MATERIALS AND METHODS

TEST CHEMICAL

The test chemical, Acephate 97% DF (Trade name *Lancer 97 DF*, manufactured by UPL Limited) was purchased from the local market at Vapi. The test chemical was dissolved in distilled water before exposing to animals.

Acute toxicity, chronic inhalation toxicity (breathing zone concentration determination, particle size analysis, haematological and biochemical analysis, histopathological evaluation) and skin sensitisation tests were performed.

HUSBANDRY PRACTICES

Caging : Polypropylene rat cages covered with stainless steel grid tops were used and autoclaved clean rice husk was used as the bedding material.

Water Bottle : Each cage was supplied with a polypropylene water bottle with a stainless steel nozzle.

Housing : One to five animals were kept in each cage depending upon the experiment (National Research Council of the National Academies, 2011).

Room Sanitation : Daily rack was cleaned and floor was swept, along with that all work tops were mopped with disinfectant (Dettol 2.5%).

Marking Method : All animals were marked with permanent ink marker prior to randomization. After randomization they were marked with unique numbers on the tail using a tattoo machine. Appropriate cage cards were attached to the cages indicating the experiment number, test chemical name, group number, sex, concentration/dose, type of study, cage number and animal number.

FOOD AND WATER

The rats were provided with food and water, *ad libitum* (except during the exposure and a day prior to sacrifice in inhalation study). The quality of food and water was regularly monitored at Jai Research Foundation. There were no known contaminants in the food and water at levels that would have interfered with the experimental results obtained.

Feed: Teklad Certified Global High Fiber Rat/Mice food manufactured by Harlan, U.S.A.

Water: UV sterilized water was filtered through Kent Reverse Osmosis water filtration system.

ENVIRONMENTAL CONDITIONS

Animals were maintained in an environmentally controlled room. The experimental room temperature and humidity were recorded daily. The temperature range was 19 to 25 ℃ and the relative humidity level during the experimental period was 30 to 70%. A minimum of 15 air changes per hour were maintained in the experimental room and 12 hours of artificial fluorescent lighting and 12 hours darkness were maintained, light hours being 6:00 to 18:00 hours. The experimental room was cleaned and mopped daily with a disinfectant (Dettol 2.5%).

ROUTE OF ADMNISTRATION

Route of exposure describes the way the chemical enters the body. Chemicals may have serious effects by one route, and minimal effects by another. Hazardous chemicals may enter the body by following routes:

- Absorption through the **respiratory tract** via inhalation.
- Absorption through the **skin** via dermal contact.
- Absorption through the **digestive tract** via ingestion. (Ingestion can occur through eating or smoking with contaminated hands or in contaminated work areas).

In the current study Acephate formulation was given through either inhalation route (chapter 1 and 2) or by dermal route (chapter 3).

PHYSICAL EXAMINATION

All animals were observed for physical examination including signs of toxicity and mortality at hourly intervals during the treatment period and at least once after the exposure on the day of treatment. Subsequently, all the animals were observed twice a day for morbidity and mortality during the experiment and clinical signs were recorded once a day.

BODY WEIGHT

Individual animal was weighed on the day of commencement of treatment and at other intervals based on the test requirement.

INHALATION TOXICITY TEST: ACUTE

Healthy young and adult Wistar rats aging between 12 to 13 weeks were selected for the experiment. The rats were received into the environmentally controlled experimental procedure room, kept under group feeding and allowed to acclimatize for minimum seven days before the commencement of the experiment. Clean, sterile and solid polypropylene cages were used to house the rats and animals were housed sex wise during the acclimatization and during experimental period. The rats were also acclimatized to rat restrainer tubes for two hours day before the exposure and were exposed to maximum achievable breathing zone concentration of Acephate 97% DF. Nominal Concentration maintained was 22.222 mg/L air.

INHALATION TOXICITY TEST: 14-DAY REPEATED DOSE

Based on the results of Acute Inhalation Toxicity test six groups (Control, low, mid, high, control recovery and high recovery concentrations) was selected for the repeated dose toxicity test. Healthy young and adult Wistar rats aging between 15 to 17 weeks were selected for the experiment. The rats were received into the environmentally controlled experimental procedure room, kept under group feeding and allowed to acclimatize for minimum seven days before the commencement of the experiment. Clean, sterile and solid polypropylene cages were used to house the rats and animals were housed sex wise during the acclimatization and during experimental period .The rats were also acclimatized to rat restrainer tubes for six hours/day for four days before the exposure. The rats were exposed with different breathing zone

concentration of Acephate 97% DF. During exposure particle size analysis was performed using seven stage cascade impactor. The rats were exposed 6 hours a day for 5 day in a week.

Haematological, biochemical and histopathological changes were studied after completion of the observation period. Haematological and biochemical analyses were conducted on blood samples collected from all the rats at the end of the treatment and recovery periods, while histopathological changes were studied by sacrificing the rats from control and high dose group at the end of the experiment and studying the effects on the organs microscopically.

PARTICLE SIZE DISTRIBUTION

The particle size distribution is the most important part of this type of the test. Humans are unable to inhale particles over 10 µm in size (Figure I). Toxicity may occur only when the particles reaches at bronchi. Hence, it should be important that the 50% particles which we generated are in the range of 1 to 3 µm.

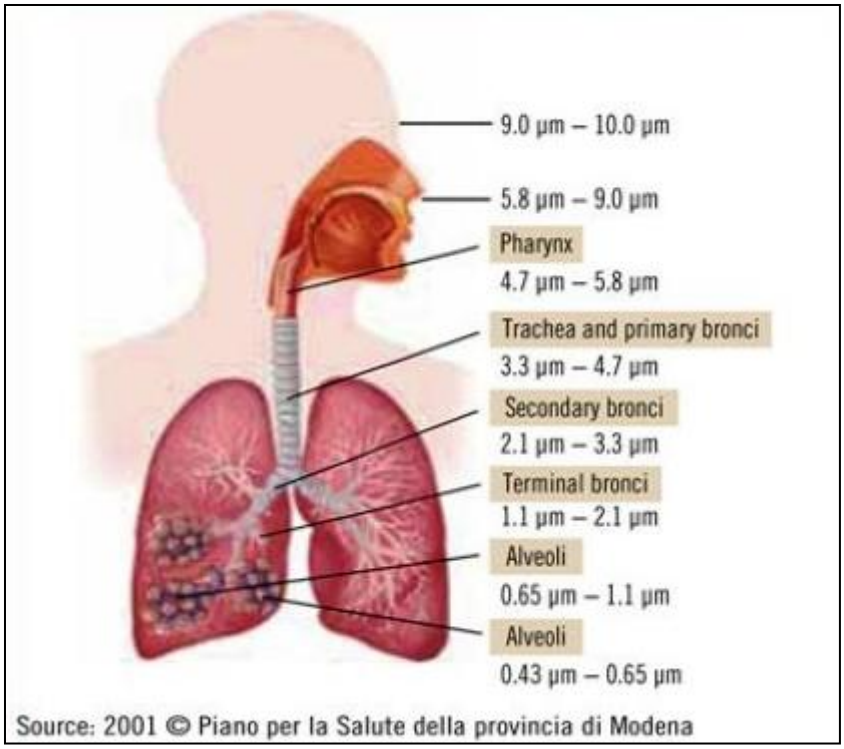

Figure I: A pictorial depiction of particle size that can enter at various parts of a human respiratory system.

In this test the particle size was determined, using a seven-stage cascade impactor. The unit (manufactured by In-Tox Products, USA) consisted of an inlet cone, seven stages, seven collection plates and a backup filter. The stages were numbered from 1 to 7 and F. The backup filter was placed in stage F. All the stages in the impactor had multiple precision-drilled orifices. The orifices of the stages were progressively smaller from top to bottom. When air was drawn through the sampler, multiple jets of air in each stage directed any airborne particles towards the surface of the collection plate for the respective stage. The base stage had an outlet nipple connected to an exhaust pump. This instrument is designed based on the human respiratory tract.

HAEMATOLOGICAL AND BIOCHEMICAL CHANGES

The rats were deprived of feed overnight and blood samples were collected by puncturing the orbital sinus plexus with the help of a fine capillary tube under mild anesthesia (Riley, 1960). The orbital sinus allows the most ease in collection of many blood samples in a short time period in rodents (Annis and Darsheimer, 1975; Suber, 1994) and allows repetitive collection of blood samples with reduced variance in clinical pathology parameters (Statland and Winkel, 1984).

Approximately 0.5 mL blood was collected in vials containing 4% K_2EDTA for haematological analysis. These vials were directly fed into a fully automated hematology analyzer *MEK-6450K* (Nihon Kohden, Japan) calibrated for rodents and analysed for the parameters *viz*., Total leukocyte count (WBC) [$x10^3/\mu L$], Erythrocyte count (RBC) [$x10^6/\mu L$], Haemoglobin (HGB) [g/dL], Haematocrit (HCT) [%], Mean corpuscular volume (MCV) [fL], Mean corpuscular haemoglobin (MCH) [pg], Concentration (MCHC) [g/dL] and Platelets count (PLT) [$x10^3/\mu L$]

CLINICAL CHEMISTRY PARAMETERS (BIOCHEMICAL)

Approximately 2.0 mL of blood was allowed to clot at room temperature for about 30 minutes and then centrifuged at 2500-3000 rpm to collect serum. These vials were directly fed into a computer controlled Automatic Analyzer *Biotecnica BT 2000 plus* (Chema Diagnostica, Italy) for various clinical chemistry parameters like Alanine aminotransferase (ALT) [IU/L], Albumin [g/dL], Aspartate aminotransferase (AST) [IU/L], Alkaline phosphatase (ALP)

[IU/L], Blood urea nitrogen (BUN) [mg/dL], Creatinine [mg/dL], Creatinine kinase (CK) [IU/L], Cholinesterase [IU/L], Glucose [mg/dL], Inorganic Phosphorus [mg/dL], Total bilirubin [µmol/L], Total protein [g/dL], Total Cholesterol [mg/dL], Urea [mg/dL], Globulin [g/dL] and also computed Albumin to Globulin ratio.

HISTOPATHOLOGICAL CHANGES

Animals were examined carefully for external abnormalities and at the end of the observation period all animals were terminally sacrificed by intraperitoneal administration of thiopentone sodium before necropsy. To detect the changes or abnormalities, thorough examination of various organs was done for which thoracic, abdominal and cranial cavities were cut opened.

All the rats from various groups were terminally sacrificed at the end of the observation period and were sacrificed by intraperitoneal administration of thiopentone sodium 150 mg/kg Body weight before subjecting to a complete necropsy. The rats were examined carefully for external abnormalities before the necropsy. The thoracic, abdominal and cranial cavities were then cut opened and thorough examinations of the organs were carried out to detect changes or abnormalities, if any.

Selected organs were collected from the control and the treated rats during necropsy and preserved in 10% neutral formalin for processing the microscopic examination. The whole process of histopathology was carried out as per the steps described by Brar *et al.*, (2000).

PREPARATION OF TISSUES

The organs collected were separated of fats and a portion of the tissue was trimmed and used for further processing. The block was identified separately, given individual identification numbers. The blocks with correct identification number were kept in fresh fixative. The tissues were washed in running tap water followed by placing in 70% alcohol, to remove formalin. The tissues were then transferred into perforated capsules for further processing

PROCESSING OF TISSUES

Following were the major steps during tissue processing

- Dehydration
- Clearing
- Infiltration
- Embedding

Dehydration

Water from the fixed tissue was removed by dehydration. To achieve this tissue was immersed and was passed through a series of increasing concentrations of alcohol (70%, 80%, 90% and 100%) for 1 hour in each concentration.

Clearing

The clearing agent used was xylene. It is the most commonly used clearing agent. It is rapid in action and is miscible in both alcohol and paraffin wax. Tissue block of 5 mm thickness were kept in two changes of xylene for 1 hour each.

Infiltration and Impregnation

Tissues were transferred to molten wax which causes diffusion of xylene into surrounding molten wax (infiltration). The wax diffuses into tissues to replace xylene (impregnation) within 2 to 3 hours. After cooling, wax provides firmness and support to tissue when these are cut on a microtome.

Embedding

It is also known as casting or blocking. Embedding is transferring of infiltrated impregnated tissue in molten wax that solidifies into a block when cools down to room temperature. Leuckhart's L pieces were used for tissue embedding. Molten wax was poured into the mould. Warm blunt forceps were used to lift the tissue from final wash and placed in bottom of mould. After cooling the hot wax, the L's were removed and hard blocks were made ready for sectioning.

PREPARATION OF SECTIONS

The blocks prepared were attached to the microtome holder. Thin sections of 4 to 5 µm were cut using rotary microtome (Leica RM2155). Paraffin wax embedded sections become slightly compressed and creased. Before, attaching to slides, creases were removed and sections were flattened by floating the sections on warm water in hot water bath maintained at below the melting point of wax. The albuminized slides prepared using Mayer's glycerol-albumin mixture, were half submerged in water and brought into contact with sections. The slide was withdrawn thus brining flattened sections with it. The excess water was drained and the slides were kept in incubator, overnight at 37 ºC.

STAINING AND MOUNTING

The slides prepared were stained using haematoxylin and eosin stain. Deparaffinization was performed by giving three serial changes in xylene for 3 to 4 minutes each. The sections were then hydrated with downgraded alcohols and water with two changes of absolute alcohol, followed by 90%, 70% and washing in tap water, each for 2 to 3 minutes and were dipped in haemotoxylin stain for 5 minutes and then washed in running tap water. The sections were then counterstained in eosin for 1 minute and then passed through 70%, 90% and two changes of absolute alcohol. The slides were then cleared with two changes of xylene for 1 minute each. Excess xylene was drained from the slides and tissue was mounted with cover slip using DPX as mountant.

LOCAL LYMPH NODE ASSAY (LLNA) IN MICE

The mouse was the species of choice for this test and it is also recommended by the OECD Test Guideline 429 (OECD, 2010). Young adult male and female mice of CBA/J strain were used. Females were nulliparous and non-pregnant. At the start of the test, animals aged 9 to 10 weeks, and the weight variation of the animals was within 20% of the mean weight.

Based on the results of solubility test performed in preliminary assay, six groups of mice comprising 4 mice per group (2 males and 2 females) were treated with Acephate 97% DF, applied at 10% (G1), 25% (G2) and 50% (G3) (w/v) in Dimethylformamide (DMF) and treated with α-Hexylcinnamaldehyde (HCA), applied at 5% (G4), 10% (G5) and 25% (G6) (v/v) in DMF, (25 µL/ear) for three consecutive days (days 0, 1 and 2). After which clinical

observations like systemic clinical sign and scoring of irritation were recorded daily. Ear thickness was measured on days 0, 2 and 5 (approximately 72 hours post last application) by digital micrometer. Body weight was recorded on days 0 and 5.

As the test material was found to be soluble in DMF the same was used as vehicle (in accordance with the OECD 429 test guideline). A preliminary assay was performed for the selection of suitable concentrations for the main study. The doses selected for the preliminary as were 10%, 25% and 50% (w/v) in DMF. No erythema was observed and ear thickness of below 25% was observed at the chosen concentrations. Therefore, Acephate 97% DF dose concentrations of 10%, 25% and 50% (w/v) in DMF were evaluated in the main study.

In mice treated with α-Hexylcinnamaldehyde at the dose concentrations of 5%, 10% and 25% (v/v) in DMF revealed ear thickness increased below 25% but at 25% (v/v) very slight erythema was observed from day 1 to day 4 while no erythema was observed in mice treated with 5% and 10% (v/v) in DMF. Therefore, dose concentration of 25% α-Hexylcinnamaldehyde (v/v) in DMF was selected in the main study as positive control.

Three groups (G2 to G4) were treated topically for three consecutive days (days 0, 1 and 2) on the dorsal surface of both ears (25 µL/ear) using a calibrated micropipette with Acephate 97% DF at concentrations of 10%, 25% and 50% (w/v) in DMF, respectively.

Mice from vehicle control (G1) and positive control groups (G5) were handled in the similar manner but received 25 µL/ear of vehicle (DMF) and 25% HCA (v/v), respectively.
The experimental schedule of the main assay was:

- Day 0: To identify and weigh each animal in the group. Open application of 25µL of the appropriate dilution of the Acephate 97% DF, the DMF alone and the positive control (25% HCA in DMF), to the dorsum of both ear of an animal.
- Days 1 and 2: Repeated the application procedure carried out on day 0.
- Days 3 and 4: No treatment was given.
- Day 5: Recorded the weight of each animal. Injected 250 µL of phosphate-buffered saline (PBS) containing approximately 20 (±1) µCi (740 kBq) of ^{3}H-methyl thymidine into all test, positive control and control mice via the tail vein. Five hours later, the animals were killed. The draining auricular lymph nodes from each ear were excised and pairs of lymph

nodes from individual animals were excised and pooled in PBS for each animal (individual animal approach). Details and diagrams of the node identification and dissection can be found in Annex I of the ICCVAM Immunotoxicology Working Group LLNA Protocol (NIH Publication, 1999).

Preparation of cell suspensions

A single cell suspension of lymph node cells (LNC) either from pooled treatment groups or bilaterally from individual animals was prepared by gentle mechanical disaggregation through 200-210 µm-mesh stainless steel gauze. Lymph node cells were washed twice with an excess of PBS and precipitated with 5% trichloroacetic acid (TCA) at 4 ^{o}C for 18h (Kimber *et al*., 1994). Plellets were re-suspended in 1 mL TCA and were then transferred to scintillation vials containing 10 mL of scintillation fluid for ^{3}H-counting.

Determination of cellular proliferation (incorporated radioactivity)

Incorporation of ^{3}H-methyl thymidine is measured by β-scintillation counting as disintegrations per minute (DPM). The incorporation will be expressed as DPM/animal (individual approach).

The animals used in this test were handled and treated in accordance with the strict guiding principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The test facility at Jai Research Foundation is AAALAC accredited and is also complies with the National Good Laboratory Practice, India. The experimental protocols were approved by the Institutional Animal Ethics Committee (JRF CPCSEA Approval No. 35/PO/bc/99/CPCSEA, Ethics No. JRF/IAEC/2013/273: JRF Research Number – RES-1-01-7480).

Statistical Analysis

Statistical comparison between two groups was evaluated by Student's't' test, with a 95% confidence limit. For multiple group comparison the data was subjected to analysis of variance (ANOVA) followed by appropriate post hoc test. All analyses of data were performed using SPSS for windows version 12.0 and a p value of ≤ 0.05 was considered statistically significant.