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Original Article

Preclinical Safety Assessment of Standardized Extract of *Centella asiatica* (L.) Urban Leaves

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ABSTRACT

Context: *Centella asiatica* (CA) leaves extract has been shown therapeutic potential. However, safety information is lacking. **Aims:** To evaluate acute oral toxicity (AOT), sub-chronic toxicity, and mutagenic potential of standardized extract of CA (L.) Urban leaves (INDCA). **Materials and Methods:** For the acute toxicity study, INDCA was orally administered to Sprague-Dawley rats at a dose range of 0–2000 mg/kg. For the repeated dose toxicity study, the rats of either sex were orally administered with INDCA at the doses of 250, 500, and 1000 mg/kg/day for a period of 90 days. The effects on body weight, food and water consumption, organ weight, hematology, clinical chemistry as well as histology were studied. The mutagenic potential of INDCA was tested using reverse mutation assay (Ames test). **Statistical Analysis Used:** Data of each parameter were analyzed by one-way ANOVA followed by Dunnett's test to compare the difference between treated groups. **Results:** The administration of INDCA did not produce mortality or significant changes in the clinical signs included but not limited to changes in the skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavior pattern. The appearance, progress, and disappearance of these signs were recorded. The lethal dose and no observable adverse effect level of INDCA were 2000 mg/kg and 1000 mg/kg, respectively. There were no significant differences in the organ weights, hematological parameters, clinical chemistry values, or gross and microscopic appearance of the organs from the treatment groups as compared to the control group. It was found to be nonmutagenic in reverse mutation assay. **Conclusions:** INDCA was found safe in AOT, sub-chronic toxicity, and mutagenicity studies when tested in rats.

Key words: Acute oral toxicity test, mutagenicity test, standardized extract of *Centella asiatica* leaves, sub-chronic toxicity test

INTRODUCTION

Since ancient times, plants (or food) derived natural products have commonly been used in folk medicine with therapeutic potential as anti-inflammatory, anti-arthritic, anti-aging, and immunomodulatory activities.[1] The rationale for the utilization of medicinal plants has rested largely on the long-term clinical experience with little or no scientific data on their efficacy and safety. However, in

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the recent past, pharmacological and toxicological effects of these plants have begun to receive attention from scientists for the verification of their claimed pharmacological and therapeutic properties.

In recent years, food chain derived natural medicines are more appealing for their potential in the management of chronic disorders. In order to acquire their maximum benefits, toxicity studies are considered necessary. This need is envisaged by many international regulatory agencies and reflected in their guidelines to ensure safe use of natural products, food supplements, or botanical medicines.[2] This critical prerequisite, especially in the form of acceptance by the Western countries, provided the impetus to carry out scientific studies in accordance with the various established regulatory guidelines applicable to the geographic requirements. The World Health Organization insists that the safety of herbal medicines is a critical component in the quality control of healthcare products.

One of the most prominent marketed food supplements that showed good therapeutic promise is based on *Centella asiatica* (Linn.) Urban (CA). CA, commonly known as gotu kola, is a small herbaceous annual plant from family Mackinlayaceae. CA is used as a medicinal herb in the traditional system of medicine including Ayurveda, African and Chinese medicines for medicinal uses in the management of many chronic conditions.[3]

CA has been reported in various animal models[4] of autoimmune diseases, wound healing, diabetic complications,[5,6] liver diseases, anxiety, and cognition.[7] The major bioactive constituents of CA are asiaticoside (AS), madecassoside, and their respective aglycons (asiatic acid and madecassic acid), collectively known as the centellosides. However, significant differences in contents of centellosides have been known between samples of CA originating from different countries.[8] Furthermore, individual centelloside possess different pharmacological active profiles.[9]

The concentration of total centelloside in the leaf extracts depends on a variety of conditions such as age of plant and nutrient supply.[10,11] Therefore, standardization based on the specific bioactive marker is utmost important to obtain therapeutic consistency. In the past, we have reported the method of preparation, characterization, and standardization of AS (a bioactive marker compound) based standardized extract of CA leaves (INDCA) and its antidepressant activity using animal model of stress specific chronic depression.[12] Furthermore, the stress-relieving properties of INDCA against behavioral disorders such as anxiety[13] and suicidal behavioral related traits[14] were also reported.

The most promising therapeutic applications of INDCA are toward management of stress-induced behavioral disorders. The management of stress-related disorders involves long-term treatment for several days or weeks for evident therapeutic effect in patients.[15] Therefore, long-term safety is an important aspect and needs to be evaluated in a systematic manner using standard protocols of preclinical toxicology.

Since INDCA has showed potential health benefits against chronic disorders, it was thought worthwhile to investigate its safety profile using standard regulatory guidelines for acute and long-term usage. Therefore, the objective of the present study was to investigate acute oral toxicity (AOT), 90-day repeated dose (sub-chronic) toxicity and mutagenicity study using well-accepted guidelines issued by Organization for Economic, Co-operation, and Development (OECD).

MATERIALS AND METHODS

Animals

The Sprague-Dawley rats; 6–8 weeks old weighing 125–150 g of both sexes, were obtained from animal house of Indian Institute of Toxicology, Pune and used for acute and 90-day (sub-chronic) toxicity studies. The females were nulliparous and nonpregnant. The rats kept for 7 days prior to dosing for acclimatization, cages were marked, and individual marking were made on fur for identification. Rats were on pelleted feed (Nav Maharashtra Chakan Oil Mills Ltd., Pune, India) and provided with pure potable water in glass bottles ad labium. The rats were maintained in ambient temperature (20 ± 3°C), relative humidity (30–70%), and 10–15 air changes per hour with 12 h: 12 h of dark and light cycle.

All experiments complied with the OECD guidelines for the testing of chemicals (OECD. 1998a) and Schedule Y in Drugs and Cosmetics Act (IInd Amendment) Rules, 2005, Ministry of Health and Family Welfare, Government of India. The protocol was approved by the Institutional Animal Ethics Committee of Indian Institute of Toxicology, Pune, which complies with the regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals.

The test compound, INDCA

The test compound, INDCA, was provided by Indus Biotech Private Limited, Pune. INDCA was prepared and standardized by high-performance liquid chromatography using earlier reported procedure[12] and found to have 45.74% of AS. INDCA was suspended in distilled water to obtain dose volume of 10 mL/kg body weight of animals for acute and sub-chronic studies. INDCA suspension was freshly prepared before administration to animals.

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Acute oral toxicity study

The acute toxicity study of INDCA was performed according to OECD guideline 423 (OECD, 1998b). INDCA was freshly formulated in distilled water corresponding to a dose volume of 10 mL/kg. The rats were grouped into two groups as follows:

- G1: Vehicle control (VC) group - distilled water - 10 mL/kg - five animals per sex
- G2: Treated group - INDCA suspended in distilled water - 2000 mg/kg - five animals per sex.

The rats were observed daily for 14 days following administration of the test compound for mortality and clinical signs of toxicity. On day 15, all animals were euthanized and underwent gross pathological examination for signs of toxicity via necropsy.

Sub-chronic (90 days repeated dose) toxicity study

The study complies with the OECD guideline for the testing of chemicals No. 408.[16] The separate VC group (G1) was maintained on distilled water. Based on lethal dose (LD50) (2000 mg/kg) obtained from acute toxicity study, animals were administered with following treatments for 90 consecutive days:

- G1: VC - distilled water - 15 animals per sex
- G2: INDCA (250 mg/kg) - 15 animals per sex
- G3: INDCA (500 mg/kg) - 5 animals per sex
- G4: INDCA (1000 mg/kg) - 15 animals per sex
- G1R: VC reversal group - 10 animals per sex
- G4R: INDCA treated reversal - 10 animals per sex.

All treated rats were daily observed for mortality and clinical signs during 90-day study period whereas reversal groups were observed for 119 days. The eyes of control and all the treated dose group animals were examined prior to the initiation of the dosing and on day 91, on day 105 and 119 (for reversal group animals) of the study. Eye examination was carried out using an ophthalmoscope (Mini 2000, HEINE Optotechnik, Herrsching, Germany) after induction of mydriasis with 0.5% solution of tropicamide. The body weight [Table 1] and feed consumption [Figure 1] of each rat were recorded at weekly intervals throughout the study period as mentioned in the guideline OECD 408. Toward the end of the exposure period in week 13 (day 91), sensory reactivity to stimuli of different types (auditory, visual and proprioceptive stimuli) and by grading different stimuli, assessment of grip strength (Digital grip strength meter, Columbus Instruments International, Columbus, OH, USA) and motor activity assessment was conducted in control and high dose group (1000 mg/kg) animals. The animals were placed in metabolic cages overnight and urine excreted by each animal was collected on day 91, on day 105, and on day 119 (for reversal group animals) of the study for urinalysis. The parameters for urinalysis included: Specific gravity, pH, occult blood, protein, bilirubin, ketones, glucose, nitrite, and urobilinogen. Using whole blood, hematological and coagulation analyses were carried out using Ac T Diff. Analyzer System (Beckman Coulter Inc., Mumbai, India). The parameters for hematological analysis included: Red blood cell count, reticulocyte count, hemoglobin, hematocrit, mean corpuscular hemoglobin (MCH), MCH concentration, mean corpuscular volume, and total leukocyte count, % cells in differential leukocyte count (including lymphocyte [L], monocyte [M], basophils [B], eosinophils [E], and prothrombin time). The biochemistry parameters and electrolytes were analyzed using Clinical Chemistry AutoAnalyzer System Vet Axcel® Chemistry Analyzer (Alfa Wassermann Diagnostic Technologies, West Caldwell, NJ, USA) and Acculyte 5P Electrolyte Analyzer System (Rapid Diagnostic Pvt., Ltd., Mumbai, India) respectively. The following parameters were measured: The parameters blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, gamma glutamyl transferase, creatine phosphokinase, lactate dehydrogenase, fasting plasma glucose, calcium (Ca), phosphorus (P), bilirubin, albumin, creatinine (CR),

Table 1: Effect of INDCA on body weights (g) of rats during 90 days repeated dose toxicity study

Weeks	Group					
	G1 VC	G2 250 mg/kg	G3 500 mg/kg	G4 1000 mg/kg	G1R VC (reversal-119)	G4R 1000 mg/kg (reversal-119)
Male						
1	91.71±11.13	89.13±7.12	90.27±7.52	91.28±8.19	90.12±9.11	90.80±7.44
12	358.66±7.53	356.23±6.84	352.97±7.89	351.61±10.66	353.98±7.37	351.51±10.31
17	-	-	-	-	383.60±12.11	370.94±8.12
Female						
1	99.13±11.07	99.31±7.80	99.79±7.65	100.91±7.39	98.09±9.53	100.65±6.62
12	259.31±8.31	255.83±5.10	261.63±3.26	252.51±2.42	258.50±5.49	255.43±3.37
17	-	-	-	-	280.34±4.80	274.04±2.66

Data were represented as mean±SD. Data on all parameters were evaluated by one-way ANOVA followed by Dunnett's test. Criteria for significance at 95% CI are dependent on the *P* value. No significance was found for either of G2, G3, G4 v/s. G1 and G4R v/s. G1R group.. *n*=15 for G1, G2, G3, and G4 whereas *n*=10 for G1R and G4R. SD = Standard deviation, VC = Vehicle control

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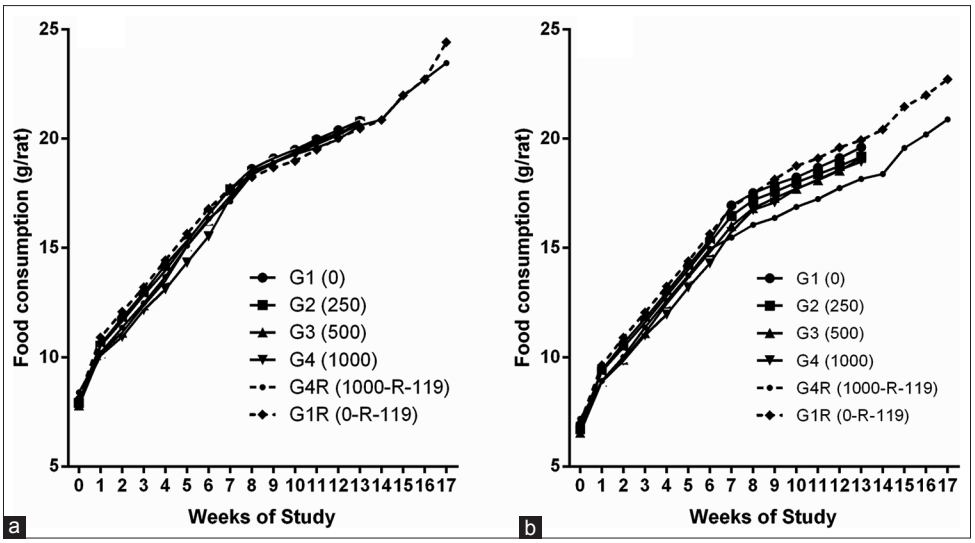


Figure 1: Mean food consumption (g/rat) during 90-day repeated dose toxicity study. (a) Mean food consumption for male rat (g/rat), (b) mean food consumption for female rat (g/rat)

sodium (Na), potassium (K), chlorine (Cl), cholesterol, and triglycerides (Trig). On day 91, all animals were euthanized and underwent gross pathological examination for signs of toxicity via necropsy. All organs, mucosa, body cavities, etc., were examined for gross pathological changes. The weights of liver, lungs, kidneys, adrenals, testes, epididymis, uterus, ovaries, thymus, spleen, brain, heart were recorded. The organ weights were recorded as absolute values, and their relative values (i.e., per cent of the body weight) were also calculated. Tissue samples of organs (as mentioned in guideline OECD 408) from all groups were preserved. Respective tissue samples from control and high dose level (1000 mg/kg) were subjected to histopathological examination via light microscopy. Eyes and testes were preserved in Davidson's fluid, and remaining tissues/organs were preserved in 10% neutral buffered formalin from control and different dose groups.

Mutagenicity study

Mutagenicity (Ames Reverse Mutation test) assay was performed in full compliance with the OECD guidelines for mutagenicity testing test No: 471[17,18] and US Food and Drug Administration (40 CFR 79.68). As no significant cytotoxic effect was observed, the five highest doses were then used in the subsequent mutagenicity evaluation. To evaluate mutagenicity, four strains of histidine-dependent *Salmonella* typhimurium (TA 97a, TA 98, TA 100, TA 1535, and TA 102) were tested in triplicate at the five highest doses (5000.00, 1666.67, 555.55, 185.18, and 61.72 µg/plate) of INDCA. Rat liver homogenate tested with mutagen 2-aminofluorene (2AF) before use. Metabolic activation was performed using a cofactor-supplemented postmitochondrial fraction (S9 fraction). Positive controls (2AF, 2-aminoanthracene, methyl methanesulfonate, 4-Nitroquinoline-N-Oxide, danthron, and sodium azide) with and without S9 activator and negative controls (VC and phosphate buffer), with and without S9 activator were included in the evaluation. This was done to ensure the test system was functioning properly (positive controls) and to obtain baseline frequencies for the various strains of bacteria used in the study (negative controls). The plates were counted after 48 h of incubation at 37°C. The mutagenic activity of the test substance was considered for positive in case of increased concentration over the range tested and a reproducible increase at one or more concentrations in number of revertant colonies per plate in at least one strain with or without metabolic activation system. The test substance was considered to be toxic if there was a decrease in the number of revertants and/or thinning or absence of background lawn.

Statistical analysis

Statistical analysis was performed using SPSS for Windows, Version 16.0 (SPSS Inc., Chicago, USA). All the data were checked for normality and values were represented as mean ± standard deviation. Data of each parameter were analyzed by one-way ANOVA followed by Dunnett's test to compare the difference between treated (G2–G6) with G1. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Acute oral toxicity study

No deaths occurred during the 14-day posttreatment evaluation period. There were neither treatment-related clinical signs of toxicity observed during the evaluation period nor was any weight loss observed in any groups.

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There were no significant changes observed in body weight in treated groups as compared to control group. Finally, no treatment-related gross pathological changes were observed in any organs of the test animals during necropsy. The results of this evaluation show that the single oral dose resulted in an LD50 of greater than 2000 mg/kg of body weight and indicates a low order of acute toxicity.

Sub-chronic (90-day repeated dose) toxicity study

No deaths occurred during the 90-day evaluation period. There were no treatment-related clinical signs of toxicity observed during the evaluation period. Ophthalmoscopic examination and functional observation tests revealed no abnormalities attributable to the treatment. There were no statistically significant differences in average daily food consumption [Figure 1] or body weight gain [Table 1] between control and treatment groups during the evaluation period.

Hematological [Tables 2 and 3] and biochemical investigations [Tables 4 and 5] conducted at the end of dosing period on day 91 and at the end of recovery period on day 105 and on day 119, revealed significant changes in the values of different parameters studied when compared with that of respective controls. G1 added as a VC group for treated dose groups of CA (250, 500, and 1000 mg/kg) for 90 days for comparison. G1R added as a reversal VC group for highest reversal dose group (1000 mg/kg) for 119 days for comparison. However, the significant increase/decrease in the values obtained did not show any adverse effect on hematological or biochemical parameters and the effect was not dose-dependent. Urinalysis results of treated dose groups were not significant as compared to that of control groups (data not shown). In comparison with respective controls on day 91, organ weight data of animals from different dose groups were not significant as compared to VC group. At the end of postdosing reversal period on day 105 and on day 119, organ weight data of animals from 1000 mg/kg reversal group were not significant as compared to that of respective controls [Tables 6 and 7].

No abnormality was detected as a part of gross pathology in any of the groups during necropsy [Table 8]. Histopathological examination revealed minimal to mild, focal lymphocytic infiltration and/or minimal focal necrosis in the liver; minimal focal to multifocal lymphocytic infiltration, necrosis and/or hydronephrosis in the kidneys [Figure 2]. The changes observed in control and high dose groups (1000 mg/kg) in histology of all evaluated organs were equivalent [Table 9]. Hence, these changes were considered as incidental. Based on the results of this study, the no observable adverse effect level (NOAEL) is 1000 mg/kg of body weight per day (the highest dose level administered).

Mutagenicity study

The tester strains fulfilled the quality check criteria. The bacterial background lawn was comparable with that of

Table 2: Effect of INDCA on hematological parameters during 90 days repeated dose toxicity study (male rats)

Parameters	G1 VC	G2 250 mg/kg	G3 500 mg/kg	G4 1000 mg/kg	G1R VC (reversal-105)	G4R 1000 mg/kg (reversal-105)	G1R VC (reversal-119)	G4R 1000 mg/kg (reversal-119)
Hb (g%)	14.30±0.94	14.24±0.72	15.64±1.04**	16.97±1.47**	15.38±1.47	15.72±0.38	14.54±1.95	16.16±1.54
RBC (×10^6/µL)	8.49±0.71	8.20±0.55	9.03±0.54	9.84±0.79**	8.96±0.76	9.03±0.24	8.36±1.22	9.28±1.03
Reticulocytes (%)	1.55±0.48	1.48±0.39	1.59±0.49	1.54±0.39	1.44±0.51	1.36±0.43	1.36±0.50	1.26±0.35
HCT (%)	42.37±2.74	41.95±2.07	45.73±2.73**	49.53±4.22**	44.82±3.84	46.04±0.98	42.86±5.54	46.36±4.85
MCV (µm^3)	50.05±2.23	51.27±1.25	50.65±2.33	50.33±1.45	50.06±1.25	50.98±1.27	51.40±2.01	49.96±1.17
MCH (pg)	16.89±0.67	17.41±0.55	17.31±0.89	17.26±0.56	17.16±0.53	17.40±0.40	17.44±0.54	17.44±0.62
MCHC (%)	33.75±0.58	33.95±0.35	34.18±0.39**	34.27±0.35**	34.28±0.47	34.10±0.37	33.92±0.75	34.90±0.65*
Platelets (×10^3/µL)	416.33±67.22	402.00±83.04	366.00±85.89	383.60±107.93	465.60±82.10	438.20±37.19	436.00±52.16	448.80±74.13
TLC (×10^3/µL)	10.96±2.01	9.75±2.74	9.89±3.60	14.99±3.96**	12.80±2.80	11.62±2.63	10.28±2.25	10.08±1.36
PT (s)	14.80±2.93	15.33±4.05	15.20±2.86	14.80±3.30	16.20±3.42	16.00±3.54	15.60±3.91	14.80±3.56
DLC								
N (%)	21.20±3.84	21.13±3.96	21.33±3.44	21.27±3.83	21.80±3.70	21.00±3.39	21.80±4.32	21.40±4.04
L (%)	75.67±3.89	75.73±3.33	75.20±3.36	75.53±3.27	74.80±3.35	75.40±2.97	74.80±3.42	75.00±3.08
E (%)	1.07±0.80	1.07±0.8	1.07±0.8	1.00±0.85	1.20±0.84	1.20±0.84	1.00±1.00	1.20±0.84
M (%)	2.07±1.03	2.07±0.8	2.40±0.83	2.20±0.86	2.20±0.84	2.40±0.55	2.40±0.55	2.40±0.55
B (%)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Data were represented as mean±SD, Data on all parameters were evaluated by one-way ANOVA followed by Dunnett's test. Criteria for significance at 95% CI are dependent on the *P* value. **P*<0.05, ***P*<0.01 as compared of a parameter value of respective VC group. *n*=15 for G1, G2, G3, and G4 whereas *n*=10 for G1R and G4R. Hb: Hemoglobin, RBC: Red blood corpuscles, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC = Mean corpuscular hemoglobin concentration, TLC = Total leukocyte (white blood corpuscles) count, PT = Prothrombin time, N = Neutrophils, L = Lymphocytes, E = Eosinophils, M = Monocytes, B = Basophils, SD = Standard deviation, DLC = Differential leukocyte count, VC = Vehicle control, CI: Confidence interval

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Table 3: Effect of INDCA on hematological parameters during 90-day repeated dose toxicity study (female rats)

Parameters	G1 VC	G2 250 mg/kg	G3 500 mg/kg	G4 1000 mg/kg	G1R VC (reversal-105)	G4R 1000 mg/kg (reversal-105)	G1R VC (reversal-119)	G4R 1000 mg/kg (reversal-119)
Hb (g %)	14.68±0.82	16.03±1.10**	14.65±1.02	13.71±0.76*	13.32±0.97	13.84±0.74	18.92±1.18	16.64±1.77**
RBC (×10^6/μL)	8.15±0.58	8.97±0.57**	8.21±0.66	7.26±1.45*	7.08±0.68	7.59±0.33	10.22±0.64	9.27±1.27
Reticulocytes (%)	1.55±0.49	1.55±0.44	1.49±0.48	1.61±0.43	1.52±0.61	1.44±0.53	1.46±0.30	1.30±0.35
HCT (%)	42.29±2.46	46.70±3.21**	42.57±2.98	38.31±6.50*	38.44±2.65	39.98±2.05	55.22±2.57	48.56±5.24**
MCV (μm^3)	51.95±1.84	52.08±1.77	51.93±1.55	53.63±4.95	54.38±1.92	52.66±0.60	54.10±2.01	52.56±1.86
MCH (pg)	18.05±0.70	17.88±0.58	17.87±0.56	20.89±11.17	18.80±0.60	18.22±0.36*	18.56±0.56	18.02±0.64
MCHC (%)	34.75±0.24	34.33±0.30**	34.41±0.35**	37.81±13.42	34.60±0.26	34.56±0.32	34.26±0.63	34.28±0.08
Platelets (×10^3/μL)	436.80±65.89	331.73±75.72**	379.20±90.90	429.07±103.33	385.60±101.77	438.40±102.42	271.80±47.68	392.00±64.52**
TLC (×10^3/μL)	9.45±1.43	8.15±2.53	8.07±1.89	8.25±2.55	7.68±2.21	9.52±1.48	7.10±2.45	8.68±2.18
PT (s)	14.47±3.18	15.47±3.02	15.47±3.16	15.13±3.62	14.80±3.70	14.60±3.05	15.40±3.58	14.00±2.74
DLC								
N (%)	21.53±3.89	21.20±2.98	22.33±2.85	21.60±3.89	20.80±3.27	21.20±3.27	21.20±3.27	20.80±3.96
L (%)	75.33±4.15	75.53±2.95	74.60±2.03	75.33±3.56	75.60±2.88	75.40±2.30	75.20±4.32	76.00±4.00
E (%)	1.00±0.85	1.13±0.83	1.00±0.85	1.00±0.85	1.20±0.84	1.20±0.84	1.20±0.84	1.20±0.84
M(%)	2.13±0.74	2.13±0.83	2.07±0.88	2.07±0.96	2.40±0.55	2.20±0.84	2.40±0.89	2.00±1.00
B (%)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Data were represented as mean±SD, Data on all parameters were evaluated by one-way ANOVA followed by Dunnett's test. Criteria for significance at 95% CI are dependent on the *P* value. **P*<0.05, ***P*<0.01 as compared of a parameter value of respective VC group. *n*=15 for G1, G2, G3, and G4 whereas *n*=10 for G1R and G4R. Hb = Hemoglobin, RBC = Red blood corpuscles, HCT = Hematocrit, MCV = Mean corpuscular volume, MCH = Mean corpuscular hemoglobin, MCHC = Mean corpuscular hemoglobin concentration, TLC = Total leukocyte (white blood corpuscles) count, PT = Prothrombin time, N = Neutrophils, L = Lymphocytes, E = Eosinophils, M = Monocytes, B = Basophils, SD = Standard deviation, DLC = Differential leukocyte count, VC = Vehicle control, CI = Confidence interval

Table 4: Effect of INDCA on blood chemistry on during 90-day repeated dose toxicity study (male rats)

Parameters	G1 VC	G2 250 mg/kg	G3 500 mg/kg	G4 1000 mg/kg	G1R VC (reversal-105)	G4R 1000 mg/kg (reversal-105)	G1R VC (reversal-119)	G4R 1000 mg/kg (reversal-119)
Total protein (g%)	7.50±0.55	7.93±0.47	7.86±0.46	7.52±0.49	7.28±0.71	7.48±0.5	7.87±0.55	6.90±0.34**
BUN (mg%)	35.87±3.94	32.73±4.03	34.53±3.78	35.20±4.92	33.60±3.91	34.20±4.60	38.40±3.97	34.80±3.42
ALT (IU/L)	41.47±4.58	40.47±4.21	40.93±5.26	39.67±4.97	43.40±1.34	43.20±4.15	38.80±8.11	40.00±7.38
AST (IU/L)	63.40±3.42	64.00±3.02	63.80±4.86	64.13±4.24	63.40±3.58	62.80±4.82	62.00±4.58	63.20±4.32
ALP (IU/L)	72.27±3.88	72.80±3.95	73.20±7.10	71.40±4.15	70.60±3.58	73.60±4.98	70.00±4.36	69.80±4.21
FPG (mg%)	97.27±8.84	97.20±8.24	96.73±10.16	94.73±8.52	93.00±6.20	97.20±6.72	95.80±7.89	99.80±6.83
Ca (mg%)	2.37±0.07	2.37±0.1	2.34±0.06	2.16±0.12**	2.26±0.12	2.12±0.06*	2.15±0.06	2.09±0.09
P (mg%)	4.13±0.45	4.24±0.44	4.31±0.31	4.27±0.38	3.84±0.55	4.04±0.25	4.12±0.36	4.46±0.32
GGT (U/L)	14.33±3.96	15.53±4.60	13.13±3.25	15.93±4.46	16.60±2.70	15.80±6.61	15.40±3.21	16.00±4.12
Bilirubin (mg%)	0.67±0.07	0.65±0.07	0.63±0.04	0.66±0.06	0.69±0.06	0.67±0.07	0.60±0.05	0.59±0.06
Albumin (g%)	3.70±0.29	3.69±0.24	3.83±0.16	3.68±0.31	3.56±0.38	3.63±0.27	3.73±0.24	3.61±0.47
CR (mg%)	0.96±0.10	0.97±0.08	0.96±0.06	0.99±0.11	0.93±0.09	0.97±0.13	0.93±0.09	0.94±0.06
CK (IU/L)	61.53±5.54	60.40±5.77	59.93±5.39	61.93±4.99	63.20±5.76	61.80±5.31	62.00±4.85	60.60±5.08
Na (mmol/l)	130.45±1.32	130.68±1.30	131.13±1.31	130.94±1.42	128.37±2.41	128.74±1.03	137.95±3.25	137.18±2.13
K (mmol/l)	4.46±0.37	4.08±0.32**	4.03±0.33**	4.60±0.72	3.71±0.29	3.52±0.18	4.91±0.60	4.62±0.30
Cl (mmol/l)	102.11±1.32	102.15±1.34	101.81±1.05	102.13±1.64	112.01±1.88	115.51±2.24**	103.42±1.68	104.04±1.34
Cholesterol (mg%)	62.80±5.33	63.40±4.91	62.53±4.49	63.47±5.11	63.40±5.50	64.20±6.22	62.80±5.67	62.00±5.92
Trig (mg%)	109.87±6.29	106.67±6.72	108.93±11.22	107.00±8.27	105.40±9.29	103.80±10.47	107.00±6.12	108.60±7.44
LDH (IU/L)	359.80±24.21	373.60±22.36	362.73±17.36	357.73±28.22	364.00±31.69	358.40±36.75	350.80±19.50	371.40±23.18

Data were represented as mean±SD Data on all parameters were evaluated by one-way ANOVA followed by Dunnett's test. Criteria for significance at 95% CI are dependent on the *P* value. **P*<0.05, ***P*<0.01 as compared of a parameter value of respective VC group. *n*=15 for G1, G2, G3, and G4 whereas *n*=10 for G1R and G4R BUN = Blood urea nitrogen, ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, ALP = Alkaline phosphatase, FPG = Fasting plasma glucose, Ca = Calcium, P = Phosphorus, GGT = Gamma-glutamyl transferase, CR = Creatinine, CK = Creatine phospho kinase, LDH = Lactate dehydrogenase, Na = Sodium, K = Potassium, Cl = Chlorine, Trig = Triglycerides, SD = Standard deviation, CI = Confidence interval

the respective VC plate up to the highest concentration of 5000 μg/plate. No substantial increases in the revertant colony count [Tables 10 and 11] in any of the five strains were reported at any of the test concentrations in the presence or absence of metabolic activation (S9 mix). Positive controls resulted in significant increases in the

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Table 5: Effect of INDCA on blood chemistry on Sprague Dawley during 90-day repeated dose toxicity study (female rats)

Parameters	G1 VC	G2 250 mg/kg	G3 500 mg/kg	G4 1000 mg/kg	G1R VC (reversal-105)	G4R 1000 mg/kg (reversal-105)	G1R VC (reversal-119)	G4R 1000 mg/kg (reversal-119)
Total protein (g%)	7.39±0.55	7.41±0.59	7.40±0.54	7.58±0.33	7.23±0.74	7.58±0.65	7.41±0.65	7.71±0.7
BUN (mg%)	33.13±4.61	34.87±3.80	33.80±5.19	34.87±5.28	34.60±4.10	34.60±4.83	36.60±4.34	33.60±4.34
ALT (IU/L)	38.00±5.37	39.40±6.39	37.27±5.89	40.13±7.69	40.60±5.73	41.80±4.92	41.40±4.77	42.40±5.41
AST (IU/L)	60.87±4.84	62.87±4.58	61.13±4.49	62.27±5.43	63.40±5.22	61.40±5.37	63.20±2.77	61.60±3.29
ALP (IU/L)	67.53±6.13	69.13±4.73	70.07±4.65	71.60±5.36	69.60±4.62	72.20±4.87	69.80±4.32	70.60±2.19
FPG (mg%)	95.40±10.94	96.40±9.39	95.60±9.09	99.27±7.90	98.00±5.24	98.80±4.71	103.60±3.36	94.20±7.50**
Ca (mg%)	2.41±0.10	2.30±0.08**	2.38±0.06	2.26±0.09**	2.25±0.06	2.28±0.13	1.40±0.14	1.13±0.15**
P (mg%)	4.15±0.43	4.27±0.36	4.16±0.40	4.15±0.31	4.42±0.43	3.98* ±0.28	4.28±0.43	4.04±0.23
GGT (U/L)	15.33±3.70	14.73±4.59	13.13±4.53	14.80±4.25	17.80±6.30	12.60±5.32	16.40±6.07	15.20±4.92
Bilirubin (mg%)	0.68±0.07	0.67±0.08	0.67±0.09	0.64±0.05	0.65±0.06	0.67±0.05	0.67±0.09	0.68±0.09
Albumin (g%)	3.76±0.26	3.72±0.31	3.67±0.32	3.67±0.33	3.78±0.12	3.93±0.06**	3.69±0.29	3.75±0.36
CR (mg%)	0.96±0.10	0.96±0.10	0.93±0.08	0.97±0.09	0.88±0.06	0.93±0.04	1.01±0.10	0.99±0.13
CK (IU/L)	58.73±5.26	60.27±5.99	60.00±4.97	58.27±4.67	60.60±4.83	57.60±5.13	63.20±4.44	60.40±5.18
Na (mmol/l)	130.62±0.98	131.66±1.16*	131.40±1.13	130.96±1.35	135.17±2.00	137.25±0.65*	141.09±2.33	145.27±2.00**
K (mmol/l)	4.98±0.51	4.36±0.39**	3.94±0.45**	4.12±0.52**	4.13±0.49	4.45±0.20	3.54±0.24	2.68±0.61**
Cl (mmol/l)	101.48±1.70	102.79±1.28*	102.17±1.14	104.60±1.53**	102.35±0.73	104.38±1.26**	106.69±1.78	107.72±2.23
Cholesterol (mg%)	63.20±4.31	61.40±4.90	63.13±3.64	65.60±2.95	63.80±4.76	65.40±4.34	63.80±5.40	65.40±2.61
Trig (mg%)	109.40±8.21	103.73±12.19	109.07±8.11	108.13±6.92	114.40±2.88	107.60±10.29	108.80±12.24	107.20±4.92
LDH (IU/L)	370.20±24.18	367.47±34.70	368.53±24.59	372.00±28.98	375.20±22.04	368.60±4.22	358.00±30.98	364.80±23.33

Data were represented as mean±SD Data on all parameters were evaluated by one-way ANOVA followed by Dunnett's test. Criteria for significance at 95% CI are dependent on the *P* value. **P*<0.05, ***P*<0.01 as compared of a parameter value of respective VC group. *n*=15 for G1, G2, G3, and G4 whereas *n*=10 for G1R and G4R BUN = Blood urea nitrogen, ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, ALP = Alkaline phosphatase, FPG = Fasting plasma glucose, Ca = Calcium, P = Phosphorus, GGT = Gamma-glutamyl transferase, CR = Creatinine, CK = Creatine phospho kinase, LDH = Lactate dehydrogenase, Na = Sodium, K = Potassium, Cl = Chlorine, Trig = Triglycerides, SD = Standard deviation, VC = Vehicle control, CI = Confidence interval

Table 6: Effect of INDCA on organ weights (g) of during 90 days repeated dose toxicity study (male rats)

Organ	G1 VC	G2 250 mg/kg	G3 500 mg/kg	G4 1000 mg/kg	G1R VC (reversal-105)	G4R 1000 mg/kg (reversal-105)	G1R VC (reversal-119)	G4R 1000 mg/kg (reversal-119)
Brain	0.587±0.025	0.599±0.031	0.591±0.029	0.579±0.048	0.599±0.023	0.587±0.012	0.594±0.024	0.601±0.008
Liver	2.831±0.337	2.988±0.363	2.875±0.237	2.983±0.316	3.081±0.403	2.969±0.481	3.059±0.245	3.049±0.208
Kidneys	0.711±0.089	0.727±0.061	0.691±0.086	0.724±0.049	0.822±0.082	0.774±0.077	0.822±0.054	0.84±0.03
Adrenals	0.0133±0.0017	0.0141±0.002	0.0134±0.0019	0.0142±0.0021	0.0145±0.002	0.0152±0.0019	0.015±0.002	0.014±0.002
Testes	0.943±0.068	0.946±0.06	0.909±0.059	0.904±0.073	0.923±0.067	0.928±0.034	0.919±0.015	0.93±0.024
Heart	0.371±0.043	0.371±0.033	0.36±0.037	0.359±0.037	0.37±0.029	0.358±0.028	0.362±0.021	0.348±0.021
Spleen	0.358±0.059	0.401±0.071	0.362±0.048	0.351±0.047	0.339±0.075	0.319±0.041	0.35±0.053	0.4±0.056
Lungs	0.559±0.044	0.526±0.078	0.518±0.057	0.532±0.081	0.518±0.076	0.517±0.068	0.563±0.063	0.527±0.061
Thymus	0.076±0.019	0.083±0.021	0.084±0.016	0.086±0.023	0.065±0.01	0.064±0.014	0.062±0.015	0.064±0.014
Epididymis	0.359±0.037	0.378±0.025	0.361±0.034	0.381±0.038	0.394±0.031	0.376±0.024	0.394±0.032	0.379±0.025

Data were represented as mean±SD. Data on all parameters were evaluated by one-way ANOVA followed by Dunnett's test. Criteria for significance at 95% CI are dependent on the *P* value. No significance was found for either of G2, G3, G4 v/s. G1 and G4R v/s. G1R group. *n*=15 for G1, G2, G3, and G4 whereas *n*=10 for G1R and G4R. SD = Standard deviation, VC = Vehicle control, CI = Confidence interval

revertant count. The spontaneous reversion rates in the negative and positive control were within the range of historical data. No biologically relevant increase in the revertant counts was observed in any of the five tester strains preincubated with the test item. The results of these investigations suggest that under the experimental conditions, INDCA did not induce gene mutation by pair changes or frameshifts in the genome of the strains used.

DISCUSSION

The interest in the use of herbal preparations in different parts of the world has been growing considerably with corresponding developments in the phytomedicinal therapy. Herbal remedies positioned themselves in various forms such as dietary supplements, mono or polyherbal drugs, dietary ingredients, etc., and have become famous and safe commercial commodities.

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Table 7: Effect of INDCA on organ weights of during 90-day repeated dose toxicity study (female rats)

Organ	G1 VC	G2 250 mg/kg	G3 500 mg/kg	G4 1000 mg/kg	G1R VC (reversal-105)	G4R 1000 mg/kg (reversal-105)	G1R VC (reversal-119)	G4R 1000 mg/kg (reversal-119)
Brain	0.786±0.039	0.799±0.037	0.786±0.02	0.807±0.037	0.777±0.023	0.783±0.032	0.75±0.019	0.765±0.016
Liver	3.086±0.311	3.035±0.247	3.164±0.319	3.197±0.34	3.479±0.271	3.226±0.296	3.436±0.122	3.342±0.229
Kidneys	0.698±0.052	0.671±0.037	0.702±0.033	0.693±0.051	0.712±0.049	0.682±0.07	0.698±0.085	0.728±0.053
Adrenals	0.0248±0.003	0.0262±0.0052	0.0257±0.004	0.0261±0.0032	0.0286±0.0046	0.0292±0.0047	0.025±0.004	0.027±0.002
Ovaries	0.0308±0.0059	0.0338±0.0042	0.0285±0.0049	0.0308±0.0051	0.0322±0.0064	0.0312±0.0067	0.0306±0.0055	0.0308±0.0043
Heart	0.371±0.028	0.362±0.028	0.369±0.028	0.377±0.023	0.394±0.039	0.377±0.026	0.357±0.019	0.361±0.014
Spleen	0.417±0.067	0.432±0.096	0.409±0.07	0.398±0.048	0.418±0.053	0.405±0.05	0.356±0.047	0.381±0.044
Lungs	0.648±0.058	0.657±0.055	0.639±0.099	0.67±0.086	0.636±0.043	0.664±0.054	0.579±0.066	0.627±0.076
Thymus	0.095±0.017	0.098±0.02	0.094±0.019	0.102±0.016	0.098±0.024	0.088±0.019	0.079±0.006	0.088±0.015
Uterus	0.203±0.049	0.201±0.038	0.214±0.033	0.207±0.049	0.218±0.029	0.227±0.014	0.18±0.032	0.203±0.048

Data were represented as mean±SD. Data on all parameters were evaluated by one-way ANOVA followed by Dunnett's test. Criteria for significance at 95% CI are dependent on the *P* value. No significance was found for either of G2, G3, G4 v/s. G1 and G4R v/s. G1R group. *n*=15 for G1, G2, G3, and G4 whereas *n*=10 for G1R and G4R. SD = Standard deviation, VC = Vehicle control, CI = Confidence interval

Table 8: Summary of gross pathology findings

Group	G1		G2		G3		G4		G1R		G4R	
Dose (mg/kg)	VC		250 mg/kg		500 mg/kg		1000 mg/kg		VC (reversal-119)		1000 mg/kg (reversal-119)	
Sex	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Organs/lesions	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD

NAD = No abnormality detected, VC = Vehicle control

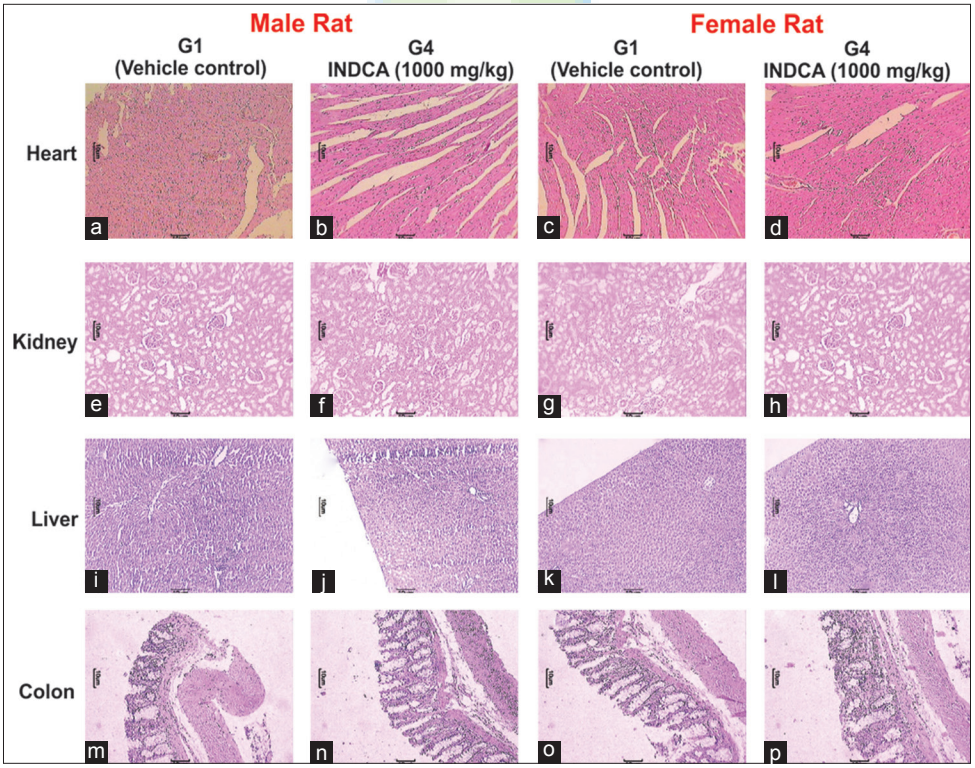


Figure 2: Histopathology of representative sections of vital organs of rats. Sections of heart (a–d), kidney (e–h), liver (i–l) and colon (m–p) tissues with G1: Vehicle control) (a, e, i, m, c, g, k and o) and G4: INDCA (1000 mg/kg) treated (b, f, j, n, d, h, l and p) rats. Hematoxylin and eosin (H and E) stain at ×40

Typically, safety studies on herbal compounds intended for oral use involve AOT study in rodents, which helps to determine the dose levels for short- and long-term repeated dose toxicity studies. Despite the alternative views on the use of LD50 data, acute studies continue to be considered valuable in establishing target organ toxicity.

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Table 9: Summary of histopathology findings

Organ and parameter	Male		Female	
	G1 VC	G4 (1000 mg/kg)	G1 VC	G4 (1000 mg/kg)
Number of animals	15	15	15	15
Adrenals dilatation	1	4	4	NAD
Aorta	NAD	NAD	NAD	NAD
Brain (infiltration)	2	1	2	4
Brain (necrosis)	2	2	0	2
Caecum	NAD	NAD	NAD	NAD
Colon	NAD	NAD	NAD	NAD
Duodenum	NAD	NAD	NAD	NAD
Epididymis	NAD	NAD	-	-
Eyes	NAD	NAD	NAD	NAD
Heart	NAD	NAD	NAD	NAD
Ileum	NAD	NAD	NAD	NAD
Jejunum	NAD	NAD	NAD	NAD
Kidneys (infiltration)	5	5	2	2
Kidneys (necrosis)	1	0	0	0
Kidneys (hydronephrosis)	1	0	0	0
Liver (infiltration)	12	13	11	12
Liver (necrosis)	0	1	2	0
Lungs (pneumonitis)	10	14	15	14
Lungs (hemorrhages)	0	1	1	2
Lymph node	NAD	NAD	NAD	NAD
Ovaries	-	-	NAD	NAD
Skin	NAD	NAD	NAD	NAD
Esophagus	NAD	NAD	NAD	NAD
Pancreas (infiltration)	1	1	0	0
Pituitary gland (cysts)	2	3	NAD	1
Prostate	NAD	NAD	-	-
Rectum	NAD	NAD	NAD	NAD
Sciatic nerve	NAD	NAD	NAD	NAD
Seminal vesicles	NAD	NAD	-	-
Skeletal muscle	NAD	NAD	NAD	NAD
Spleen	NAD	NAD	NAD	NAD
Spinal cord (necrosis)	NAD	NAD	NAD	1
Sternum bone marrow	NAD	NAD	NAD	NAD
Stomach	NAD	NAD	NAD	NAD
Testes	NAD	NAD	-	-
Trachea (infiltration)	7	7	5	2
Thymus	NAD	NAD	NAD	NAD
Thyroid/parathyroid gland (cysts)	2	2	4	4
Urinary bladder	NAD	NAD	NAD	NAD

NAD = No abnormality detected, VC = Vehicle control

INDCA was found to be safe during AOT study and 90-day repeated dose toxicity with LD50 more than 2000 mg/kg and NOAEL was 1000 mg/kg, respectively. Reversal groups were incorporated to assess the reversibility of toxicity or the potential that toxicity will develop or progress after cessation of test compound administration. INDCA treated reversal groups was found to be analogous with that of respective control groups and did not show any adverse effects after cessation of treatment in reversal groups.

In the AOT study, a compound is considered as safe if no death occurs, and no clinical signs are observed at the highest dose tested as per OECD guideline 423. INDCA did not show any toxic reactions at a dose of 2000 mg/kg. Thus, LD50 of INDCA was more than 2000 mg/kg. In general, the decrease in the body weight gain is a simple and sensitive index of toxicity after exposure to potentially toxic substances.[19,20] In the present study, INDCA did not show significant changes in body weights as compared to the control group. This suggests that INDCA did not hamper the normal growth of experimental animals. The necropsy performed after 14 days showed no significant changes in organ gross anatomy in all treatment groups when compared with control. Therefore, this extract can be considered as safe when tested for acute toxicity.

In the 90-day repeated dose toxicity study, INDCA did not appear to affect the behavior of the rats at the administrated oral doses of 250, 500, and 1000 mg/kg. It is reported that some agents like immunosuppressive compounds may be associated with the occurrence of hematologic toxicity, such as anemia, due to bone marrow suppression or hemolysis, leukopenia, and thrombocytopenia.[21] In the current study, it was found that repeated administration of INDCA did not adversely affect levels of any of the hematological parameters.

It is known fact that the levels of ALT, AST, CR, and BUN are good indicators of liver and kidney functions.[22] There was no adverse effect on biochemical parameters of the groups treated with INDCA. It suggests that the repeated administration of INDCA does not have toxic effects on liver and kidney.

The dose of 30 mg/kg (oral, once a day) was reported as pharmacologically active dose of INDCA in animal models of stress-induced behavioral symptoms such as depression[12] and suicidal behavioral-related traits.[14] Recently, INDCA has also been reported efficacy at its optimal dose of 30 mg/kg in epilepsy,[23] migraine,[24] and benign prostate hyperplasia.[25] The recorded NOAEL in the present study (1000 mg/kg, oral) is more than 30 times as that of effective dose (30 mg/kg, oral) indicates excellent safety margin of INDCA.

The Ames test is a widely employed method that uses bacteria to test whether a given chemical can cause cancer. More formally, it is a biological assay to assess the mutagenic potential of chemical compounds.[26] INDCA did not exhibit signs of significant mutagenicity (Ames Reverse Mutation Study) either in the presence or absence of a metabolic activator. It was evaluated over a fairly broad concentration range (61.72–5000 μg/plate) with no indication of a dose-related mutagenic effect.

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Table 10: Summary data of histidine revertants in *Salmonella* (experiment number: 1)

Treatment	Dose (μg/plate)	Mean number of revertants (mean±SD)					
		S-9	TA1535	TA97a	TA98	TA100	TA102
INDCA	5000	–	7.00±1.00	101.00±4.00	27.00±2.00	114.33±4.51	220.67±3.06
		+	8.67±1.53	122.67±5.03	34.67±3.06	132.67±4.04	238.33±3.51
	1666.67	–	8.33±1.53	113.00±4.36	30.33±1.53	127.33±7.02	229.67±4.16
		+	10.33±1.53	138.67±4.51	42.33±2.52	143.33±5.51	263.67±4.51
	555.55	–	9.67±1.53	129.67±3.51	40.33±6.11	140.67±4.51	253.00±4.00
		+	12.00±1.00	156.67±6.11	55.67±7.77	160.33±2.52	276.00±6.56
	185.18	–	11.33±2.08	138.33±4.04	49.33±4.16	152.00±5.57	262.67±5.69
		+	13.67±1.53	171.00±3.61	65.00±5.57	177.33±5.03	290.33±2.52
	61.72	–	13.33±2.08	144.67±4.51	58.67±5.03	163.67±5.51	270.33±7.51
		+	17.00±1.00	184.33±4.93	75.00±7.00	186.67±3.51	306.33±4.04
DMSO (solvent control)	100 μl	–	11.33±1.53	107.00±4.36	32.00±2.00	124.00±6.00	231.00±5.00
		+	14.67±1.53	140.33±4.51	42.33±2.08	149.33±3.06	264.33±4.04
MMS (μl/plate)	1	–	-	-	-	2220.67±225.44	4919±383.14
4-NQNO	0.5	–	-	570.33±92.95	289.67±42.78	-	-
Sodium azide	0.5	–	1144.00±65.28	-	-	-	-
2-AF	10	+	-	1298.33±89.51	1331.33±114.27	1299.33±124.02	-
Danthron	30	+	-	-	-	-	1253.33±80.83
2-AA	0.5	+	224.00±51.07	-	-	-	-

The data were represented as mean±SD. Data were analyzed by unpaired *t*-test. – = Represents plates without metabolic activation (without S9 mix), + = Represents plates with metabolic activation (with S9 mix). Data of each strain were compared with respective positive control group. 4-NQNO = 4-nitroquinoline-N-Oxide, 2-AF = 2-aminofluorene, MMS = Methyl methane sulfonate, 2-AA = 2-aminofluorene, DMSO = Dimethyl sulfoxide

Table 11: Summary data of histidine revertants in *Salmonella* (experiment number: 2)

Treatment	Dose (μg/plate)	Number of revertants (mean±SD)					
		S-9	TA1535	TA97a	TA98	TA100	TA102
INDCA	5000	–	7.33±0.58	90.33±3.51	19.00±2.00	137.33±6.03	236.67±5.51
		+	10.33±1.53	106.33±4.73	33.00±4.58	158.00±6.56	276.33±5.69
	1666.67	–	9.67±0.58	94.00±3.61	25.33±3.51	144.67±3.79	254.33±4.04
		+	12.00±1.00	111.00±3.00	34.00±3.00	166.00±4.58	282.67±3.51
	555.55	–	12.00±1.00	102.33±3.51	34.67±3.51	153.33±4.51	260.33±3.51
		+	15.33±1.53	122.33±4.04	47.00±3.61	181.00±7.55	290.67±7.09
	185.18	–	13.33±1.53	110.00±6.56	40.00±3.00	161.33±5.51	272.67±5.03
		+	18.00±1.00	131.33±6.03	54.00±4.00	187.67±6.03	297.00±6.56
	61.72	–	15.00±1.00	119.00±2.00	52.33±3.51	167.33±4.73	276.67±6.03
		+	19.00±1.00	142.33±4.04	65.00±3.00	194.67±6.03	306.33±6.66
DMSO (solvent control)	100 μl	–	12.00±1.00	98.33±4.04	30.67±2.52	150.67±3.06	257.33±4.51
		+	15.33±1.53	122.00±4.58	42.00±3.00	173.33±5.51	288.33±6.66
MMS (μl/plate)	1	–	-	-	-	2042.00±260.07	5204.33±200.04
4-NQNO	0.5	–	-	554.33±61.61	339.67±54.65	-	-
Sodium azide	0.5	–	1216.33±85.56	-	-	-	-
2-AF	10	+	-	1334.00±100.95	1377.33±107.06	1313.33±140.48	-
Danthron	30	+	-	-	-	-	1270.33±76.05
2-AA	0.5	+	175.33±39.31	-	-	-	-

The data were represented as mean±SD. Data were analyzed by unpaired *t*-test. – = Represents plates without metabolic activation (without S9 mix), + = Represents plates with metabolic activation (with S9 mix), Data of each strain were compared with respective positive control group. 4-NQNO = 4-nitroquinoline-N-Oxide, 2-AF = 2-aminofluorene, MMS = Methyl methane sulfonate, 2-AA = 2-aminofluorene, SD = Standard deviation, DMSO = Dimethyl sulfoxide

CONCLUSIONS

The results of present study showed that single and 90-day repeated dose oral administration of the INDCA produced no significant toxic effects with the median LD50 >2000 mg/kg, the NOAEL of 1000 mg/kg/day for both genders, and no mutagenicity potential. The present study demonstrated excellent safety margins of INDCA and suggested potential for further development after appropriate clinical studies.

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Conflicts of interest

There are no conflicts of interest.

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