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Characterization and Preclinical Toxicity Assessment of Intranasal Administration of Standardized Extract of *Centella asiatica* (L.) Urban Leaves (INDCA-NS) in Laboratory Rats

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Abstract

The objective of the present work was to characterize and assess the intranasal toxicity of a solution of total triterpenoids - based standardized extract of *Centella asiatica* (L.) Urban leaves (INDCA-NS). The acute and 28-day repeated-dose toxicity of intranasal administrated INDCA-NS in Wistar rats was evaluated. For the acute intranasal toxicity study, a single dose of 100 µg/rat/day was administered to five male and female rats and observed for mortality and signs of toxicity for 14 days. In the subacute toxicity study, the INDCA-NS (10, 30, and 100 µg/rat/day) were intranasally administered to a separate group of five male and female rats for 28 days, followed by a 14-day reversal period for behavioral, biochemical, and histological parameters. The effect of subacute administration of INDCA-NS on the ability to smell in rats was evaluated by a buried food test. Treatment with INDCA-NS did not result in mortality or treatment-related adverse signs during single or 28-day repeated-dose administration. In addition, intranasal INDCA-NS did not affect the sense of smell, as observed in the buried food test. Intranasal NDCA-NS was found safe in rats with a Maximum Tolerated Dose (MTD) of more than 100 µg/rat/day and No-Observed Adverse Effect Level (NOAEL) of more than 100 µg/rat/day in male and female rats during acute and subacute intranasal toxicity study respectively.

Keywords: Acute Toxicity, *Centella asiatica* (L.) Urban Leaves, Intranasal, Subacute Toxicity, Total Triterpenoids

1. Introduction

Centella asiatica (L.) Urban (CA), or Gotu kola, is a tropical, medicinal plant prevalent in Southeast Asian countries such as India, Sri Lanka, China, Indonesia, Malaysia, South Africa, and Madagascar[1]. Its uses are well documented in traditional Chinese, Indonesian, and ayurvedic medicine for wound healing and anti-inflammatory capabilities[2] and high blood pressure lowering, memory and longevity promoting potential[3].

CA is a common herb utilized by natural healing therapists to revitalize the nerves and brain cells and manage psychological disorders such as depression[4]. The CA and its extracts were reported to provide excellent neuroprotection, as observed in the scientific studies[5,6]. Furthermore, the clinical efficacy of oral supplementation of CA leaves in reducing symptoms and managing mood, anxiety, and stress-related disorders has been reported[7-10].

The broad range of medicinal properties of CA against nervous system disorders has been attributed

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to its major group of phytoconstituents, triterpenoids, including asiaticoside, madecassoside, asiatic acid, and madecassic acid[4,11,12]. In addition, the medicinal potential of oral administration of CA leaves, CA extract and triterpenoids-based standardized extracts of CA leaves (INDCA) for prophylaxis and treatment of stress, anxiety, migraine, mood-related disorders, neuropathy, spinal cord injury, and other neuropsychiatric conditions has been reviewed in the past[5,6,13,14].

Recently, the intranasal administration route is emerging as the most promising for safer or faster brain delivery[15-17]. The intranasal route directly delivers the content to the brain, avoids the side effects, prevents systemic absorption, and thus enhances the efficacy of agents in managing psychology[18,19] and neurological disorders[20]. Therefore, intranasal administration of triterpenoid-based standardized extract of CA leaves (INDCA-NS) can be explored to manage many neuropsychiatric disorders.

Recently, anti-nociceptive activity INDCA-NS against migraine-like pain[21,22] and chronic unpredictable mild stress[23] has been reported in laboratory animals. However, toxicity information of INDCA-NS will be essential before clinical exploration to manage neuropsychiatric and stress-induced conditions in a clinical setup. Therefore, the present study investigated acute and subacute toxicity of intranasal administration of INDCA-NS in rats using international guidelines recommended by the Organisation for Economic Co-operation and Development (OECD).

2. Materials and Methods

2.1 Experimental Animals

Wistar rats (125-230 g, either sex, 6 to 7 weeks of age) were purchased from Vivo Bio Tech Ltd. Telangana, India, and used for the study. The rats were housed in sterilized solid bottom polypropylene cages with stainless steel grill tops. The cages were provided with food and water bottle arrangements with clean and sterilized paddy husk bedding. Rats were provided with filtered potable water in sterilized bottles with stainless steel sipper tubes. The rats were acclimatized for a minimum of six days before dosing. The markings were done on cages and individual rats for identification. Rats were fed on pelleted feed (M/s Altromin Spezialfutter GmbH & Co. KG, Germany). The rats were maintained at ambient temperature (19-25 °C) and relative humidity (30-70 %) with 12 h: 12 h of the dark: light cycle. The female rats were nulliparous and non-pregnant. The study protocols were approved by the Institutional Animal Ethics Committee (IAEC) of INTOX Pvt Ltd, Pune, India (Approval number: 17501/17502).

2.2 The Test Solution, INDCA-NS

The INDCA powder (an active ingredient) and INDCA-NS (the test solution) were supplied by Indus Biotech Limited. Pune. The INDCA-NS, a solution of INDCA powder (1 mg/ml), was made in saline and a preservative (benzalkonium chloride). The maximum volume administered intranasal administration in the rats is reported as 50 µl per rat[24]. Therefore, INDCA-NS (1 mg/ml, i.e., 1000 µg/ml) was suitably diluted further to prepare for different doses of INDCA-NS in the same volume to maintain 25 µl solution per nostril.

2.2.1 Characterization of INDCA and INDCA-NS

The samples of INDCA for HPLC analysis were prepared by making a stock solution by dissolving 10 mg of INDCA powder in a minimum amount of methanol and making the volume 10 ml with methanol. The aliquot of 1.5 ml is withdrawn and made up the volume to 10 ml with methanol. The reference standard solutions were prepared by dissolving 1.5 mg of each standard (asiaticoside, madecassoside, asiatic acid and madecassic acid) in a minimum amount of methanol and made up the volume to 10 ml with methanol. The samples of INDCA-NS for HPLC analysis were prepared by withdrawing an aliquot of 1.5 ml of INDCA-NS (1 mg/ml) and making up the volume to 10 ml with methanol.

The INDCA and INDCA-NS samples were analyzed for marker compounds, total and individual triterpenoids, namely asiaticoside, madecassoside, asiatic acid and madecassic acid by HPLC with the following details: HPLC system: Jasco HPLC LC 2000 equipped with AS-2055 auto-sampler, detector- UV-2075, Data acquisition software: JASCO ChromPass, column: C18 (150 mm X 4.6mm, 5µ), The mobile phase: (A) distilled water and (B) acetonitrile, at 0 min (75: 25), 0 to 30 min (45:55), 30 to 35 min (45:55), 35 to 45 min (75:25) using gradient elution. Flow rate :1.4 ml/min, at 220 nm, runtime: 45 min. The results were expressed as a percentage of individual and total triterpenoids (sum of % of asiaticoside, madecassoside, asiatic acid and madecassic

acid) by comparing HPLC fingerprinting of INDCA and INDCA-NS with individual reference standards.

2.3 Acute Intranasal Toxicity (AINT) Study

The present study evaluated the acute toxicity of INDCA-NS as per OECD Guideline (test No. 423) using the intranasal route of administration[25]. The 25 µl INDCA-NS was intranasally instilled per nostrils twice for one day (total = 100 µl per rat) to five rats per sex. The observation period of 14 days was followed for any signs of morbidity or mortality.

2.4 Subacute (28-Day Repeated Dose) Intranasal Toxicity Study

The OECD guideline for the 28-day repeated dose oral toxicity in rodents (Test No. 407)[26] was used to evaluate subacute intranasal toxicity of INDCA-NS. The sixty rats were randomly divided into six groups (5 rats per group of each sex, a total of 60 rats). The INDCA-NS (1 mg/ml) was suitably diluted to prepare different doses (10, 30 or 100 µg per day). The volume of 25 µl was suitably diluted solutions were instilled twice a day at approximately the same time within 4 h intervals.

The final delivered doses of INDCA-NS are 100 µg (highest dose), 30 µg (middle dose) and 10 µg (lowest dose) per rat per day in two equally divided doses with the concurrent groups of vehicles as per the following scheme:

- G1 - VC (Saline, 100 µl/rat/day, 28 days) - 5 rats/sex
- G2 - INDCA-NS-10 (INDCA-NS, 10 µg/rat/day, 28 days) - 5 rats/sex
- G3 - INDCA-NS-30 (INDCA-NS, 30 µg/rat/day, 28 days) - 5 rats/sex
- G4 - INDCA-NS-100 (INDCA-NS, 100 µg/rat/day, 28 days) - 5 rats/sex
- G1R - VC-R (VC reversal) (Saline, 100 µl/rat/day, 43 days) - 5 rats/sex
- G4R - INDCA-NS-100-R (INDCA-NS, 100 µg/rat/day for 28 days) followed by 14 days of vehicle (saline) at 100 µl/rat/day, from day 29 to day 43) - 5 rats/sex

2.4.1 Clinical Signs, Mortality, Body Weight, and Food Consumption

Clinical signs and mortality were checked daily for all rats. Every rat was checked for detailed clinical examination before initiation of treatment and weekly during the treatment and reversal period. Each rat's body weight and food consumption were recorded weekly throughout the study period.

2.4.2 Buried Food Test

All rats were assessed for a buried food test to assess their ability to smell[27]. The buried food test is based on the animal's natural tendency to use olfactory cues for foraging and is used to confirm the ability to smell different odors. The latency or the time to find buried food (one pallet) that was hidden underneath a layer of bedding after fasting overnight during buried food test was considered as measure of smell ability[27]. The latency time of smell of rats was measured during buried food test, before the initiation of treatment and weekly during the treatment and the reversal period as follow: Rats were fasted for 18 to 20 h before the test and provided with water *ad-libitum*. On the test day, each rat was placed in a clean cage containing 3 cm deep clean bedding and was acclimatized to the cage for 5 min. Post acclimatization for 5 minutes, the rat was transferred to an empty cage. The measured quantity of food (2 pellets) was buried approximately 1 cm beneath the bedding surface at one corner of the cage. The surface of the bedding was smoothened. Each rat was re-introduced to the cage, and the timer started. The timer was stopped, and the time was recorded once the rat found the buried food. If the animal failed to find the buried food after 15 min, the test was stopped, and a time of 15 min was recorded[27].

2.4.3 Hematology, Clinical Chemistry and Urinalysis

Blood samples (for hematology and serum chemistry) and urine samples (for urinalysis) were collected from all the rats towards the end of the 28-day study and the end of the 14-day reversal period (for reversal groups) before the scheduled necropsy. For hematological assessment, Hemoglobin (Hb), Packed Cell Volume (PCV), Red Blood Corpuscles (RBC), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin Concentration (MCHC), Total Leukocyte (white blood corpuscles) Count (TLC), % cells in Differential Leukocyte Count (DLC - Neutrophils (N), Lymphocytes (L), Monocytes (M), Eosinophils (E), Basophils (B) and Platelet Count, Reticulocytes, Activated Partial Thromboplastin Time (APTT), Prothrombin

time (Pt) were determined. Similarly, for serum biochemistry - total protein, albumin, globulin, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), blood glucose, Blood Urea Nitrogen (BUN), urea, creatinine, total cholesterol, Gamma Glutamyl Transpeptidase (GGT), total bile acid, triglycerides, total bilirubin, Sodium (Na), Potassium (K), Calcium (Ca), Phosphorus (P), Albumin/Globulin (A/G) ratio (calculated) were assessed. Qualitative urinalysis was performed for color, appearance, specific gravity, pH, protein, glucose, ketone, bilirubin, urobilinogen, nitrite, occult blood, leucocytes, and microscopically analyzed for epithelial cells, crystals, casts, and other abnormal constituents if any.

2.4.4 Necropsy, Organ Weights, and Histopathology

On completion of the treatment, all rats were sacrificed by exsanguinations under CO_2-induced deep anesthesia, and a necropsy was performed on each rat. Organs (adrenals, brain, ovaries, heart, uterus, liver, spleen, kidneys, testes, epididymides, and thymus) were isolated to determine their absolute and relative weights. The histopathological evaluation was performed with light microscopy after preservation (using 10% neutral buffered formalin), fixation (with paraffin wax) and staining with Hematoxylin and Eosin (H&E). Testes and eyes were collected in modified Davidson's fixative solution. Histopathological examination was conducted on all rats from groups G1 (VC) and G4 (INDCA-NS-100) sacrificed at termination. Histopathological examination was conducted on the brain (cerebrum, cerebellum, midbrain), spinal cord, eyes, pituitary, thyroid, parathyroid, spleen, thymus, adrenals, pancreas, trachea (at least two levels including 1) longitudinal section through the carina and 2) transverse section, lungs (all lobes at one level, including main bronchi), larynx (3) levels including one level for the base of the epiglottis), heart, aorta, esophagus, stomach, duodenum, jejunum, terminal ileum (with Peyer's patch), colon, rectum, liver, kidneys, urinary bladder, prostate, seminal vesicle, epididymis, testes, ovaries, uterus with a cervix, vagina, skin, sciatic nerve, bone marrow (smear), mammary gland (females), mesenteric lymph node, axillary lymph node, salivary gland, mandibular lymph node and specifically on nasal passage at four levels (including a level to include the nasopharyngeal duct and the nasal associated lymphoid tissue and including all macroscopically abnormal tissues.

2.5 Statistical Analysis

All values were represented as mean ± Standard Deviation (SD). The data of each parameter was analyzed by one-way ANOVA followed by treatment group comparisons by Dunnett's test (INDCA-NS treated v/s VC) and Student's t-test (INDCA-NS-100-R v/s VC-R). Statistical analysis was performed using SPSS version 23.0 (SPSS Inc, Chicago, Illinois, USA). Statistical significance at $p < 0.05$ was considered significant.

3. Results

3.1 The Characterization of INDCA and INDCA-NS

The HPLC chromatograms of INDCA powder, INDCA-NS, and reference standards of marker triterpenoid compounds, namely Asiaticoside, Madecassosides, Asiatic Acid, and Madecassic Acid, are presented in Figure 1. The chromatogram of INDCA powder showed two major peaks, namely C1 and C2, corresponding to RT at 5.442 and 8.258 mins, respectively. These peaks were identified as Madecassoside and Asiaticoside by comparing with HPLC of standard Madecassoside at RT 5.533 min and standard Asiaticoside at RT 8.242 min. The peaks for Madecassic Acid and Asiatic Acid were not observed in the HPLC of INDCA (peaks of standard Madecassic Acid at RT 20.717 min and standard Asiatic Acid at RT 25.992 min). The total triterpenoid content in the sample of INDCA was 60.85%, with 23.39% Madecassosides and 37.46% Asiaticoside, whereas other markers, namely Asiatic Acid and Madecassic Acid, were absent.

The chromatogram of the INDCA-NS solution showed two major peaks, namely C1 and C2, corresponding to RT at 5.292 and 8.133 mins. The peak C1 was identified as Madecassoside, and C2 was identified as Asiaticoside by comparing with HPLC of standard Madecassoside at RT 5.533 min. and standard Asiaticoside at RT 8.242 min. The peaks for Madecassic Acid and Asiatic Acid were not observed in INDCA compared with HPLC of standard Madecassic Acid (RT 20.717 min) and standard Asiatic Acid (RT 25.992 min). The total triterpenoids content in INDCA-NS was 61.4%, with 26.24% Madecassosides and 35.17% Asiaticoside, whereas other markers, namely Asiatic Acid and Madecassic Acid, were absent.

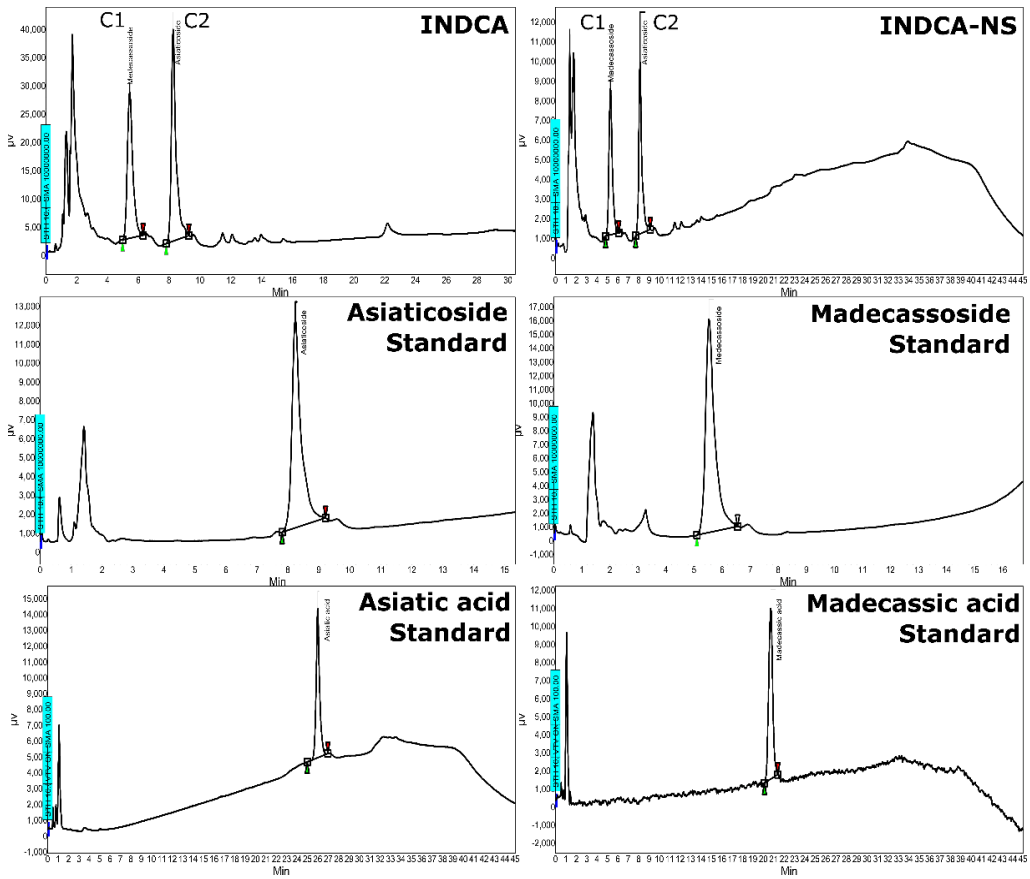


Figure 1. HPLC fingerprints of INDCA and INDCA-NS with the reference standards of marker compounds (asiaticoside, madecassoside, asiatic acid and madecassic acid)

3.2 Acute Intranasal Toxicity (AINT) Study

The acute toxicity study of intranasal administration of INDCA-NS on rats of both sexes revealed the Maximum Tolerated Dose (MTD) greater than 100 µl/rat/day. INDCA-NS did not reveal any abnormal clinical signs among the rats treated at 100 µl/rat dose. All the treated rats survived through the 14 days of the observation period. The differences in body weight gain after the 14-day post-dosing period were not significant. No abnormalities were found in gross pathology and macroscopic examination of rats sacrificed at the termination of the study.

3.3 Subacute (28-Day Repeated Dose) Toxicity Study

3.3.1 Clinical Signs, Mortality, Body Weight, and Food Consumption

No mortality was observed in rats from VC and all INDCA-NS treated groups throughout the dosing period of 28 days and the post-dosing reversal period. No abnormal clinical signs were found in INDCA-NS and VC groups during the entire study period and 14 days of the reversal period. Functional observation tests were conducted on rats after 28 days and did not reveal any remarkable and treatment-related incidence of neurological or behavioral abnormalities. Necropsy did not reveal any treatment-attributable abnormalities. The body weights of rats from INDCA-NS treated groups were not significantly different from those of the VC group of respective sex (Table 1). The average daily food consumption from the treated group of rats did not show a significant difference as compared to the VC group during the study period.

3.3.2 Buried food test

The buried food test showed no significant change in the time required to find the buried food in INDCA-NS treated groups compared to the VC group. It reveals that the ability to smell volatile odors was not affected during the treatment and reversal period (Table 2).

Table 1. Effect of INDCA-NS on body weights gain (g) of rats (28-day repeated-dose intranasal treatment)

Tine in Weeks	Body weight gain (g)					
	VC	INDCA-NS-10	INDCA-NS-30	INDCA-NS-100	VC-R	INDCA-NS-100-R
Male						
1	16.64 ± 5.61	17.62 ± 4.17	14.03 ± 9.82	19.39 ± 8.65	17.92 ± 3.32	19.78 ± 8.02
2	13.84 ± 7.88	9.08 ± 3.63	10.37 ± 6.85	6.58 ± 3.87	14.16 ± 7.44	9.96 ± 5.36
3	4.19 ± 1.43	7.20 ± 3.01	5.83 ± 3.07	5.78 ± 1.93	5.90 ± 3.34	5.79 ± 1.45
4	10.77 ± 1.71	8.46 ± 2.92	8.90 ± 2.48	8.90 ± 0.61	9.41 ± 1.84	7.72 ± 1.53
5	-	-	-	-	4.21 ± 2.24	4.86 ± 1.29
6	-	-	-	-	5.57 ± 1.75	4.70 ± 1.08
Female						
1	11.13 ± 2.11	9.81 ± 2.17	10.41 ± 3.69	11.32 ± 1.85	8.71 ± 5.54	10.84 ± 4.31
2	6.84 ± 2.03	6.69 ± 2.82	8.37 ± 3.97	6.34 ± 1.68	8.80 ± 5.24	6.91 ± 2.62
3	7.66 ± 1.87	6.89 ± 1.91	8.54 ± 3.12	8.10 ± 4.31	9.41 ± 8.06	6.63 ± 2.85
4	3.59 ± 1.63	3.31 ± 1.33	4.23 ± 1.57	4.31 ± 1.45	4.34 ± 1.49	3.40 ± 1.13
5	-	-	-	-	5.09 ± 2.29	4.99 ± 1.89
6	-	-	-	-	2.52 ± 1.46	3.12 ± 0.92

Data were represented as Mean ± Standard Deviation (SD). Comparisons with respective days are as follows: INDCA-NS-10, INDCA-NS-30, and INDCA-NS-100 v/s. VC; INDCA-NS-100-R v/s. VC-R, n= 5/sex

Table 2. Effect of INDCA-NS on time to find buried food (min) in rats (28-day repeated-dose intranasal treatment)

Days	Time to find buried food (min)					
	VC	INDCA-NS-10	INDCA-NS-30	INDCA-NS-100	VC-R	INDCA-NS-100-R
Male						
0	3.60 ± 4.72	2.40 ± 1.34	2.20 ± 2.17	4.60 ± 4.51	2.00 ± 1.73	2.40 ± 2.07
8	3.00 ± 1.87	2.00 ± 1.73	6.00 ± 4.06	7.20 ± 7.12	2.60 ± 1.14	8.20 ± 4.97
15	6.40 ± 5.32	5.00 ± 2.55	7.00 ± 5.43	9.00 ± 5.43	6.60 ± 0.89	4.80 ± 4.44
22	7.40 ± 4.39	5.80 ± 3.11	5.40 ± 4.98	3.80 ± 5.72	4.60 ± 3.29	3.00 ± 3.94
29	4.60 ± 1.14	5.00 ± 2.35	6.60 ± 2.79	4.40 ± 3.51	5.80 ± 2.86	2.60 ± 1.82
36					4.00 ± 3.74	3.60 ± 4.77
43					4.20 ± 1.10	5.60 ± 4.16
Female						
0	1.40 ± 0.55	2.80 ± 2.17	1.20 ± 0.45	1.20 ± 0.45	2.00 ± 1.00	1.40 ± 0.55
8	1.40 ± 0.89	3.00 ± 4.47	1.40 ± 0.89	1.40 ± 0.89	1.40 ± 0.55	1.80 ± 1.79
15	3.20 ± 2.39	3.60 ± 2.61	1.00 ± 0.00	2.40 ± 1.14	1.60 ± 0.55	1.40 ± 0.89
22	4.00 ± 3.67	3.20 ± 3.83	1.60 ± 0.89	1.40 ± 0.89	3.40 ± 2.51	1.40 ± 0.89
29	4.60 ± 2.70	1.60 ± 0.89	1.80 ± 0.84	4.80 ± 3.63	2.80 ± 2.95	3.40 ± 2.88
36					1.60 ± 0.89	1.60 ± 0.55
43					1.60 ± 0.89	1.60 ± 0.55

Data were represented as Mean ± Standard Deviation (SD). Comparisons with respective days are as follows: INDCA-NS-10, INDCA-NS-30, and INDCA-NS-100 v/s. VC; INDCA-NS-100-R v/s. VC-R, n = 5/sex

3.3.3 *Hematology, Clinical Chemistry and Urinalysis*

The subacute intranasal treatment of INDCA-NS did not reveal any significant treatment-related differences in hematological (Table 3 and Table 4) and blood chemistry (Table 5 and Table 6) on day 29 and day 43 (at the end of the reversal period) parameters as compared with respective VC group in separate groups of male and female rats. However, few significant changes were observed in males (PT, albumin, total bile acid and A/G ratio) and females ((% E, glucose, cholesterol, Na and K) in rats from the recovery groups (INDCA-NS-1000-R group v/s VC-R). All the parameters, including those with significant differences, were within the normal historical control of the test facility (for example, albumin - 1.0 to 1.50 g/dL, A/G ratio - 0.2 to 0.3, Na - 138 to 158 mmol/L, total bile acid - 8.59 to 40.46 µmol/L, glucose - 43 to 128 mg/dL, total cholesterol - 31 to 76 mg/dL and K - 4.2 to 6.0 mmol/L). Therefore, these changes were found incidental and of no toxicological significance.

The urinalysis data did not reveal any abnormal observations in INDCA-NS treated groups. The qualitative and semi-quantitative urine parameters such as color, appearance, specific gravity, pH, urobilinogen, protein, glucose, ketones, nitrite, occult blood, bilirubin, leucocytes, and microscopic examination for epithelial cells, crystals, casts, abnormal constituents' observations recorded and no significant difference was found between the INDCA-NS and respective VC groups.

Table 3. Effect of INDCA-NS on hematological parameters (28-day repeated-dose intranasal treatment) - Male rats

Parameters	Group					
	VC	INDCA-NS-10	INDCA-NS-30	INDCA-NS-100	VC-R	INDCA-NS-100-R
Hb (g/dl)	16.08 ± 1.04	16.18 ± 1.65	17.50 ± 1.22	16.60 ± 0.73	16.32 ± 0.95	15.72 ± 0.49
PCV (%)	45.26 ± 4.46	46.62 ± 6.26	49.70 ± 3.67	47.40 ± 1.62	46.10 ± 2.80	45.06 ± 1.04
RBC (x10^6 /mm^3)	8.56 ± 0.57	9.02 ± 0.52	9.17 ± 0.66	8.93 ± 0.49	8.92 ± 0.68	8.65 ± 0.45
MCH (pg)	18.34 ± 0.59	19.80 ± 0.95	18.98 ± 0.60	18.98 ± 0.70	18.34 ± 0.59	18.22 ± 1.09
MCV (fl)	52.42 ± 2.44	56.16 ± 2.82	53.34 ± 1.89	53.08 ± 1.81	51.82 ± 1.72	52.22 ± 2.44
MCHC (g/dl)	35.04 ± 0.61	35.38 ± 0.76	35.76 ± 0.98	35.72 ± 0.42	35.44 ± 0.17	34.92 ± 0.73
TLC (x 10^3 /mm^3)	4.15 ± 1.42	4.28 ± 0.99	2.82 ± 1.40	4.40 ± 1.97	4.24 ± 1.64	3.30 ± 1.04
DLC						
N (%)	25.78 ± 4.72	17.14 ± 7.20	16.30 ± 6.92	19.42 ± 10.10	24.54 ± 4.34	24.54 ± 6.61
L (%)	72.38 ± 5.20	80.82 ± 9.43	82.06 ± 7.73	78.00 ± 9.19	72.52 ± 5.58	72.58 ± 7.89
M (%)	0.35 ± 0.32	0.49 ± 0.50	0.09 ± 0.19	0.25 ± 0.24	0.42 ± 0.38	0.39 ± 0.38
E (%)	1.47 ± 1.48	0.75 ± 1.03	1.56 ± 1.45	2.11 ± 1.40	2.50 ± 1.71	2.06 ± 1.30
B (%)	0.02 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.23 ± 0.50	0.01 ± 0.02	0.00 ± 0.00
Platelets (x 10^3/ mm^3)	534.00 ± 188.54	472.60 ± 134.66	321.20 ± 189.51	638.60 ± 184.37	516.40 ± 155.70	633.40 ± 152.09
Reticulocytes (%)	1.89 ± 0.56	1.97 ± 0.63	1.69 ± 0.19	1.71 ± 0.30	2.84 ± 0.88	2.87 ± 0.97
APTT (sec)	12.90 ± 2.13	14.42 ± 3.15	17.28 ± 1.19	15.60 ± 2.69	15.78 ± 2.58	16.70 ± 1.50
PT (sec)	17.72 ± 2.07	15.50 ± 2.89	15.48 ± 1.37	15.52 ± 1.88	20.42 ± 2.55	16.46* ± 2.26

Data were represented as Mean ± Standard Deviation (SD). * P < 0.05. Comparisons with respective days are as follows: INDCA-NS-10, INDCA-NS-30, and INDCA-NS-100 v/s. VC; INDCA-NS-100-R v/s. VC-R, n= 5/sex. Hb: Hemoglobin; PCV: Packed Cell Volume, RBC: Red Blood Corpuscles, MCH: Mean Corpuscular Hemoglobin, MCV: Mean Corpuscular Volume, MCHC: Mean Corpuscular Hemoglobin Concentration, TLC: Total leukocyte (White Blood Corpuscles) count, N: Neutrophils, L: Lymphocytes, M: Monocytes, E: Eosinophils, B: Basophils, APTT: Activated Partial Thromboplastin Time, PT.: Prothrombin time

Table 4. Effect of INDCA-NS on haematological parameters (28-day repeated-dose intranasal treatment)- Female rats

Parameters	Group					
	VC	INDCA-NS-10	INDCA-NS-30	INDCA-NS-100	VC-R	INDCA-NS-100-R
Hb (g/dl)	15.52 ± 0.45	15.60 ± 0.35	15.36 ± 0.26	15.48 ± 0.61	15.54 ± 0.34	14.64 ± 1.52
PCV (%)	43.66 ± 1.83	43.68 ± 1.37	43.30 ± 0.71	43.68 ± 1.93	43.72 ± 0.94	41.42 ± 2.42
RBC (x10^6 /mm^3)	7.93 ± 0.39	8.38 ± 0.18	7.82 ± 0.33	7.94 ± 0.30	8.25 ± 0.23	7.92 ± 0.48
MCH (pg)	19.66 ± 0.93	18.60 ± 0.39	19.70 ± 0.74	19.48 ± 0.53	18.88 ± 0.23	18.48 ± 1.16
MCV (fl)	55.16 ± 2.43	52.10 ± 1.38	55.48 ± 2.02	55.00 ± 1.31	52.98 ± 0.62	52.34 ± 1.49
MCHC (g/dl)	35.54 ± 0.54	35.72 ± 0.43	35.68 ± 0.43	35.44 ± 0.46	35.64 ± 0.36	35.32 ± 1.80
TLC (x 10^3 /mm^3)	6.24 ± 1.27	3.86 ± 0.39	3.65 ± 1.34	3.82 ± 0.63	2.44 ± 0.81	3.53 ± 1.76
DLC						
N (%)	23.96 ± 6.70	16.98 ± 2.10	21.56 ± 5.25	21.28 ± 2.52	17.30 ± 6.64	18.20 ± 8.07
L (%)	72.50 ± 7.51	80.12 ± 1.38	76.70 ± 6.26	76.00 ± 2.25	79.46 ± 7.53	80.40 ± 8.68
M (%)	0.51 ± 0.25	0.62 ± 0.39	0.42 ± 0.44	0.66 ± 0.25	0.34 ± 0.38	0.00 ± 0.00
E (%)	3.02 ± 0.98	2.29 ± 1.60	1.31 ± 1.91	2.03 ± 1.15	2.88 ± 1.20	1.60 ± 1.34*
B (%)	0.01 ± 0.02	0.01 ± 0.01	0.00 ± 0.00	0.03 ± 0.05	0.33 ± 0.74	0.00 ± 0.00
Platelets (x 10^3 / mm^3)	718.60 ± 230.04	726.00 ± 202.83	784.20 ± 85.36	861.60 ± 109.21	435.80 ± 251.01	650.60 ± 276.44
Reticulocytes (%)	3.75 ± 0.92	3.25 ± 1.26	3.10 ± 1.81	4.34 ± 1.62	3.64 ± 0.38	2.91 ± 0.94
APTT (sec)	11.92 ± 0.62	15.58 ± 4.71	14.58 ± 1.08	14.18 ± 2.44	15.38 ± 4.05	16.38 ± 2.61
PT (sec)	15.20 ± 0.37	15.96 ± 1.40	15.46 ± 0.69	16.18 ± 0.84	17.64 ± 4.19	18.80 ± 2.40

Data were represented as Mean ± Standard Deviation (SD). * P < 0.05. Comparisons with respective days are as follows: INDCA-NS-10, INDCA-NS-30, and INDCA-NS-100 v/s. VC; INDCA-NS-100-R v/s. VC-R, n = 5/sex. Hb: Hemoglobin; PCV: Packed Cell Volume, RBC: Red Blood Corpuscles, MCH: Mean Corpuscular Hemoglobin, MCV: Mean Corpuscular Volume, MCHC: Mean Corpuscular Hemoglobin Concentration, TLC: Total leukocyte (White Blood Corpuscles) count, N: Neutrophils, L: Lymphocytes, M: Monocytes, E: Eosinophils, B: Basophils, APTT: Activated Partial Thromboplastin Time, PT.: Prothrombin time

Table 5. Effect of INDCA-NS on blood chemistry (28-day repeated-dose intranasal treatment) - Male rats

Parameters	Group					
	VC	INDCA-NS-10	INDCA-NS-30	INDCA-NS-100	VC-R	INDCA-NS-100-R
Total Protein (g/dL)	6.08 ± 0.42	6.34 ± 0.27	6.38 ± 0.18	6.36 ± 0.29	6.78 ± 0.13	6.98 ± 0.24
Albumin (g/dL)	1.24 ± 0.17	1.32 ± 0.13	1.28 ± 0.08	1.32 ± 0.11	1.26 ± 0.09	1.40 ± 0.07*
Globulin (g/dL)	4.84 ± 0.32	5.02 ± 0.27	5.10 ± 0.19	5.04 ± 0.26	5.52 ± 0.08	5.58 ± 0.22
ALT(IU/L)	43.00 ± 10.61	40.20 ± 7.50	39.20 ± 6.22	42.20 ± 4.21	44.20 ± 4.32	51.40 ± 5.59
AST (IU/L)	80.60 ± 7.23	73.60 ± 10.33	75.80 ± 6.83	83.40 ± 14.01	74.00 ± 6.20	86.20 ± 10.62
ALP (IU/L)	125.60 ± 16.86	121.60 ± 20.62	105.80 ± 13.08	110.00 ± 30.31	97.00 ± 4.00	98.80 ± 17.40
Glucose (mg/dL)	77.00 ± 7.97	84.80 ± 11.56	89.00 ± 10.84	77.20 ± 4.60	75.20 ± 8.93	82.00 ± 12.98
BUN (mg/dL)	16.00 ± 1.41	19.40 ± 1.52	22.20 ± 3.56	21.00 ± 2.74	20.20 ± 2.77	20.40 ± 1.95
Urea (mg/dL)	34.24 ± 3.03	41.52 ± 3.25	47.51 ± 7.63	44.94 ± 5.86	43.23 ± 5.94	43.66 ± 4.17
Creatinine (mg/dL)	0.43 ± 0.03	0.47 ± 0.02	0.57 ± 0.06	0.53 ± 0.06	0.53 ± 0.06	0.60 ± 0.13
Cholesterol (mg/dL)	55.80 ± 4.97	54.60 ± 8.47	60.00 ± 6.28	60.00 ± 8.22	59.40 ± 5.41	61.80 ± 5.07

Table 5 continued...

Parameters	Group					
	VC	INDCA-NS-10	INDCA-NS-30	INDCA-NS-100	VC-R	INDCA-NS-100-R
GGT (IU/L)	6.00 ± 0.71	5.80 ± 0.45	5.40 ± 0.55	5.80 ± 0.84	6.20 ± 0.84	5.80 ± 0.45
Total bile acid (µmol/L)	29.32 ± 22.00	13.78 ± 3.25	12.82 ± 8.30	21.76 ± 9.96	35.28 ± 7.28	25.31± 6.16*
Triglyceride (mg/dL)	73.80 ± 22.33	89.80 ± 44.00	87.00 ± 22.96	74.80 ± 15.88	89.00 ± 19.30	89.40 ± 22.50
Total Bilirubin (mg/dL)	0.02 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.08	0.18 ± 0.04
Na (mmol/l)	145.20 ± 1.48	145.00 ± 1.00	144.40 ± 2.19	144.60 ± 0.89	147.20 ± 2.17	147.00 ± 2.12
K (mmol/l)	5.20 ± 0.57	5.44 ± 0.21	5.20 ± 0.44	5.60 ± 0.48	5.54 ± 0.43	5.66 ± 0.43
CA (mg/dL)	10.36 ± 0.29	10.58 ± 0.50	10.52 ± 0.36	10.76 ± 0.34	10.56 ± 0.18	11.34 ± 0.89
P (mg/dL)	7.04 ± 0.36	7.22 ± 0.79	7.52 ± 0.64	7.76 ± 0.97	6.52 ± 0.27	7.04 ± 0.50
A/G ratio	0.26 ± 0.03	0.26 ± 0.03	0.25 ± 0.02	0.26 ± 0.03	0.23 ± 0.02	0.25 ± 0.01*

Data were represented as Mean ± Standard Deviation (SD). * $P < 0.05$. Comparisons with respective days are as follows: INDCA-NS-10, INDCA-NS-30, and INDCA-NS-100 v/s. VC; INDCA-NS-100-R v/s. VC-R, n= 5/sex. ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, ALP: Alkaline Phosphatase, BUN: Blood Urea Nitrogen, GGT: Gamma Glutamyl Transferase, Na: Sodium, K: Potassium, Ca: Calcium, P: Phosphorus, A/G ratio: Albumin to Globulin ratio

Table 6. Effect of INDCA-NS on blood chemistry (28-day repeated-dose intranasal treatment) - Female rats

Parameters	Group					
	VC	INDCA-NS-10	INDCA-NS-30	INDCA-NS-100	VC-R	INDCA-NS-100-R
Total Protein (g/dL)	6.90 ± 0.20	6.90 ± 0.23	6.90 ± 0.21	7.08 ± 0.36	6.92 ± 0.28	7.04 ± 0.35
Albumin (g/dL)	1.54 ± 0.13	1.40 ± 0.07	1.56 ± 0.09	1.54 ± 0.15	1.46 ± 0.05	1.52 ± 0.11
Globulin (g/dL)	5.36 ± 0.18	5.50 ± 0.20	5.34 ± 0.19	5.54 ± 0.23	5.46 ± 0.27	5.52 ± 0.27
ALT(IU/L)	31.20 ± 7.60	27.40 ± 5.18	30.20 ± 6.53	30.40 ± 4.16	30.80 ± 3.35	28.80 ± 6.26
AST (IU/L)	69.00 ± 7.00	74.60 ± 8.91	76.40 ± 6.27	72.00 ± 6.67	67.00 ± 9.54	76.20 ± 4.32
ALP (IU/L)	62.20 ± 24.35	62.00 ± 11.90	55.40 ± 3.05	55.00 ± 9.97	35.00 ± 5.79	41.60 ± 13.15
Glucose (mg/dL)	81.40 ± 8.99	79.40 ± 7.64	84.80 ± 15.64	86.20 ± 3.90	90.00 ± 6.44	73.40± 7.83*
BUN (mg/dL)	17.80 ± 2.77	19.60 ± 3.29	21.20 ± 2.17	22.80 ± 3.83	18.80 ± 3.27	19.00 ± 3.08
Urea (mg/dL)	38.09 ± 5.94	41.94 ± 7.03	45.37 ± 4.64	48.79 ± 8.20	40.23 ± 7.00	40.66 ± 6.60
Creatinine (mg/dL)	0.50 ± 0.03	0.58 ± 0.06	0.58 ± 0.03	0.62 ± 0.08	0.57 ± 0.02	0.60 ± 0.07
Cholesterol (mg/dL)	51.40 ± 5.08	51.60 ± 10.60	51.00 ± 8.92	51.80 ± 17.28	44.80 ± 3.70	34.80 ± 7.40*
GGT (IU/L)	6.40 ± 0.89	6.40 ± 0.55	6.20 ± 0.84	5.80 ± 0.45	5.20 ± 0.84	6.00 ± 1.00
Total bile acid (µmol/L)	18.04 ± 9.10	8.68 ± 3.99	18.39 ± 12.78	19.19 ± 18.67	17.65 ± 4.31	30.87 ± 16.71
Triglyceride (mg/dL)	70.20 ± 17.43	65.20 ± 22.87	55.40 ± 11.15	64.80 ± 17.01	55.00 ± 6.12	53.20 ± 21.92
Total Bilirubin (mg/dL)	0.05 ± 0.06	0.00 ± 0.00	0.08 ± 0.05	0.00 ± 0.00	0.12 ± 0.04	0.16 ± 0.05
Na (mmol/l)	144.80 ± 0.84	143.80 ± 1.48	143.60 ± 0.89	145.40 ± 0.89	141.80 ± 0.45	143.80 ± 1.79*
K (mmol/l)	4.98 ± 0.25	5.08 ± 0.45	4.74 ± 0.42	4.90 ± 0.33	5.24 ± 0.15	4.96 ± 0.18*
CA (mg/dL)	10.92 ± 0.16	10.78 ± 0.30	10.74 ± 0.17	11.02 ± 0.28	10.44 ± 0.19	10.42 ± 0.31

Table 6 continued...

Parameters	Group					
	VC	INDCA-NS-10	INDCA-NS-30	INDCA-NS-100	VC-R	INDCA-NS-100-R
P (mg/dL)	5.94 ± 0.59	5.74 ± 0.26	5.60 ± 0.32	5.58 ± 0.86	4.34 ± 0.48	4.58 ± 0.31
A/G ratio	0.29 ± 0.03	0.25 ± 0.01	0.29 ± 0.02	0.28 ± 0.02	0.27 ± 0.02	0.28 ± 0.01

Data were represented as Mean ± Standard Deviation (SD). Comparisons with respective days are as follows: INDCA-NS-10, INDCA-NS-30, and INDCA-NS-100 v/s. VC; INDCA-NS-100-R v/s. VC-R, n= 5/sex. ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, ALP: Alkaline Phosphatase, BUN: Blood Urea Nitrogen, GGT: Gamma Glutamyl Transferase, Na: Sodium, K: Potassium, Ca: Calcium, P: Phosphorus, A/G ratio: Albumin to Globulin ratio

3.3.4 Necropsy, Organ Weights and Histopathology

The mean values of the relative organ weights of adrenals, testes, ovaries, kidneys, liver, brain, thymus, heart, spleen, epididymis, and uterus of rats treated with INDCA-NS did not show significant differences with respective VC groups (Table 7 and Table 8).

All the organs examined after necropsy did not show any gross pathological alterations in male and female rats of INDCA-NS treated groups as compared to respective VC groups. The summary of observations from histopathological examination of different organs from INDCA-NS-100 treated and VC groups has been tabulated in Table 9. The microscopic examination of other tissues revealed isolated observations of lymphocytic aggregation in lungs (1/5 in VC-male), corticular vacuolation (2/5 and 1/5 in VC-male and INDCA-NS-100-males, respectively) and accessory tissue in adrenals (1/5 in INDCA-NS-100-female); periportal lymphoid cell infiltration in the liver (2/5 in VC-male); basophilic tubules (1/5 in VC-males) in kidneys, lymphocytic infiltration in the renal pelvis (1/5 each in VC-female and INDCA-NS-100-females); dilation of the renal pelvis (1/5 in INDCA-NS-100-males); lymphoid infiltration in the prostate (1/5 each in VC-male and INDCA-NS-100-male); submucosal lymphocytic infiltration in the glandular stomach (1/5 in VC-female); lymphocytic aggregation in submucosal area of the rectum (1/5 in VC- male); a squamous cyst in mammary glands in females (2/5 in VC-females). The observed histopathological findings were incidental with minimal severity and randomly distributed between both treatment groups without any correlation with treatment or sex, so they were not considered of toxicological importance.

Table 7. Effect of INDCA-NS on relative organ weights (g) (28-day repeated-dose intranasal treatment) - Male rats

Parameters	Group					
	VC	INDCA-NS-10	INDCA-NS-30	INDCA-NS-100	VC-R	INDCA-NS-100-R
Adrenals	0.015 ± 0.001	0.016 ± 0.002	0.016 ± 0.002	0.018 ± 0.001	0.016 ± 0.002	0.017 ± 0.004
Testes	1.22 ± 0.14	1.16 ± 0.11	1.23 ± 0.14	1.22 ± 0.10	1.04 ± 0.09	1.11 ± 0.11
Kidneys	0.79 ± 0.08	0.72 ± 0.04	0.70 ± 0.05	0.73 ± 0.05	0.68 ± 0.09	0.65 ± 0.03
Liver	3.07 ± 0.18	2.86 ± 0.13	2.80 ± 0.31	3.01 ± 0.14	2.59 ± 0.27	2.52 ± 0.18
Brain	0.81 ± 0.07	0.81 ± 0.05	0.82 ± 0.09	0.82 ± 0.05	0.70 ± 0.05	0.72 ± 0.03
Thymus	0.20 ± 0.03	0.21 ± 0.03	0.17 ± 0.02	0.19 ± 0.04	0.16 ± 0.02	0.17 ± 0.02
Heart	0.36 ± 0.01	0.34 ± 0.03	0.35 ± 0.03	0.35 ± 0.01	0.33 ± 0.01	0.34 ± 0.02
Spleen	0.22 ± 0.02	0.22 ± 0.02	0.20 ± 0.02	0.20 ± 0.03	0.20 ± 0.04	0.20 ± 0.02
Epididymis	0.39 ± 0.03	0.39 ± 0.03	0.42 ± 0.05	0.40 ± 0.02	0.37 ± 0.04	0.41 ± 0.03

Data were represented as Mean ± Standard Deviation (SD). Comparisons with respective days are as follows: INDCA-10, INDCA-30, and INDCA-100 v/s. VC; INDCA-100-R v/s. VC-R, n= 5/sex.

Table 8. Effect of INDCA-NS on relative organ weights (g) (28-day repeated-dose intranasal treatment) - Female rats

Organ	Group					
	VC	INDCA-NS-10	INDCA-NS-30	INDCA-NS-100	VC-R	INDCA-NS-100-R
Adrenals	0.029 ± 0.004	0.030 ± 0.001	0.033 ± 0.002	0.029 ± 0.003	0.032 ± 0.006	0.031 ± 0.003
Ovaries	0.064 ± 0.004	0.065 ± 0.004	0.071 ± 0.006	0.053 ± 0.006	0.051 ± 0.010	0.056 ± 0.009
Kidneys	0.79 ± 0.05	0.74 ± 0.06	0.77 ± 0.07	0.74 ± 0.04	0.71 ± 0.05	0.70 ± 0.05
Liver	3.14 ± 0.29	2.97 ± 0.25	2.93 ± 0.19	2.84 ± 0.20	2.81 ± 0.26	2.78 ± 0.16
Brain	1.05 ± 0.03	1.10 ± 0.03	1.09 ± 0.08	1.07 ± 0.06	0.92 ± 0.04	0.93 ± 0.05
Thymus	0.28 ± 0.03	0.22 ± 0.05	0.27 ± 0.03	0.26 ± 0.01	0.20 ± 0.06	0.23 ± 0.05
Heart	0.38 ± 0.03	0.38 ± 0.01	0.40 ± 0.00	0.37 ± 0.03	0.36 ± 0.03	0.34 ± 0.03
Spleen	0.28 ± 0.03	0.26 ± 0.03	0.26 ± 0.04	0.27 ± 0.03	0.24 ± 0.03	0.22 ± 0.02
Uterus	0.24 ± 0.07	0.27 ± 0.04	0.30 ± 0.06	0.26 ± 0.05	0.26 ± 0.10	0.29 ± 0.12

Data were represented as Mean ± Standard Deviation (SD). Comparisons with respective days are as follows: INDCA-NS-10, INDCA-NS-30, and INDCA-NS-100 v/s. VC; INDCA-NS-100-R v/s. VC-R, n= 5/sex.

Table 9. Summary of histopathology findings

Parameter	Male		Female	
	G1	G5	G1	G5
	VC	INDCA-NS-100	VC	INDCA-NS-100
Number of rats Examined	5	5	5	5
Lungs: Aggregation, lymphocytic, minimal	1	NAD	NAD	NAD
Adrenal: Vacuolation, corticular, minimal	2	1	NAD	NAD
Liver: Infiltration, lymphoid, periportal, minimal	2	NAD	NAD	NAD
Kidneys: Basophilic tubules, minimal	1	NAD	NAD	NAD
Kidneys: Infiltration, lymphocytic, pelvis, minimal	NAD	NAD	1	1
Kidneys: Dilation of pelvis, unilateral	NAD	1	NAD	NAD
Prostate: Infiltration, lymphoid, minimal	1	1	-	-
Stomach: Infiltration, lymphocytic, submucosal, minimal	NAD	NAD	1	NAD
Rectum: Lymphocytic aggregation, submucosal, minimal	1	NAD	NAD	NAD
Mammary gland (female): Squamous cyst	-	-	2	0
Nasal Passage: Hyperplasia, goblet cell, minimal	5	5	5	5

NAD = No Abnormality Detected.

In addition, detailed histopathology of the Nasal Passage (NP) mucosa from level-1 to level-4 was performed. The photomicrographs of representative sections of the mucosa of the nasal passage are presented in Figure 2. There were no significant histological changes in nasal mucosa except minimal goblet cell hyperplasia in the mucosal lining of nasal passage at NP level-1 in most rats (an expected adaptive response).

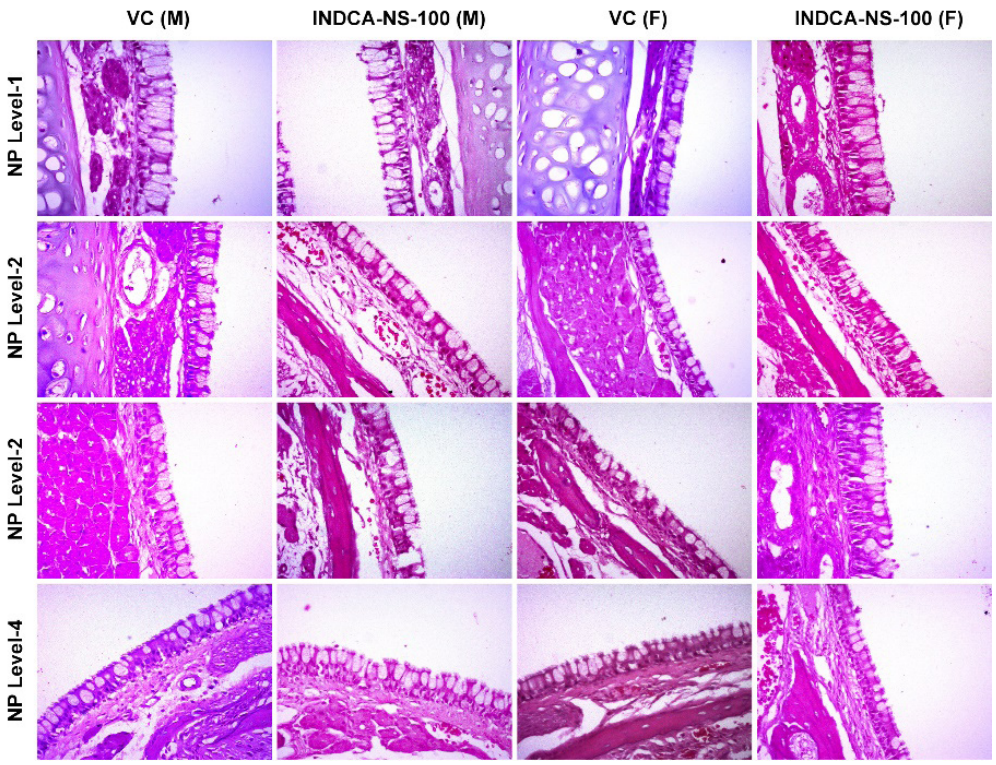


Figure 2. Photomicrograph of sections of the Nasal Passage (NP) of rats on 28-days subacute intranasal administration of vehicle and INDCA-NS (100 µl/rat/day). Magnification 40 X, Stain: Haematoxylin and Eosin. VC – Vehicle control.

4. Discussion

The use of complementary medicines to treat neuropsychiatric and stress related illnesses,such as anxiety and depression, has attracted considerable interest in recent years[28-30]. The leaves extracts of many plants have been reported to have efficacy against stress-related disorders[31,32]. At the same time, many leaves extracts had been evaluated for potential toxicities in animals before human consumption as a dietary supplement or complementary medicine[33-35]. CA leaves and extracts have a long history of medicinal use in traditional Chinese and Indian medicines and are scientifically explored in the modern scientific literature for neuropsychiatric disorders[6]. Total triterpenes from CA leaves have been reported to have anti-depressant[11,36], anxiolytic[37] and stress-relieving potential[37]. In addition, preclinical safety[38] and efficacy against animal models of depression, anxiety and stress-related disorders[37,39-43] of INDCA (standardized CA leaves extract, with major triterpenes, namely Asiaticoside and Madecassoside has been reported. Therefore, the total triterpenoid content of the sample of INDCA-NS used for the present study was analyzed and found to have 61.4% of total triterpenoids (35.17% Asiaticoside and 26.24% Madecassoside with the absence of Asiatic Acid and Madecassic Acid).

In addition, the present study reported the results of the safety assessment of INDCA-NS in rats conducted in accordance with OECD guidelines. The single and 28-day repeated intranasal exposure of INDCA-NS (100 µg/rat/day) with a 14-day recovery period did not show significant toxic effects compared with respective VC. In the past, the lifespan of 13.2 days for a rat is reported to be equivalent to 1 year of a human lifespan[44]. Therefore, the absence of toxicity during 28 days of subacute intranasal exposure to INDCA-NS in rats in the present study can be considered equivalent to nearly two years of human safety.

The possibility of systemic absorption and potential impact on the Hypothalamic-Pituitary-Adrenal (HPA) axis suppression has been implicated in nasal corticosteroids[45,46] The importance of coordinated functional interactions of the HPA axis in promoting adaptive responses to stress from anxiety or fear has

been emphasized in past studies in animal models[47,48]. The role of the HPA axis in the mechanism of orally administrated INDCA against anxiety and depression has been suggested in the past[11,12]. However, subacute intranasal administration of INDCA-NS did not reveal any behavioral abnormalities in functional observation tests in the present study.

In addition, detailed hematological, clinical chemistry, and urinalysis evaluations in separate groups of male and female rats in the present study suggested the absence of toxicity during the 28-day repeated dose administration of INDCA-NS. Few significant observations in rats from the INDCA-NS-1000-R group (recovery group) as compared to VC-R groups were observed in males (PT, albumin, total bile acid and A/G ratio) and females (% E, glucose, cholesterol, Na and K). These changes were observed only in the recovery group and not during the treatment period in INDCA-NS groups of either sex. There were no dose- or sex-dependent correlations. Furthermore, these changes did not accompany the corresponding changes in other related parameters (e.g., decreased PT with APTT levels, % E with other hematological parameters, and changes in albumin and A/G ratio with total protein levels). Serum bile acid levels are known to be inversely correlated with serum triglycerides[49,50]. However, no significant change in triglyceride levels was observed in the present study in any tested doses in either sex. Besides, a significant increase in Na and a decrease in K levels were not accompanied by any toxic effects in urinalysis, gross pathology, and histopathology of kidneys. Besides, all the values were found to be in the historical control ranges at the test facility. The body weights, gross pathology of organs and relative organ weights data showed no significant toxic effects of INDCA-NS on subacute internasal administration during treatment or recovery. Therefore, these isolated observations were considered incidental and had no toxicological significance.

As INDCA-NS was administrated by the nasal route, detailed histopathology of nasopharyngeal tissues up to 4 was performed. The minimal goblet cell hyperplasia in the respiratory epithelium lining the nasal septum at level 1 of the nasal passage in rats was observed in the present study. Such hyperplasia is an expected adaptive response to exposure to the irritant[51,52] and not a toxicological indication. Similarly, few incidental observations of other tissues in the microscopic examination aligned with the reported background lesions of congenital origin[52]. Therefore, changes in microscopic observations were considered incidental.

More than 200 commonly prescribed medicines, especially those consumed by the intranasal route, are associated with olfactory dysfunction[53-55]. Intranasal administration of some medications such as zinc[56] and macrolide antibiotics[57] are reported with olfactory dysfunction, leading to anosmia (loss of sense of smell). In the present study, intranasal subacute administration of INDCA-NS did not induce any olfactory dysfunction or toxic effect on smell sensation, as evident by the observations during the buried food test. The rats can distinguish between specific odors and the ability to sense of smell[58]. The buried food test evaluates the animal's natural tendency to use olfactory cues for foraging and is used to confirm the ability to smell volatile odors[27,59]. The latency to uncover a small piece of palatable food hidden beneath a layer of cage bedding within a limited time indicates normal olfactory function[27].

The subacute oral[60] and intranasal[23] administration of INDCA in rats were reported to prevent social isolation-induced stress and chronic unpredictable mild stress, respectively. The safety of INDCA-NS in terms of olfactory function in the present study will be a robust basis for developing a safe solution against stress-related disorders. Furthermore, the relationship between an olfactory function with stress[61,62], mood[63], and cognitive performance[64] has been suggested.

Overall, Intranasal administration of INDCA-NS did not show significant toxicity at MTD (acute) and NOAEL (28-day repeated dose toxicity study) of 100 µg/rat/day. The total surface area of the human nose is about ten times that of the rat nose[65]. Therefore, the dose of 1000 µg per day (10 times NOAEL in rats) can be considered as a safe human equivalent dose (HED) for subacute administration of INDCA-NS. However, suitable clinical studies on INDCA-CS in relevant patient populations will be needed.

5. Conclusion

The acute and subacute (28-day repeated dose) intranasal INDCA-CA exposure was found safe in laboratory rats with MTD greater than 100 µg/rat (AINT study), NOAEL greater than 100 µg/day/rat (subacute, 28-day repeated-dose toxicity study) and HED of 1000 µg/day, without affecting sense of smell.

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