																				
Ethanol	100 µL	312	288	300	4+	4+	4+	300.00	12.00	-	100 µL	280	288	280	4+	4+	4+	282.67	4.62	-
Positive Control (PC)*																				
PC	1 µL	1920	2040	1992	4+	4+	4+	1984.00	60.40	6.61	30	1264	1600	1720	4+	4+	4+	1528.00	236.37	5.41

*Positive Controls – 3- Methyl Methane Sulphonate (-), Danthron (+)
Bacterial Background Lawn Evaluation Codes: 4+: Thick lawn (Normal)

Table 18: Experiment No.1 Individual Plate Count of Histidine Revertant Colonies (Pre-incubation method) [TA102].

INDIVIDUAL PLATE COUNT OF HISTIDINE REVERTANT COLONIES - (PRE INCUBATION METHOD)

Strain : TA1535 Experiment No. : 2

Treatment	(-) Without Metabolic Activation										(+) With Metabolic Activation									
	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control
Ageratum conyzoides extract powder	5000	19	15	10	4+	4+	4+	14.67	4.51	0.81	5000	13	9	12	4+	4+	4+	11.33	2.08	0.67
	1500	12	12	9	4+	4+	4+	11.00	1.73	0.61	1500	12	14	18	4+	4+	4+	14.67	3.06	0.86
	500	13	11	16	4+	4+	4+	13.33	2.52	0.74	500	8	6	14	4+	4+	4+	9.33	4.16	0.55
	150	14	10	12	4+	4+	4+	12.00	2.00	0.67	150	11	15	18	4+	4+	4+	14.67	3.51	0.86
	50	10	9	16	4+	4+	4+	11.67	3.79	0.65	50	16	12	13	4+	4+	4+	13.67	2.08	0.80
	15	7	12	18	4+	4+	4+	12.33	5.51	0.69	15	10	12	7	4+	4+	4+	9.67	2.52	0.57
Vehicle Control																				
Ethanol	100 µL	20	18	16	4+	4+	4+	18.00	2.00	-	100 µL	15	20	16	4+	4+	4+	17.00	2.65	-
Positive Control (PC)*																				
PC	2	744	576	600	4+	4+	4+	640.00	90.86	35.56	10	576	552	736	4+	4+	4+	621.33	100.03	36.55

*Positive Controls - Sodium Azide (-), 2- Aminoanthracene (+)
Bacterial Background Lawn Evaluation Codes: 4+: Thick lawn (Normal)

Table 19: Experiment No.2 Individual Plate Count of Histidine Revertant Colonies (Pre-incubation method) [TA1535].

INDIVIDUAL PLATE COUNT OF HISTIDINE REVERTANT COLONIES - (PRE INCUBATION METHOD)

Strain : TA97a Experiment No. : 2

Treatment	(-) Without Metabolic Activation										(+) With Metabolic Activation									
	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control
Ageratum conyzoides extract powder	5000	116	98	114	4+	4+	4+	109.33	9.87	0.77	5000	102	124	136	4+	4+	4+	120.67	17.24	0.83
	1500	128	106	138	4+	4+	4+	124.00	16.37	0.87	1500	124	124	98	4+	4+	4+	115.33	15.01	0.80
	500	128	110	130	4+	4+	4+	122.67	11.02	0.86	500	134	136	110	4+	4+	4+	126.67	14.47	0.88
	150	140	134	136	4+	4+	4+	136.67	3.06	0.96	150	118	128	126	4+	4+	4+	124.00	5.29	0.86
	50	136	144	128	4+	4+	4+	136.00	8.00	0.96	50	96	150	94	4+	4+	4+	113.33	31.77	0.78
	15	136	146	120	4+	4+	4+	134.00	13.11	0.94	15	136	144	128	4+	4+	4+	136.00	8.00	0.94
Vehicle Control																				
Ethanol	100 µL	152	136	138	4+	4+	4+	142.00	8.72	-	100 µL	138	144	152	4+	4+	4+	144.67	7.02	-
Positive Control (PC)*																				
PC	1	984	896	952	4+	4+	4+	944.00	44.54	6.65	20	1592	1008	976	4+	4+	4+	1192.00	346.78	8.24

*Positive Controls - ICR-191 (-), 2-Aminofluorene (+)
Bacterial Background Lawn Evaluation Codes: 4+: Thick lawn (Normal)

Table 20: Experiment No.2 Individual Plate Count of Histidine Revertant Colonies (Pre-incubation method) [TA97a].

INDIVIDUAL PLATE COUNT OF HISTIDINE REVERTANT COLONIES - (PRE INCUBATION METHOD)

Strain : TA98 Experiment No. : 2

Treatment	(-) Without Metabolic Activation										(+) With Metabolic Activation									
	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control
Ageratum conyzoides extract powder	5000	38	42	35	4+	4+	4+	38.33	3.51	0.99	5000	44	48	35	4+	4+	4+	42.33	6.66	1.08
	1500	48	39	31	4+	4+	4+	39.33	8.50	1.02	1500	36	42	40	4+	4+	4+	39.33	3.06	1.00
	500	48	43	36	4+	4+	4+	42.33	6.03	1.09	500	41	38	33	4+	4+	4+	37.33	4.04	0.95
	150	37	42	43	4+	4+	4+	40.67	3.21	1.05	150	44	46	50	4+	4+	4+	46.67	3.06	1.19
	50	44	39	46	4+	4+	4+	43.00	3.61	1.11	50	39	40	44	4+	4+	4+	41.00	2.65	1.04
	15	35	44	44	4+	4+	4+	41.00	5.20	1.06	15	46	40	42	4+	4+	4+	42.67	3.06	1.08
Vehicle Control																				
Ethanol	100 µL	35	42	39	4+	4+	4+	38.67	3.51	-	100 µL	40	36	42	4+	4+	4+	39.33	3.06	-
Positive Control (PC)*																				
PC	0.5	680	712	760	4+	4+	4+	717.33	40.27	18.55	20	736	712	768	4+	4+	4+	738.67	28.10	18.78

*Positive Controls – 4-Nitroquinoline-N-Oxide (-), 2-Aminofluorene (+)
Bacterial Background Lawn Evaluation Codes: 4+: Thick lawn (Normal)

Table 21: Experiment No.2 Individual Plate Count of Histidine Revertant Colonies (Pre-incubation method) [TA98].

INDIVIDUAL PLATE COUNT OF HISTIDINE REVERTANT COLONIES - (PRE INCUBATION METHOD)

Strain : TA100 Experiment No. : 2

Treatment	(-) Without Metabolic Activation										(+) With Metabolic Activation									
	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control
Ageratum conyzoides extract powder	5000	128	138	144	4+	4+	4+	136.67	8.08	0.94	5000	118	126	124	4+	4+	4+	122.67	4.16	0.84
	1500	146	120	144	4+	4+	4+	136.67	14.47	0.94	1500	158	152	140	4+	4+	4+	150.00	9.17	1.03
	500	146	130	150	4+	4+	4+	142.00	10.58	0.97	500	128	138	132	4+	4+	4+	132.67	5.03	0.91
	150	122	118	158	4+	4+	4+	132.67	22.03	0.91	150	146	130	164	4+	4+	4+	146.67	17.01	1.00
	50	138	154	148	4+	4+	4+	146.67	8.08	1.00	50	160	120	132	4+	4+	4+	137.33	20.53	0.94
	15	136	160	142	4+	4+	4+	146.00	12.49	1.00	15	146	122	144	4+	4+	4+	137.33	13.32	0.94
Vehicle Control																				
Ethanol	100 µL	138	154	146	4+	4+	4+	146.00	8.00	-	100 µL	144	166	128	4+	4+	4+	146.00	19.08	-
Positive Control (PC)*																				
PC	1 µL	1136	872	976	4+	4+	4+	994.67	132.99	6.81	20	960	928	1104	4+	4+	4+	997.33	93.75	6.83

*Positive Controls – 3- Methyl Methane Sulphonate (-), 2-Aminofluorene (+)
Bacterial Background Lawn Evaluation Codes: 4+: Thick lawn (Normal)

Table 22: Experiment No.2 Individual Plate Count of Histidine Revertant Colonies (Pre-incubation method) [TA100].

INDIVIDUAL PLATE COUNT OF HISTIDINE REVERTANT COLONIES - (PRE INCUBATION METHOD)

Strain : TA102 Experiment No. : 2

Treatment	(-) Without Metabolic Activation										(+) With Metabolic Activation									
	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control	Conc. (µg/plate)	R1	R2	R3	Bacterial Lawn #			Mean	± S. D.	Multiples of Vehicle Control
Ageratum conyzoides extract powder	5000	280	280	292	4+	4+	4+	284.00	6.93	0.99	5000	276	280	256	4+	4+	4+	270.67	12.86	0.94
	1500	304	288	268	4+	4+	4+	286.67	18.04	1.00	1500	252	288	236	4+	4+	4+	258.67	26.63	0.90
	500	304	256	304	4+	4+	4+	288.00	27.71	1.00	500	268	272	260	4+	4+	4+	266.67	6.11	0.93
	150	272	256	248	4+	4+	4+	258.67	12.22	0.90	150	288	264	288	4+	4+	4+	280.00	13.86	0.98
	50	276	232	260	4+	4+	4+	256.00	22.27	0.89	50	292	256	276	4+	4+	4+	274.67	18.04	0.96
	15	280	244	252	4+	4+	4+	258.67	18.90	0.90	15	304	268	240	4+	4+	4+	270.67	32.08	0.94
Vehicle Control																				
Ethanol	100 µL	256	288	316	4+	4+	4+	286.67	30.02	-	100 µL	324	260	276	4+	4+	4+	286.67	33.31	-
Positive Control (PC)*																				
PC	1 µL	1320	1608	1728	4+	4+	4+	1552.00	209.69	5.41	30	1384	1344	1456	4+	4+	4+	1394.67	56.76	4.87

*Positive Controls – 3- Methyl Methane Sulphonate (-), Danthron (+)
Bacterial Background Lawn Evaluation Codes: 4+: Thick lawn (Normal)

Table 23: Experiment No.2 Individual Plate Count of Histidine Revertant Colonies (Pre-incubation method) [TA102

EXPERIMENT NO. 1

Test / Control Item	Dose µg/mL	No. of Nuclei Analysed in Each Replicate	%MN Mean ± SD		RPD	%Cytotoxicity
			mean	SD		
Vehicle Control **Analytical grade water**	1% v/v	10000	1.72	0.22	-	-
Positive Control **Cyclophosphamide monohydrate**	6.25	10000	9.39*	1.57	47.6	52.4
Test Item **Ageratum conyzoides extract powder**	312.5	10000	1.56	0.19	46.8	53.2
	156.25	10000	1.57	0.10	86.6	13.4
	78.125	10000	1.19	0.37	91.6	8.4

* $p < 0.05$, SD – Standard deviation, RPD – Relative population doubling, MN – Micronucleated events

EXPERIMENT NO. 2

Test / Control Item	Dose µg/mL	No. of Nuclei Analysed in Each Replicate	%MN (mean ± SD)		RPD	%Cytotoxicity
			mean	SD		
Vehicle Control **Analytical grade water**	1% v/v	10000	1.65	0.38	-	-
Positive Control **Mitomycin C**	160 ng/mL	10000	5.52*	0.74	45.9	54.1
Test Item **Ageratum conyzoides extract powder**	312.5	10000	1.57	0.25	46.9	53.1
	156.25	10000	1.59	0.34	87.3	12.7
	78.125	10000	1.62	0.17	92.6	7.4

* $p < 0.05$, SD – Standard deviation, RPD – Relative population doubling, MN – Micronucleated events

EXPERIMENT NO. 3

Test / Control Item	Dose µg/mL	No. of Nuclei Analysed in Each Replicate	%MN (mean ± SD)		RPD	%Cytotoxicity
			mean	SD		
Vehicle Control **Analytical grade water**	1% v/v	10000	1.71	0.16	-	-
Positive Control **Vinblastine**	3.5 ng/mL	10000	8.61*	0.34	44.8	55.2
Test Item **Ageratum conyzoides extract powder**	312.5	10000	1.67	0.36	45.3	54.7
	156.25	10000	1.60	0.43	84.0	16.0
	78.125	10000	1.35	0.21	90.4	9.6

* $p < 0.05$, SD – Standard deviation, RPD – Relative population doubling, MN – Micronucleated events

Table 24: Results summary of *In Vitro* Mammalian Cell Micronucleus Test using Human Lymphoblastoid Cell Line (TK6).

The number of histidine revertant colonies in all tester strains TA1535, TA97a, TA98, TA100 and TA102 in the presence and absence of metabolic activation system were comparable with vehicle control group.

The test item did not induce any concentration-dependent increase or 2-fold (3-fold for TA1535) increase in revertant frequencies when compared with the vehicle control group, according to the criteria of evaluation of findings of this study.

Concurrent positive controls demonstrated sensitivity of the assay with and without metabolic activation.

In Vitro Mammalian Cell Micronucleus Test using Human Lymphoblastoid Cell Line (TK6)

To measure and interpret the cytotoxicity, values of relative population doubling for the vehicle control and positive control groups and the three treatment levels for each of the three experiments were observed.

For experiment No. 1 (With Metabolic Activation, 3 hours exposure), the % cytotoxicity observed was 53.2, 13.4 and 8.4% at the concentrations of 312.5, 156.25 and 78.125 µg/ml respectively. For experiment No. 2 (Without Metabolic Activation, 3 hours exposure), the % cytotoxicity observed was 53.1, 12.7 and 7.4o/o at the concentrations of 312.5, 156.25 and 78.125 µg/ml respectively. For experiment No. 3 (Without Metabolic Activation, 24 hours exposure), the % cytotoxicity observed was 54.7, 16.0 and 9.6% at the concentrations of 312.5, 156.25 and 78.125 µg/ml respectively. *A conyzoides* extract induced 53.2, 53.1 and 54.7% cytotoxicity to the TK6 cells at the highest concentration in experiment no. 1,2 and three respectively.

Comparison of the % incidence of micronuclei for each of the three experiments was recorded. In experiment no.1, the percentage incidence (Mean ± SD) of micronuclei was 1.56 ± 0.19, 1.57± 0.10 and 1.19 ± 0.37% at test concentrations of 312.5, 156.25 and 78.125 µg/ml respectively. In Experiment no. 2, the percentage incidence of micronuclei was 1.57 ± 0.25,1.59 ± 0.34 and 1.62 ± 0.17% at test concentrations of 312.5, 156.25 and 78.125 µg/mL respectively. In experiment no.3, the percentage incidence of micronuclei corresponding to these dose levels was 1.67 ± 0.36, 1.60 ± 0.43 and 1.35 ± 0.21°/o respectively at test concentrations of 312.5, 156.25 and 78.125 µg/ml. *A conyzoides* extract, with or without metabolic activation system, did not increase the incidence of micronuclei over the tested range.

Positive Controls: Sensitivity of the test system and activity of S9 mix were adequately demonstrated in the positive control group.

Discussion

This paper is the first to report on the mutagenicity and chronic toxicity of *A. conyzoides*.

In the Ames test with pre-incubation, *A. conyzoides* extract was evaluated for its ability to induce reverse mutation at selected histidine loci in five tester strains of salmonella typhimurium in the presence and absence of metabolic activation system (S9). *A conyzoides* extract did not induce cytotoxicity in any of the tester strains at doses up to 5000 µg/plate. Numbers of histidine revertant colonies in all tester strains were comparable with the vehicle control group, indicating a lack of genotoxic potential.

The *in vitro* mammalian cell micronucleus test using Human Lymphoblastoid Cell Line (TK6) was performed to evaluate the potential of the test item and/or its metabolites to cause clastogenic and aneugenic effects. TK6 cells were exposed to the test item at concentrations of up to 312.5 µg/ml with and without metabolic activation for 3 hours and 24 hours exposure. The incidence of micronuclei for each of the three experiments conducted in this study did not reveal any significant and/or dose-related increase in micronucleation, indicating an absence of clastogenic and aneugenic potential.

In the chronic oral toxicity study, *A conyzoides* Extract even at 2000 mg/kg b.w did not induce any adverse effects. The No-Observed-Adverse-Effect level (NOAEEL) of *A. conyzoides* extract in rats was therefore greater than 2000 mg/kg b.w/day.

Previous sub-chronic and acute and sub-chronic toxicity studies with *A. conyzoides* in rats concluded that hydro-alcoholic leaves extracts of the plant were relatively the safest [21]. In the acute toxicity test, rats were administered 5000 mg/kg daily for 14 days and observed 1h post-administration for 14 days. No toxicity or mortality was observed in this study. For the sub-chronic toxicity test, 500 and 1000 mg/kg b.w/day was administered orally for 28 days and relative organ weights and various haematological and biochemical parameters were assessed. There was no mortality or toxicity recorded other than an increase in liver size in the group receiving 1000 mg/kg.

While investigating the plant for anti-inflammatory and anti-coccidal properties, similar safety patterns were reported with no observed acute toxicity [22,23]. In a parallel report, a methanolic extract of A. conyzoides administered to mice appeared safe at doses ranging from 10 to 1000 mg/kg, while doses above 1600 mg/kg were lethal [24]. Despite the reported hepatotoxic effect of PA in *A. conyzoides*, a *n*-hexane and acetone extract of the plant effectively repaired hepatic damage induced by acetaminophen, restoring normal values of hepatic damage markers including albumin, alanine transaminase and conjugated and unconjugated bilirubin levels [17]. A further study demonstrated the potential of an ethanolic extract of the plant in providing protection against sodium arsenite-induced toxicity.

Together with these previous findings, our current results indicate that extracts, fractions and isolated compounds from *A. conyzoides* are safe within therapeutic dosage ranges.

References

1. Chahal R, Nanda A, Akkol EK, et al. Ageratum conyzoides L. and its secondary metabolites in the management of different fungal pathogens. Molecules. 2021; 26: 2933.

2. Adebayo AH, Zang GZ, Fan JT, et al. Biochemical haematological and histopathological studies of extract of Ageratum conyzoides L. in Sprague Dawley rats. J Med Plant Res. 2010; 4: 264-2272.
3. Okunade, Adewole L. Ageratum conyzoides L. Asteraceae. Fitoterapia. 2002; 73: 1-16.
4. Galati EM, Miceli N, Taviano MF, et al. Anti-inflammatory and antioxidant activity of Ageratum conyzoides. Pharmaceutical Biol. 2001; 39: 336-339.
5. Moura AC, Silva EL, Fraga MC, et al. Antiinflammatory and chronic toxicity study of the leaves of Ageratum conyzoides L. in rats. Phytomed. 2005; 12: 138-142.
6. Quellet M, Percival MD. Effect of inhibitor time-dependency on selectivity towards cyclooxygenase isoforms. Biochem J. 1995; 306: 247-251.
7. Tandan SK. Pharmacological Effects of Ageratum Conyzoides Roots. Indian J Pharmaceutical Sci. 1994; 56: 182-182.
8. Lake BG. Coumarin metabolism toxicity and carcinogenicity relevance for human risk assessment. Food Chem Toxicol. 1999; 37: 423-453.
9. Samarth RM, Samarth M, Matsumoto Y. Medicinally important aromatic plants with radioprotective activity. Future Science OA. 2017; 3: FSO247.
10. Whittle SR, Turner A. Antibacterial activities of Ageratum conyzoides. J Biochem Pharmacol. 1981; 30: 1191.
11. Widodo GP, Sukandar EY, Adnyana IK. A coumarin from Ageratum leaves Ageratum conyzoides L. Intern Pharmacol. 2008; 4: 56-59.
12. Samy RP, Ignacimuthu S, Raja DP. Preliminary screening of ethnomedicinal plants from India. J Ethnopharmacol. 1999; 66: 235-240.
13. Chah KF, Eze SA, Emuelosi SE, et al. Antibacterial and wound healing properties of methanolic extracts of some Nigerian medicinal plants. J Ethno pharmacol. 2006; 104: 164-167.
14. Kusman IT, Pradini GW, Maruf IF, et al. The Potentials of Ageratum conyzoides and Other Plants from Asteraceae as an Antiplasmodial and Insecticidal for Malaria Vector An Article Review. Infect Drug Resist. 2023; 16: 7109-7138.
15. Destaa T, Afeworka M, Unnithana CR, et al. Isolation and structural elucidation of toxic pyrrolizidine alkaloids from Ageratum conyzoides collected from Vod disease affected communities. Int J Pharm Technol. 2014; 6: 6281-6290.
16. Igboasoiyi AC, Ezenwa NK, Oladimeji HO. Studies on the Toxicity of Ageraturn conyzoides. J Pharm and Tox. 2007; 2: 743-747.
17. Verma PK, Sultana M, Raina R, et al. Hepatoprotective Effects of Ageratum conyzoides L. on biochemical indices induced by acetaminophen toxicity in Wistar rats. J Appl Pharmaceutical Sci. 2013; 3: S23-S27.
18. Clayton P, Venkatesh R, Shama, et al. Efficacy of a Topical Application of Ageratum conyzoides on Increasing Hair Growth and in Males and Females A Randomised Double-blind Placebo c ontrolled Study. Trichol Cosmetol Open J. 2022; 5: 10-15.
19. Clayton P, Bogoda N, Rao A. Efficacy of an Oral Ageratum Conyzoides Formulation on Increasing Hair Growth and Decreasing Hair Loss in Males and Females A Randomised Double-Blind Placebo-Controlled Study. Trichol Cosmetol Open J. 2023; 6: 1-6.
20. Palmer PA, Bryson JA, Clewell AE, et al. A comprehensive toxicological safety assessment of an extract of Ageratum conyzoides. Regulatory Toxicol Pharmacol. 2019; 103: 140-149.
21. Diallo A, Eklu-Gadegkeku K, Agbono A, et al. Acute and sub-chronic 28-day oral toxicity studies of hydroalcohol leaf extract of Ageratum conyzoides L Asteraceae. Trop J Pharm Res. 2010; 9: 5.
22. Nweze NE, Obiwulu LS. Anticoccidial effects of Ageratum conyzoides. J Ethnopharmacol. 2009; 122: 6-9.
23. Moreira MD, Picanço MC, Barbosa LC, et al. Compounds from Ageratum conyzoides isolation structural elucidation and insecticidal activity. Pest Management Science formerly Pesticide Science. 2007; 63: 615-621.
24. Adesanwo JK, Egbomeade CO, Noronkoia DO, et al. Chemical toxicity and antibacterial studies on methanol extracts of Melanthera scandens Ageratum conyzoides Aspilia Africana and Synedrella nodiflora. J Explor Res Pharmacol. 2019; 4: 1-7.