CHAPTER 3

EVALUATION OF THE ALLERGIC CONTACT DERMATITIS POTENTIAL OF ACEPHATE FORMULATION BY MOUSE LOCAL LYMPH NODE ASSAY

INTRODUCTION

Allergic contact dermatitis (ACD) is an adverse health effect that frequently develops in workers and consumers exposed to skin-sensitising chemicals and products resulting in lost workdays (ICCVAM, 2009). Though several aspects pertaining to the adverse health effects of the current test chemical, Acephate 97% DF has been evaluated over the years (Harrington, 2002; CDC, 2009; USEPA, 2012; Kumar and Upadhay, 2013) its skin sensitization potential remains to be assessed. The study is more pertinent since the pesticide in question is largely been sprayed by the applicators, without proper protective gears, in the field to control the pest population and hence the chance for dermal exposure is more than frequent.

Though, there are several conventional animal models to evaluate the ACD potential of a chemical namely Buehler Test (Buehler, 1965), Guinea Pig Maximisation Test - GPMT (Magnusson and Kligman, 1970) and the mouse Local Lymph Node Assay - LLNA (Kimber and Basketter, 1992), the former two methods use only one concentration for induction and elicitation, and hence the dose response information obtained thorough these methods is limited. Therefore, the murine LLNA developed by Kimbler and coworkers (1986) is largely accepted as a standard protocol to evaluate ACD of a chemical. This method is based on the fact that dermal sensitization requires the elicitation of an immune response. This immune response requires proliferation of a lymphocyte subpopulation. The LLNA relies on the detection of increased DNA synthesis via tritiated thymidine incorporation. Sensitization is measured as a function of lymph node cell proliferative response induced in a draining lymph node following repeated topical exposure of the test animal to the test article. . Moreover, studies have revealed that there is enough qualitative concordance of the LLNA with human data (NIEHS, 1999; Dean *et al*., 2001).

Furthermore, in 1999, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine local lymph node assay as a valid test method to assess the skin sensitisation potential of most types of substances (ICCVAM, 1999). ICCVAM concluded that the LLNA provided several advantages over the guinea pig method, including elimination of potential pain and distress, use of fewer animals, less time required to perform and availability of dose-response information. The international regulatory authorities subsequently accepted the LLNA as an alternative test method for ACD testing. It is now widely accepted and commonly used around the world (Hutchings *et al.*, 2001; Skoet *et al.*, 2003) which give further credence to the presently selected animal model to study ACD potential of Acephate 97% DF.

STUDY OBJECTIVE

The objective of this test was to evaluate the skin sensitisation potential of Acephate 97% DF in male and female mouse by the local lymph node assay as per test guideline 429 of OECD (OECD 2010). Analysis was also made to understand existence of the sex sensitivity in eliciting allergic contact dermatitis by the selected test substance.

JUSTIFICATION FOR SELECTION OF THE TEST SYSTEM

The male and female CBA/J strain mouse is a readily available rodent animal and suitable for the establishment of allergic contact dermatitis. There is a considerable amount of published information available concerning sensitisation reactions in mice to assist in the assessment of the significance to human of any treatment induced changes.

INSTRUMENTS AND EQUIPMENT USED IN THE EXPERIMENT

Weighing Balances	:	1. Adair Dutt, ADGR-202 (Range: 0.01 mg to 210 g) 2. Electronic Weighing Scale, SMART (Range: 5 g to 3 kg)
Micropipette	:	Eppendorf and Qualipipette (Glaxo) (capacity 2-20 μL, 10-100 μL, 100-1000 μL & 1-5 mL)

Syringe	:	BD 1 mL Disposable Syringe
Needle	:	"B-D" Precision Glide™ needle, size: 26 G½ (0.45 x 13 mm)
Gauze	:	Jayant Test Sieves (stainless steel, size: 210 µ, serial NO. 525/3/05)
Refrigerated Centrifuge	:	ThermoScientific, Germany
pH Analyser	:	QuicKchecK™ pH meter, Orion 106pH/ATC
Refrigerator	:	Videocon Refrigerator
Liquid Scintillation Analyser (LSA)	:	PerkinElmer (TRI-CARB®3100 TR)
Tattooing Machine	:	"AIMS" Tattoo Identification System (AIMS, Inc., U.S.A.)
Centrifuge Tubes	:	Tarsons, 15 mL graduated centrifuge tube
Vertical Cylinder Autoclave	:	Medica Inst. Mfg Co., 10 lbs Pressure (115 °C) for 10 minutes
Micrometer	:	Mitutoyo product

SOLVENT AND CHEMICAL

Trichloroacetic Acid	:	Minimum assay 98%, SQ grade, Thermo Fisher Scientific India Pvt. Ltd.
Phosphate Buffered Saline	:	Minimum assay 98%, SQ grade, Himedia Laboratories Pvt. Ltd.
Absorbent Paper	:	"Whatman ®" Absorbent Paper
Floor disinfectant	:	Dettol 2.5%
Distilled Water	:	Millipore Elix 10 water Purification System
Dimethyl Formamide (DMF)	:	Thermo Fisher Scientific Pvt. Ltd
α-Hexylcinnamaldehyde (HCA)	:	Sigma-Aldrich Co. LLC.

ANIMALS

Total of Thirty seven male and thirty seven female healthy CBA/J mice *(Mus musculus)*, obtained from the Animal Breeding Facility of Jai Research foundation, Vapi, India, and were used for the study. For preliminary test total of twenty four mice were used (two male and two female per group; six groups) and fifty mice were used for main study (five male and five female per group; five groups). Females were nulliparous and non-pregnant. The mice were examined for good health at the time of receipt and acclimatised to the controlled environmental conditions for a period of eight days. At the start of the exposure the male mice weighed between 22.4 g – 37.1 g, while the female mice weighed between 18.5 g and 27.0 g; the mice were between 9 and 10 weeks old. Before the start of the study, mice were randomised and assigned to the required number of groups using in-house developed validated computer software.

This test was undertaken in compliance with the 'Guidelines for Laboratory Animals Facility' issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. These guidelines promote the humane care of animals used in research by providing specifications that will enhance animal well-being and experimental quality for the advancement of biological knowledge that is relevant to humans and animals.

Project proposal for the experimentation was approved by Institutional Animal Ethics Committee (IAEC), Jai Research Foundation. JRF is also accredited with Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) that promotes the humane treatment of animals in science (JRF CPCSEA Approval No. 35/PO/bc/99/CPCSEA, Ethics No. JRF/IAEC/2013/273: JRF Research Number – RES-1-01-7480).

ACCLIMATISATION

Mice were received into the experimental procedure room after veterinary examination for health condition and allowed to acclimatise to the laboratory conditions for a period of 8 days prior to commencement of the experiment.

HUSBANDRY PRACTICES

Caging	:	Solid floor polypropylene mice cages (size: 290 mm x 220 mm x 140 mm). Each cage was fitted with a top grill having provision for keeping rodent pellet feed and water bottles. The bottom of the cages was layered with clean sterilized rice husk as the bedding material. Animals were group-housed during acclimatisation. On the days of test item application (Day 0, 1 and 2), the animals were housed in individual cages. From day 3 the

		animals were group-housed 5 mice/cage. On day 5, animals were housed in metabolic cages.
Water Bottle	:	Each cage was supplied with a polypropylene water bottle (capacity 300 mL) with a stainless steel nozzle.
Room Sanitation	:	Each day, floor of experimental room was swept and all work tops and floor were mopped with a disinfectant solution (Dettol 2.5%).

RANDOMIZATION

After acclimatisation animals were randomized into five groups using in-house developed, validated computer software.

ANIMAL IDENTIFICATION

The animals were identified with appropriate labels attached to the cages indicating the test item name, group number, sex, dose, cage number and animal number. Animals (except preliminary assays animals) were tattooed on paw after randomization.

FOOD AND WATER

The mice were provided with food and water *ad libitum*. The quality of food and water is 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.

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

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

ENVIRONMENTAL CONDITIONS

Mice were maintained in an environmentally controlled room. The experimental room temperature and humidity were recorded daily. The temperature range was 20 to 23 °C and the relative humidity level during the experimental period was 65 to 67%. A minimum of 15 air changes per hour was maintained in the experimental room. In the experimental room, 12 hours of artificial fluorescent lighting and 12 hours darkness were maintained, light hours being 0600 hrs to 1800 hrs. The experimental room was cleaned and mopped daily with a disinfectant (Dettol 2.5%).

EXPERIMENTAL ROOM PROCEDURES

The following activities were performed daily during the experimental period in animal room:

- animal observations
- food replenishment
- floor sweeping and mopping
- recording room temperature and relative humidity

VEHICLE SELECTION AND JUSTIFICATION

The Acephate 97% DF was not found to be soluble in the first recommended vehicle acetone: olive oil (4:1) and was found to be soluble in the subsequent recommended vehicle dimethylformamide (DMF) at a concentration of up to 50% (v/v). Therefore, as per the accepted norms (Kimber *et al.*, 1994; ICCVAM, 2010) DMF was selected for the study.

Fresh test solutions were prepared everyday prior to application. Required quantity of the test item was weighed and required volume of the vehicle was added into the vial and mixed thoroughly, then the test item was serially diluted to get the desired concentration. The test solutions were used within 1 hour after preparation and were not subjected to analytical verification.

For positive control α-Hexylcinnamaldehyde (HCA) (internally validated positive control) in required volume was mixed with the vehicle to get the desired concentration (25%). A concurrent vehicle control group was treated with DMF.

PRELIMINARY ASSAY

The test item formed solution with DMF up to the dose concentration of 50% (w/v).

To assess the irritant potential of the test item through ear thickness measurement and erythema, a preliminary assay was carried out.

In the preliminary test, six groups of mice comprising 4 mice per group (2 male and 2 female) were treated with Acephate 97% DF, applied at 10% (G1), 25% (G2) and 50% (G3) (w/v) in DMF and treated with α-Hexylcinnamaldehyde, applied at 5% (G4), 10% (G5) and 25% (G6) (v/v) in DMF (McGarry, 2007), (25 µL/ear) for three consecutive days (days 0, 1 and 2). Clinical observations, including systemic clinical signs and scoring of irritation, were recorded daily during the experiment. Ear thickness was measured on days 0, 2 and 5 (at 72 hours post last application). Body weight was recorded on days 0 and 5.

DOSE SELECTION FOR FURTHER STUDY

In the preliminary assay, mice treated with Acephate 97% DF at any of the selected dose levels did not elicit signs of skin sensitization. Therefore, all the three doses were further selected for the main study of LLNA as per the accepted guidelines for ACD (Reeder *et al.*, 2007; ICCVAM, 2009).

The positive control α-Hexylcinnamaldehyde at a dose level of 25% (v/v) induced mild erythema on day 1 to day 4. Therefore, as suggested by Basketter *et al*. (1996), the dose concentration of 25% (v/v) HCA in DMF was evaluated in the main study of LLNA.

TREATMENT

Mice from 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 group (G1) and positive control group (G5) were handled in the same manner but received 25 μL/ear of vehicle (DMF) and 25% α-Hexylcinnamaldehyde (v/v) in vehicle (DMF), respectively.

ADMINISTRATION OF ^{3}H-Methyl Thymidine

On day 5 (approximately 72 h after the last treatment), all mice from the vehicle control, positive control and all the treatment groups were injected with 250 μL of sterile phosphate buffered saline (PBS) containing approximately 20 ± 1 μCi (740 KBq) of ^{3}H-methyl thymidine via the tail vein.

BODY WEIGHT

Body weights of individual mice were recorded on the first day of dosing (day 0) and prior to administration of ^{3}H-methyl thymidine (day 5). Group mean body weights were calculated. (Appendix 3.1)

OBSERVATIONS

Individual animals were observed carefully for clinical signs and local irritation at the site of application and systemic toxicity. All the observations were systematically recorded for individual mice. Local irritation responses were made as per criteria given below (Homey *et al.*, 1998; Woolhiser *et al.*, 1998; Hayes and Meade, 1999; OECD, 2002; OECD, 2010; Vohr and Ahr, 2005; Patterson *et al.*, 2007):

Observation	**Score**
No erythema	**0**
Very slight erythema (barely perceptible)	**1**
Well defined erythema	**2**
Moderate to severe erythema	**3**
Severe erythema to eschar formation preventing grading of erythema	**4**

COLLECTION OF LYMPH NODES

On day 5, 5 hours post-administration of ^{3}H-methyl thymidine, all mice from the vehicle control, positive control and all the treatment groups were euthanised by CO_2 asphyxiation. The draining auricular lymph nodes from each mouse were excised and collected in petri dishes filled with phosphate buffered saline (PBS).

PREPARATION OF CELL SUSPENSION

The draining auricular lymph nodes of individual mice were collected in separate petri dishes containing PBS. A single cell suspension of lymph node cells was prepared by gentle mechanical disaggregation through 200 to 210 µm-mesh stainless steel gauze with the plunger of the syringe and collected in a petridish. The gauze was washed with PBS into the petridish and a single cell suspension was transferred into a 15 mL graduated centrifuge tube. The single cell suspension was finally made up to 10 mL with PBS used to rinse the petridish. The cell suspension was centrifuged 200 g for 10 minutes in a centrifuge at 4 °C.

After centrifugation, the supernatant was removed by aspiration using a micropipette leaving 1 to 2 mL of supernatant above each pellet. Each pellet was gently agitated before making up to 10 mL with PBS; this procedure was repeated twice. After the final wash, the supernatant was removed leaving a minimal volume (approximate 0.5 mL) of supernatant above each pellet.

Each pellet was agitated before re-suspending with 3 mL of 5% trichloroacetic acid (TCA) and kept for precipitation of macromolecules in the refrigerator for approximately 18 hours. After incubation with 5% TCA at 4 ± 1 °C, each precipitate was recovered by centrifugation (190 to 200 g) for 10 minutes and the supernatant was removed.

The precipitate was re-suspended in 1 mL of 5% TCA. Each precipitate was transferred to a scintillation vial with 10 mL of scintillation fluid (Hionic flour) and thoroughly mixed. The vials were loaded into a β–scintillation counter and after minimum 30 minutes, ^{3}H-TdR incorporation was measured. Background ^{3}H-TdR level was measured into 1 mL aliquots of 5% TCA.

DETERMINATION OF CELLULAR PROLIFERATION

Incorporation of ^{3}H-methyl thymidine was measured by β-scintillation counting as disintegrations per minute (DPM) for each mouse and expressed as DPM/mouse. The total radioactivity of each sample was counted using Liquid Scintillation Analyser (LSA) after 30 minutes of mixing with scintillation fluid and required quench corrections were made.

The quench curve was established using extended quench standards of 253100 ± 1.6% DPM β source using LSA. Computer constructed quench curve was derived from the above commercially available series of scaled standards which automatically converts Counts per Minute (CPM) to DPM.

LIQUID SCINTILLATION ANALYZER PARAMETERS

LSA	:	PerkinElmer (TRI-CARB®3100 TR)
Detector	:	High Performance Photo Multiplier Tube (PMT)
Method	:	Conventional DPM
Counting Time	:	10 minutes (2 sigma value of counting reaction 0.5%)
Counting Region	:	0 – 18.6 keV
SNC DPM (standard)	:	258000 DPM (^{3}H)

EVALUATION OF RESULTS

The proliferate response of lymph nodes from each mouse was expressed as the number of radioactive disintegrations per minute (DPM) per mouse, calculated by subtracting out background DPM (measured in 1 mL of 5% TCA aliquot).

The DPM/mouse, along with an appropriate measure of inter-animal variability (*i.e.*, mean ± standard deviation), were calculated for each test group and vehicle and positive control groups. Final results were expressed as the Stimulation Index (SI) (Kimber *et al.*, 1994) which is calculated as a ratio of the mean DPM of test group divided by mean DPM of vehicle control group.

The test item was not regarded as a skin sensitiser as the SI for a dose group was ≤ 3 together with consideration of a dose-response relationship.

EC_3 value (theoretical concentration resulting in a SI value of 3) was determined by linear interpolation of points on the dose-response curve, immediately above and below the 3-fold threshold (Basketter *et al.*, 1999). The equation used for calculation of EC_3 was:

$$EC_3 = c + \left[\frac{(3-d)}{(b-d)}\right](a-c)$$

Where, a = the lowest concentration giving stimulation index > 3;

b = the actual stimulation index caused by a;

c = the highest concentration failing to produce a stimulation index of 3; and

d = the actual stimulation index caused by c.

If EC_3 value is found to be ≤ 2%, the test item is classified under Category 1A while if EC_3 value is found to be > 2% it is classified under Category 1B based on the Globally Harmonized System of Classification and Labelling of Chemicals (GHS, 2013). If the test item produces a SI < 3 in the LLNA, it is considered negative for contact sensitisation potential and therefore, an EC_3 was not determined.

In addition to an assessment of the magnitude of the SI, statistical analysis was carried out for the assessment of the dose response relationship and pair-wise comparison made between the treatment and the solvent/vehicle control group. All the parameters characterised by continuous data such as body weight and radioactive disintegrations per minute (DPM) were subjected to Bartlett's test to meet the homogeneity of variance before conducting Analysis of Variance (ANOVA). To compare vehicle and positive control data, Student's't' test was performed to calculate significance.

RESULTS AND DISCUSSION

PRELIMINARY TEST

In the preliminary test, six groups of mice comprising 4 mice per group (2 male and 2 female) were treated with Acephate 97% DF, applied at 10% (G1), 25% (G2) and 50% (G3) (w/v) in DMF and treated with α-Hexylcinnamaldehyde, 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).

In the preliminary test, dose concentrations of Acephate 97% DF at 10%, 25% and 50% (w/v) in DMF revealed no erythema and also the increase in ear thickness was within the accepted level of below 25%. Therefore, Acephate 97% DF dose concentrations of 10%, 25% and 50% (w/v) in DMF were evaluated in the main study of LLNA as per Reeder *et al.* (2007) and ICCVAM (2009).

In the case of α-Hexylcinnamaldehyde, the tested concentrations of 5% and 10% and 25% (v/v) in DMF revealed no significant increase in ear thickness, but at 25% HCA (v/v), mild to moderate erythema was observed on day 1 to day 4. Therefore, a dose of 25% (v/v) in DMF was selected as positive control for the main study of LLNA.

MAIN STUDY OF LLNA

CLINICAL OBSERVATIONS

No clinical signs were observed in any mice from the vehicle control, positive control and treated groups at 10%, 25% and 50% (w/v) in DMF of Acephate 97% DF (OECD 1987; OECD, 2000; ICCVAM, 2009) (Appendix 3.2).

No erythema was observed in any treated mice at 10%, 25% and 50% (w/v) in DMF of Acephate 97% DF on day 0 to day 5. Very slight erythema (barely perceptible) (on days 1 to 4) was observed in dose group of 25% HCA in all mice (10/10 mice) (Table 3.1).

BODY WEIGHT

The mean body weight of positive control as well as Acephate 97% DF treated mice was comparable to that of the control groups. (Table 3.2)

EAR THICKNESS MEASUREMENTS

There was no noteworthy increase (more than 25%) observed in ear thickness on days 2 and 5 in all treated groups as well as in vehicle and positive control groups. (Table 3.3,3.4, Figure 3.1,3.2, Appendix 3.3,3.4)

GROUP MEAN DPM

Male Mice: Proliferative responses in the draining lymph nodes were monitored by measuring the incorporation of ^{3}H-methyl thymidine. These analyses revealed group mean DPM values of 376.30, 435.20, 593.60 and 599.90 for the vehicle control (DMF), 10%, 25% and 50% (w/v) in DMF dose concentrations of Acephate 97% DF, respectively. The DPM value for positive control (25% α-Hexylcinnamaldehyde) was found to be 1623.20.
The DPM values observed at 25% HCA treated group were increased in a statistically significant manner, when compared to control values(Table 3.5, Appendix 3.5).

Female Mice: Proliferative responses in the draining lymph nodes were monitored by measuring the incorporation of ^{3}H-methyl thymidine. These analyses revealed group mean DPM values of 493.10, 683.38, 762.60 and 770.70 for the vehicle control (DMF), 10%, 25% and 50% (w/v) in DMF dose concentrations of Acephate 97% DF, respectively. The DPM value for positive control (25% α-Hexylcinnamaldehyde) was found to be 2680.90.
The DPM values observed at 25% HCA treated group were increased in a statistically significant manner, when compared to control values. .(Table 3.5, Appendix 3.2)

STIMULATION INDEX (SI VALUE) AND EC_3 VALUE

Male Mice: Stimulation Index (SI) values calculated for groups treated with Acephate 97% DF were found to be 1.16, 1.58 and 1.59 at the dose concentration of 10%, 25% and 50% (w/v) in DMF respectively and 4.31 for the 25% HCA treated positive control group.
Since the SI obtained for Acephate 97% DF at 10%, 25% and 50% (w/v) in DMF showed less than three-fold increase over the control value, an EC_3 calculation was not carried out.

Female Mice: Stimulation Index (SI) values calculated for groups treated with Acephate 97% DF were found to be 1.39, 1.55 and 1.56 at the dose concentration of 10%, 25% and 50% (w/v) in DMF, respectively and 5.44 for the 25% HCA treated positive control group. However, as observed in the case of male mice, the SI obtained for female mice treated with Acephate 97% DF showed less than three-fold increase over the control value, here too EC_3 calculation was not carried out(Figure 3.3).

INTERPRETATION OF RESULTS

A Stimulation Index (SI) of three or more (SI value of treated groups over the control) indicates potential to cause skin sensitisation.

The SI obtained for Acephate 97% DF at all the tested concentrations showed a less than three-fold increase over the control value of male and female mice. Therefore, Acephate 97% DF did not demonstrate dermal sensitisation potential in the local lymph node assay. The SI of 4.31 for male mice and 5.44 for female mice obtained for the concurrent positive control, α-Hexylcinnamaldehyde, showed greater than a three-fold increase over the control value indicating a positive response in agreement with the historical control (ICCVAM, 2009) for this known weak sensitiser. This confirmed the reliability of this test procedure.

Stimulation indices (SI) for the three Acephate 97% DF treatment groups were 1.16, 1.58 and 1.59 for male and 1.39, 1.55 and 1.56 for females, respectively. SI for the HCA treated group was 4.31 for male and 5.44 for females. The results revealed no significant difference between samples means or variability between the sexes and the SI values observed for both male and female mice of positive control were within the range of historical control data for female mice. These data provide initial support for the use of male mice in the LLNA and should be followed by further experimentation.

Table 3.1

DERMAL IRRITATION SCORES

Sex: Male

Group no.	Dose Concentration (%)	Mouse no.	Erythema scores on Day					
			0	1	2	3	4	5
G1	Control (DMF)	1	0	0	0	0	0	0
		2	0	0	0	0	0	0
		3	0	0	0	0	0	0
		4	0	0	0	0	0	0
		5	0	0	0	0	0	0
G2	10% Acephate 97% DF (w/v) in DMF	11	0	0	0	0	0	0
		12	0	0	0	0	0	0
		13	0	0	0	0	0	0
		14	0	0	0	0	0	0
		15	0	0	0	0	0	0
G3	25% Acephate 97% DF (w/v) in DMF	21	0	0	0	0	0	0
		22	0	0	0	0	0	0
		23	0	0	0	0	0	0
		24	0	0	0	0	0	0
		25	0	0	0	0	0	0
G4	50% Acephate 97% DF (w/v) in DMF	31	0	0	0	0	0	0
		32	0	0	0	0	0	0
		33	0	0	0	0	0	0
		34	0	0	0	0	0	0
		35	0	0	0	0	0	0
G5	25% HCA (v/v) in DMF	41	0	1	1	1	1	0
		42	0	1	1	1	1	0
		43	0	1	1	1	1	0
		44	0	1	1	1	1	0
		45	0	1	1	1	1	0

Key: 0 = No erythema, 1 = Very slight erythema (barely perceptible), HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide

Table 3.1 (Continued)

DERMAL IRRITATION SCORES

Sex: Female

Group no.	**Dose Concentration (%)**	**Mouse no.**	**Erythema scores on Day**					
			0	**1**	**2**	**3**	**4**	**5**
G1	Control (DMF)	6	0	0	0	0	0	0
		7	0	0	0	0	0	0
		8	0	0	0	0	0	0
		9	0	0	0	0	0	0
		10	0	0	0	0	0	0
G2	10% Acephate 97% DF (w/v) in DMF	16	0	0	0	0	0	0
		17	0	0	0	0	0	0
		18	0	0	0	0	0	0
		19	0	0	0	0	0	0
		20	0	0	0	0	0	0
G3	25% Acephate 97% DF (w/v) in DMF	26	0	0	0	0	0	0
		27	0	0	0	0	0	0
		28	0	0	0	0	0	0
		29	0	0	0	0	0	0
		30	0	0	0	0	0	0
G4	50% Acephate 97% DF (w/v) in DMF	36	0	0	0	0	0	0
		37	0	0	0	0	0	0
		38	0	0	0	0	0	0
		39	0	0	0	0	0	0
		40	0	0	0	0	0	0
G5	25% HCA (v/v) in DMF	46	0	1	1	1	1	0
		47	0	1	1	1	1	0
		48	0	1	1	1	1	0
		49	0	1	1	1	1	0
		50	0	1	1	1	1	0

Key: 0 = No erythema, 1 = Very slight erythema (barely perceptible), HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide

TABLE 3.2

SUMMARY OF BODY WEIGHT

Sex: Male

Group no.	Dose Concentration (%)	No. of Mice Used	Mean Body Weight (g)	Mean Body Weight (g)
			Day 0	Day 5
G1	Control (DMF)	5	31.22 ± 3.57	32.26 ± 4.03
G2	10% Acephate 97% DF (w/v) in DMF	4	31.48 ± 3.72	32.20 ± 3.91
G3	25% Acephate 97% DF (w/v) in DMF	5	31.06 ± 3.91	31.94 ± 4.26
G4	50% Acephate 97% DF (w/v) in DMF	5	30.04 ± 4.52	31.58 ± 4.62
G5	25% HCA (v/v) in DMF	5	31.04 ± 1.59	31.94 ± 1.50

Note : Values are mean with standard deviation.

Key:

Day 0 = Commencement of Topical Application

Day 5 = Prior to administration of ^{3}H-methyl Thymidine

HCA = α-Hexylcinnamaldehyde

DMF = Dimethylformamide

TABLE 3.2 (Continued)

SUMMARY OF BODY WEIGHT

Sex: Female

Group no.	Dose Concentration (%)	No. of Mice Used	Mean Body Weight (g)	Mean Body Weight (g)
			Day 0	Day 5
G1	Control (DMF)	5	23.32 ± 2.36	24.54 ± 3.11
G2	10% Acephate 97% DF (w/v) in DMF	5	22.90 ± 2.28	24.08 ± 2.37
G3	25% Acephate 97% DF (w/v) in DMF	5	22.62 ± 2.62	23.68 ± 2.75
G4	50% Acephate 97% DF (w/v) in DMF	5	23.00 ± 2.42	24.22 ± 2.68
G5	25% HCA (v/v) in DMF	5	23.34 ± 1.18	24.36 ± 1.11

Note: Values are in mean with standard deviation.

Key:
Day 0 = Commencement of Topical Application
Day 5 = Prior to administration of ^{3}H-methyl Thymidine
HCA = α-Hexylcinnamaldehyde
DMF = Dimethylformamide

Table 3.3

SUMMARY OF EAR THICKNESS

Sex : Male

Group no.	Dose Concentration (%)	No. of Animal Used	Mean Ear Thickness (mm) on day					
			0		2		5	
			Left	Right	Left	Right	Left	Right
G1	Control (DMF)	5	0.275 ± 0.009	0.277 ± 0.007	0.286 ± 0.008	0.288 ± 0.008	0.283 ± 0.008	0.286 ± 0.007
G2	10% Acephate 97% DF (w/v) in DMF	5	0.276 ± 0.009	0.277 ± 0.009	0.298 ± 0.006	0.299 ± 0.007	0.291 ± 0.007	0.290 ± 0.008
G3	25% Acephate 97% DF (w/v) in DMF	5	0.260 ± 0.005	0.260 ± 0.005	0.289 ± 0.005	0.289 ± 0.005	0.281 ± 0.005	0.280 ± 0.004
G4	50% Acephate 97% DF (w/v) in DMF	5	0.269 ± 0.006	0.267 ± 0.007	0.308 ± 0.006	0.306 ± 0.008	0.300 ± 0.005	0.297 ± 0.007
G5	25% HCA (v/v) in DMF	5	0.267 ± 0.006	0.266 ± 0.006	0.314 ± 0.008	0.314 ± 0.008	0.306 ± 0.008	0.305 ± 0.006

Note: Values are in mean with standard deviation.

Key: Day 0 = Commencement of topical application, Day 5 = Prior to administration of ^{3}H–methyl thymidine, HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide

Table 3.3 (Continued)

Sex: Female

Group no.	Dose Concentration (%)	No. of Animal Used	Mean Ear Thickness (mm) on day					
			0		2		5	
			Left	Right	Left	Right	Left	Right
G1	Control (DMF)	5	0.256 ± 0.008	0.256 ± 0.009	0.267 ± 0.008	0.268 ± 0.008	0.262 ± 0.009	0.262 ± 0.010
G2	10% Acephate 97% DF (w/v) in DMF	5	0.247 ± 0.005	0.246 ± 0.003	0.265 ± 0.005	0.266 ± 0.002	0.257 ± 0.004	0.257 ± 0.003
G3	25% Acephate 97% DF (w/v) in DMF	5	0.245 ± 0.004	0.246 ± 0.007	0.272 ± 0.004	0.274 ± 0.002	0.266 ± 0.005	0.266 ± 0.006
G4	50% Acephate 97% DF (w/v) in DMF	5	0.252 ± 0.005	0.252 ± 0.008	0.290 ± 0.006	0.290 ± 0.007	0.279 ± 0.007	0.278 ± 0.009
G5	25% HCA (v/v) in DMF	5	0.249 ± 0.009	0.250 ± 0.007	0.296 ± 0.010	0.297 ± 0.010	0.284 ± 0.009	0.286 ± 0.009

Note: Values are in mean with standard deviation.

Key: Day 0 = Commencement of topical application, Day 5 = Prior to administration of ^{3}H–methyl thymidine, HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide

Table 3.4

SUMMARY OF PERCENT EAR THICKNESS CHANGE

Sex: Male

Group no.	Dose Concentration (%)	No. of Animals Used	Mean Ear Thickness (percent change)			
			Left Ear Thickness (% Change)		Right Ear Thickness (% Change)	
			Day 0 - 2	Day 0 - 5	Day 0 – 2	Day 0 – 5
G1	Control (DMF)	5	4.008 ± 0.681	2.915 ± 0.443	3.970 ± 0.671	3.036 ± 0.579
G2	10% Acephate 97% DF (w/v) in DMF	5	7.920 ± 1.350	5.226 ± 0.791	8.271 ± 1.323	4.932 ± 0.675
G3	25% Acephate 97% DF (w/v) in DMF	5	10.833 ± 0.399	7.918 ± 0.542	10.984 ± 0.307	7.687 ± 0.633
G4	50% Acephate 97% DF (w/v) in DMF	5	14.823 ± 0.435	11.782 ± 1.158	14.673 ± 0.533	11.023 ± 1.741
G5	25% HCA (v/v) in DMF	5	17.768 ± 0.810	14.689 ± 0.848	18.016 ± 0.746	14.421 ± 0.869

Note: Values are in mean with standard deviation.

Key: Day 0 = Commencement of topical application
Day 5 = Prior to administration of ^{3}H–methyl thymidine
HCA = α-Hexylcinnamaldehyde
DMF = Dimethylformamide

Table 3.4 **(Continued)**

Sex: Female

Group no.	Dose Concentration (%)	No. of Animals Used	Mean Ear Thickness (percent change)			
			Left Ear Thickness (% Change)		Right Ear Thickness (% Change)	
			Day 0 - 2	Day 0 - 5	Day 0 – 2	Day 0 – 5
G1	Control (DMF)	5	4.145 ± 0.281	2.262 ± 0.491	4.459 ± 0.561	2.024 ± 0.377
G2	10% Acephate 97% DF (w/v) in DMF	5	7.384 ± 0.561	4.224 ± 0.723	7.965 ± 1.436	4.302 ± 0.222
G3	25% Acephate 97% DF (w/v) in DMF	5	11.020 ± 0.939	8.316 ± 0.438	11.333 ± 2.270	8.129 ± 0.656
G4	50% Acephate 97% DF (w/v) in DMF	5	15.017 ± 0.982	10.799 ± 0.651	15.106 ± 1.521	10.324 ± 0.464
G5	25% HCA (v/v) in DMF	5	18.911 ± 0.795	14.248 ± 0.452	18.861 ± 0.976	14.141 ± 0.476

Note: Values are in mean with standard deviation.

Key: Day 0 = Commencement of topical application
Day 5 = Prior to administration of ^{3}H–methyl thymidine
HCA = α-Hexylcinnamaldehyde
DMF = Dimethylformamide

Table 3.5

SUMMARY OF DPM AND SI VALUE

Sex: Male

Group no.	Dose Concentration (%)	No. of Mice Used	Group Mean DPM	Stimulation Index (SI)
G1	Control (DMF)	5	376.30 ± 88.64	1.00
G2	10% Acephate 97% DF (w/v) in DMF	5	435.20 ± 121.86	1.16
G3	25% Acephate 97% DF (w/v) in DMF	5	593.60 ± 183.83	1.58
G4	50% Acephate 97% DF (w/v) in DMF	5	599.90 ± 251.29	1.59
G5	25% HCA (v/v) in DMF	5	1623.20 ± 499.89*	4.31

Note : Values are mean with standard deviation.

Key : HCA = α-Hexylcinnamaldehyde

DMF = Dimethylformamide

DPM = Disintegration per minute

Stimulation Index = Mean DPM of test group divided by mean DPM of solvent/vehicle control group

* = Significantly higher than control ($p \leq 0.01$)

Table 3.5 (Continued)

SUMMARY OF DPM AND SI VALUE

Sex: Female

Group no.	Dose Concentration (%)	No. of Mice Used	Group Mean DPM	Stimulation Index (SI)
G1	Control (DMF)	5	493.10 ± 160.28	1.00
G2	10% Acephate 97% DF (w/v) in DMF	5	683.38 ± 267.31	1.39
G3	25% Acephate 97% DF (w/v) in DMF	5	762.60 ± 380.01	1.55
G4	50% Acephate 97% DF (w/v) in DMF	5	770.70 ± 321.69	1.56
G5	25% HCA (v/v) in DMF	5	2680.90 ± 390.50*	5.44

Notes : 1. Values are mean with standard deviation.

2. DPM value of Animal number 38 was 122 and it considered as outlier.

Key :

HCA	=	α-Hexylcinnamaldehyde
DPM	=	Disintegration per minute
DMF	=	Dimethylformamide
Stimulation Index	=	Mean DPM of test group divided by mean DPM of solvent/vehicle control group
*	=	Significantly higher than control ($p \leq 0.01$)

Figure 3.1

COMPARISON OF % EAR THICKNESS CHANGE ON DAY 2 BETWEEN MALE AND FEMALE

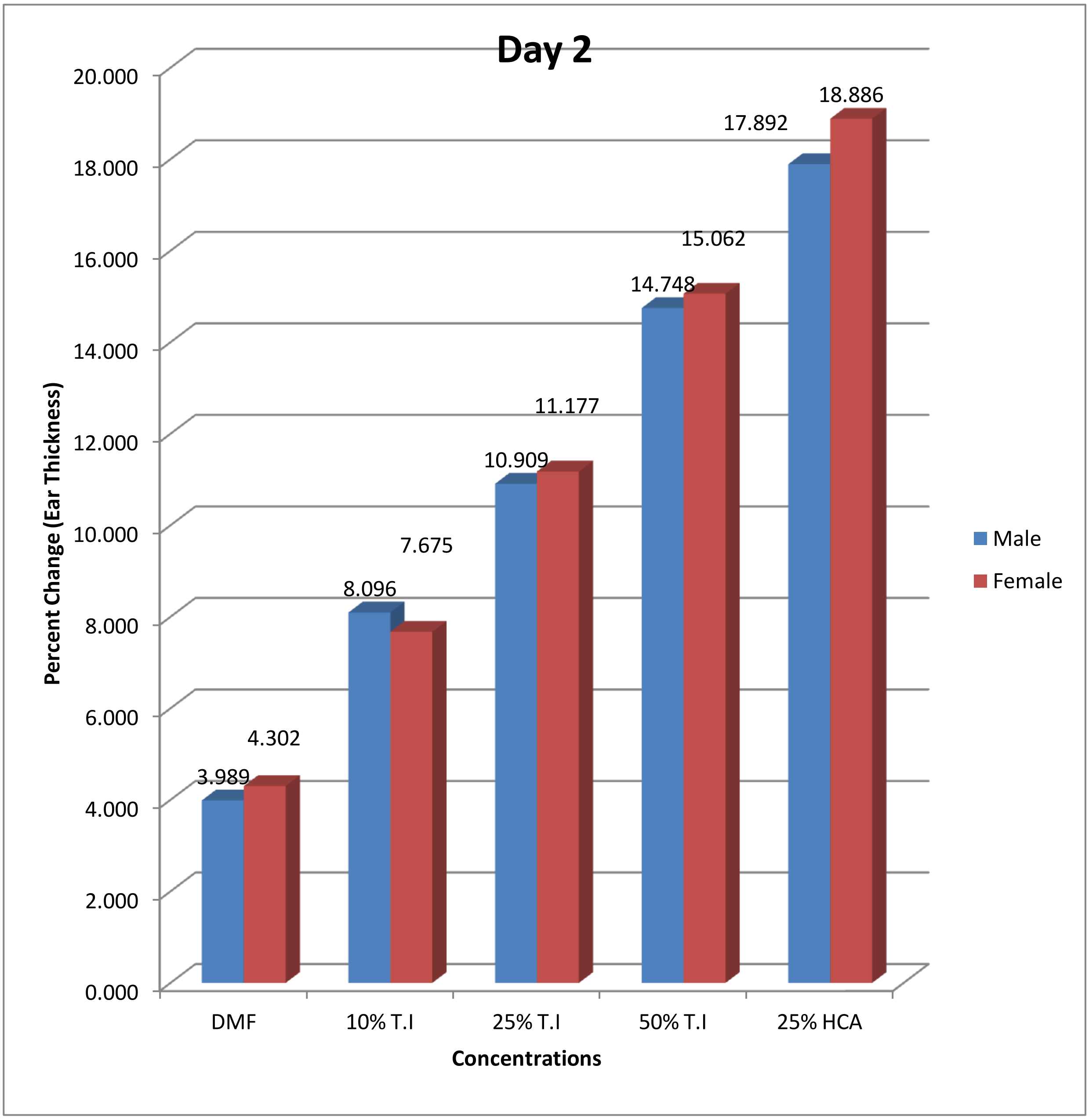


Figure 3.2

COMPARISON OF % EAR THICKNESS CHANGE ON DAY 5 BETWEEN MALE AND FEMALE

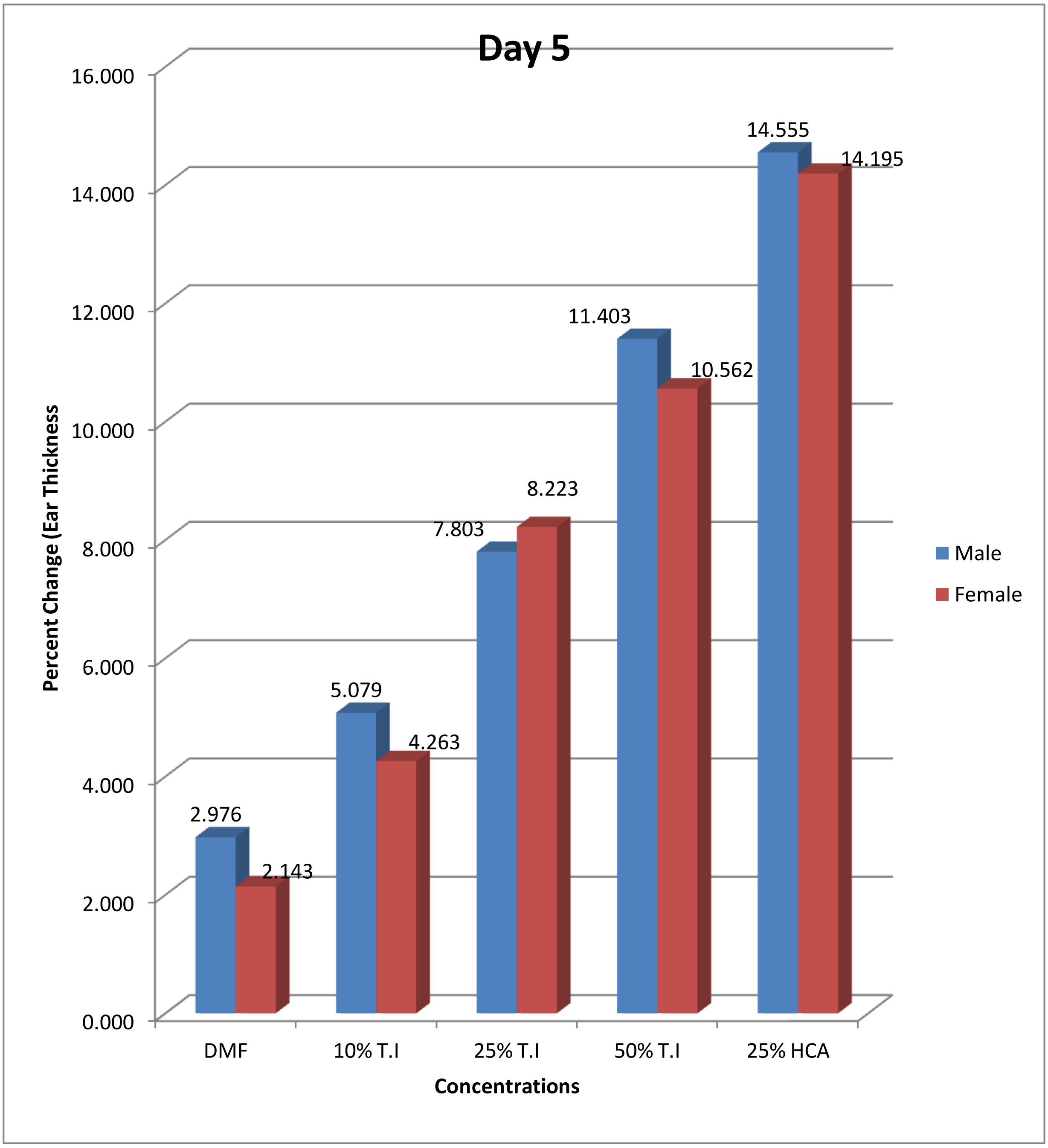
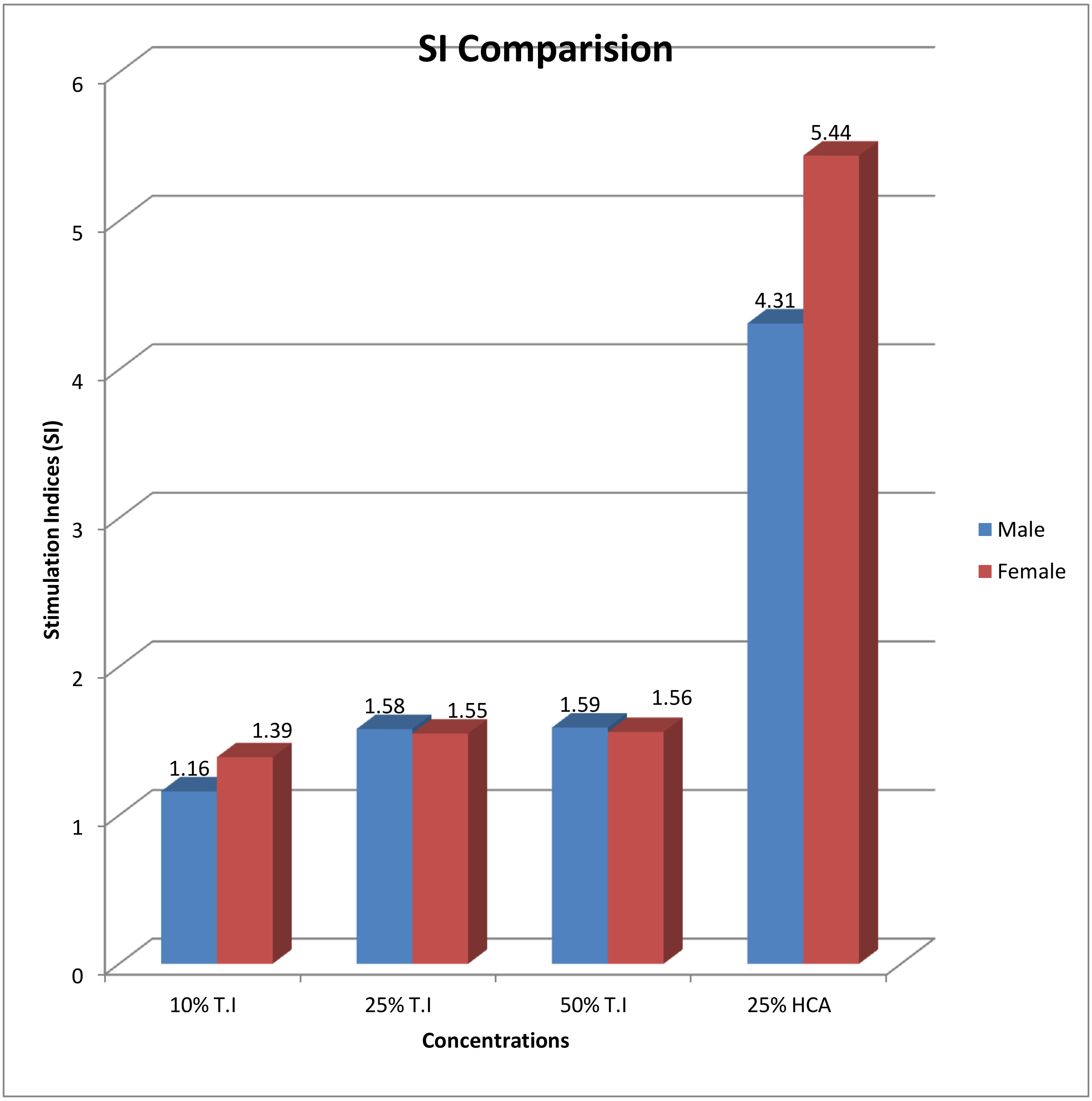


Figure 3.3

COMPARISON OF SI VALE BETWEEN MALE AND FEMALE



Appendix 3.1

BODY WEIGHT OF INDIVIDUAL MOUSE

Sex: Male

Group NO.	Dose Concentration (%)	Mouse NO.	Body Weight (g)		Percent Change
			Day 0	Day 5	
G1	Control (DMF)	1	30.8	31.6	2.60
		2	31.0	32.2	3.87
		3	29.7	29.9	0.67
		4	27.5	28.6	4.00
		5	37.1	39.0	5.12
G2	10% Acephate 97% DF (w/v) in DMF	11	37.6	38.6	2.66
		12	32.3	33.1	2.48
		13	29.7	30.6	3.03
		14	29.5	29.9	1.36
		15	28.3	28.8	1.77
G3	25% Acephate 97% DF (w/v) in DMF	21	26.5	27.0	1.89
		22	36.9	38.1	3.25
		23	31.9	32.6	2.19
		24	31.3	33.0	5.43
		25	28.7	29.0	1.05
G4	50% Acephate 97% DF (w/v) in DMF	31	32.2	33.5	4.04
		32	31.2	32.9	5.45
		33	30.2	31.7	4.97
		34	22.4	23.8	6.25
		35	34.2	36.0	5.26
G5	25% HCA (v/v) in DMF	41	33.5	33.9	1.19
		42	31.5	33.0	4.76
		43	30.9	31.6	2.27
		44	29.7	30.2	1.68
		45	29.6	31.0	4.73

Key: Day 0 = Commencement of Topical Application, Day 5 = Prior to administration of ^{3}H – methyl Thymidine, HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide

Appendix 3.1 (Continued)

Sex: Female

Group NO.	Dose Concentration (%)	Mouse NO.	Body Weight (g)		Percent Change
			Day 0	Day 5	
G1	Control (DMF)	6	24.0	25.2	5.00
		7	22.9	23.4	2.18
		8	21.8	22.9	5.05
		9	20.9	21.6	3.35
		10	27.0	29.6	9.63
G2	10% Acephate 97% DF (w/v) in DMF	16	25.9	27.2	5.02
		17	23.9	24.7	3.35
		18	23.1	24.9	7.79
		19	21.8	22.6	3.67
		20	19.8	21.0	6.06
G3	25% Acephate 97% DF (w/v) in DMF	26	18.5	19.6	5.95
		27	25.2	26.6	5.56
		28	23.9	24.8	3.77
		29	23.8	25.1	5.46
		30	21.7	22.3	2.76
G4	50% Acephate 97% DF (w/v) in DMF	36	24.0	25.3	5.42
		37	23.9	24.4	2.09
		38	23.0	24.9	8.26
		39	18.9	19.7	4.23
		40	25.2	26.8	6.35
G5	25% HCA (v/v) in DMF	46	24.5	25.7	4.90
		47	24.0	24.9	3.75
		48	23.9	24.6	2.93
		49	22.7	23.8	4.85
		50	21.6	22.8	5.56

Key: Day 0 = Commencement of Topical Application, Day 5 = Prior to administration of ^{3}H – methyl Thymidine, HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide

Appendix 3.2

CLINICAL OBSERVATIONS OF INDIVIDUAL MOUSE OTHER THAN IRRITATION RESPONSE

Sex: Male

Group NO.	Dose Concentration (%)	Mouse NO.	Clinical Observation on Day					
			0	1	2	3	4	5
G1	Control (DMF)	1	1	1	1	1	1	1
		2	1	1	1	1	1	1
		3	1	1	1	1	1	1
		4	1	1	1	1	1	1
		5	1	1	1	1	1	1
G2	10% Acephate 97% DF (w/v) in DMF	11	1	1	1	1	1	1
		12	1	1	1	1	1	1
		13	1	1	1	1	1	1
		14	1	1	1	1	1	1
		15	1	1	1	1	1	1
G3	25% Acephate 97% DF (w/v) in DMF	21	1	1	1	1	1	1
		22	1	1	1	1	1	1
		23	1	1	1	1	1	1
		24	1	1	1	1	1	1
		25	1	1	1	1	1	1
G4	50% Acephate 97% DF (w/v) in DMF	31	1	1	1	1	1	1
		32	1	1	1	1	1	1
		33	1	1	1	1	1	1
		34	1	1	1	1	1	1
		35	1	1	1	1	1	1
G5	25% HCA (v/v) in DMF	41	1	1	1	1	1	1
		42	1	1	1	1	1	1
		43	1	1	1	1	1	1
		44	1	1	1	1	1	1
		45	1	1	1	1	1	1

Key: 1 = Normal, HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide

Appendix 3.2 (Continued)

Sex: Female

Group NO.	Dose Concentration (%)	Mouse NO.	Clinical Observation on Day					
			0	**1**	**2**	**3**	**4**	**5**
G1	Control (DMF)	6	1	1	1	1	1	1
		7	1	1	1	1	1	1
		8	1	1	1	1	1	1
		9	1	1	1	1	1	1
		10	1	1	1	1	1	1
G2	10% Acephate 97% DF (w/v) in DMF	16	1	1	1	1	1	1
		17	1	1	1	1	1	1
		18	1	1	1	1	1	1
		19	1	1	1	1	1	1
		20	1	1	1	1	1	1
G3	25% Acephate 97% DF (w/v) in DMF	26	1	1	1	1	1	1
		27	1	1	1	1	1	1
		28	1	1	1	1	1	1
		29	1	1	1	1	1	1
		30	1	1	1	1	1	1
G4	50% Acephate 97% DF (w/v) in DMF	36	1	1	1	1	1	1
		37	1	1	1	1	1	1
		38	1	1	1	1	1	1
		39	1	1	1	1	1	1
		40	1	1	1	1	1	1
G5	25% HCA (v/v) in DMF	46	1	1	1	1	1	1
		47	1	1	1	1	1	1
		48	1	1	1	1	1	1
		49	1	1	1	1	1	1
		50	1	1	1	1	1	1

Key: 1 = Normal, HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide

Appendix 3.3

EAR THICKNESS VALUE FOR INDIVIDUAL MOUSE

Sex: Male

Group Nº	Dose Concentration (%)	Mouse Nº	Ear Thickness (mm)					
			Day 0		Day 2		Day 5	
			Left	Right	Left	Right	Left	Right
G1	Control (DMF)	1	0.260	0.266	0.273	0.277	0.269	0.274
		2	0.278	0.281	0.288	0.295	0.285	0.288
		3	0.275	0.274	0.286	0.284	0.284	0.285
		4	0.281	0.284	0.290	0.293	0.289	0.292
		5	0.283	0.281	0.295	0.292	0.290	0.289
G2	10% Acephate 97% DF (w/v) in DMF	11	0.284	0.282	0.303	0.305	0.298	0.294
		12	0.285	0.287	0.305	0.308	0.297	0.299
		13	0.278	0.278	0.298	0.297	0.293	0.293
		14	0.269	0.271	0.293	0.295	0.283	0.285
		15	0.266	0.265	0.292	0.292	0.283	0.280
G3	25% Acephate 97% DF (w/v) in DMF	21	0.259	0.257	0.286	0.285	0.279	0.276
		22	0.256	0.259	0.284	0.288	0.277	0.281
		23	0.269	0.266	0.297	0.294	0.288	0.285
		24	0.261	0.265	0.290	0.295	0.283	0.284
		25	0.257	0.255	0.286	0.283	0.278	0.276
G4	50% Acephate 97% DF (w/v) in DMF	31	0.261	0.259	0.300	0.297	0.294	0.290
		32	0.273	0.272	0.313	0.314	0.306	0.307
		33	0.276	0.277	0.316	0.316	0.303	0.300
		34	0.269	0.266	0.308	0.304	0.301	0.294
		35	0.264	0.262	0.305	0.301	0.297	0.292
G5	25% HCA (v/v) in DMF	41	0.266	0.261	0.311	0.305	0.305	0.301
		42	0.265	0.268	0.315	0.318	0.307	0.308
		43	0.277	0.276	0.325	0.325	0.317	0.314
		44	0.267	0.263	0.316	0.312	0.307	0.302
		45	0.259	0.264	0.304	0.312	0.294	0.299

Key: HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide

Appendix 3.3 (Continued)

Sex: Female

Group Nº	Dose Concentration (%)	Mouse Nº	Ear Thickness (mm)					
			Day 0		Day 2		Day 5	
			Left	Right	Left	Right	Left	Right
G1	Control (DMF)	6	0.259	0.261	0.270	0.272	0.266	0.265
		7	0.269	0.270	0.279	0.280	0.275	0.277
		8	0.251	0.248	0.262	0.259	0.256	0.253
		9	0.252	0.250	0.263	0.263	0.259	0.255
		10	0.249	0.253	0.259	0.265	0.253	0.258
G2	10% Acephate 97% DF (w/v) in DMF	16	0.245	0.249	0.261	0.264	0.255	0.259
		17	0.240	0.243	0.259	0.263	0.251	0.253
		18	0.249	0.246	0.268	0.267	0.258	0.257
		19	0.253	0.250	0.271	0.268	0.262	0.261
		20	0.246	0.244	0.265	0.268	0.259	0.255
G3	25% Acephate 97% DF (w/v) in DMF	26	0.245	0.251	0.270	0.277	0.264	0.269
		27	0.249	0.256	0.276	0.276	0.271	0.276
		28	0.238	0.239	0.267	0.271	0.257	0.260
		29	0.246	0.243	0.275	0.273	0.267	0.264
		30	0.248	0.243	0.273	0.274	0.269	0.263
G4	50% Acephate 97% DF (w/v) in DMF	36	0.253	0.246	0.295	0.289	0.281	0.273
		37	0.256	0.263	0.293	0.301	0.286	0.289
		38	0.251	0.253	0.288	0.289	0.276	0.278
		39	0.243	0.242	0.280	0.280	0.269	0.267
		40	0.256	0.256	0.292	0.291	0.283	0.283
G5	25% HCA (v/v) in DMF	46	0.242	0.245	0.289	0.293	0.276	0.278
		47	0.247	0.244	0.291	0.286	0.282	0.279
		48	0.253	0.255	0.303	0.304	0.289	0.292
		49	0.261	0.261	0.309	0.310	0.297	0.299
		50	0.240	0.246	0.286	0.294	0.276	0.280

Key: HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide

Appendix 3.4

INDIVIDUAL PERCENT EAR THICKNESS CHANGE

Sex: Male

Group NO.	Dose Concentration (%)	Mouse NO.	Left Ear Thickness (% Change)		Right Ear Thickness (% Change)	
			Day 0 - 2	Day 0 – 5	Day 0 - 2	Day 0 - 5
G1	Control (DMF)	1	5.000	4.135	3.462	3.008
		2	3.597	4.982	2.518	2.491
		3	4.000	3.650	3.273	4.015
		4	3.203	3.169	2.847	2.817
		5	4.240	3.915	2.473	2.847
G2	10% Acephate 97% DF (w/v) in DMF	11	6.690	8.156	4.930	4.255
		12	7.018	7.317	4.211	4.181
		13	7.194	6.835	5.396	5.396
		14	8.922	8.856	5.204	5.166
		15	9.774	10.189	6.391	5.660
G3	25% Acephate 97% DF (w/v) in DMF	21	10.425	10.895	7.722	7.393
		22	10.938	11.197	8.203	8.494
		23	10.409	10.526	7.063	7.143
		24	11.111	11.321	8.429	7.170
		25	11.284	10.980	8.171	8.235
G4	50% Acephate 97% DF (w/v) in DMF	31	14.943	14.672	12.644	11.969
		32	14.652	15.441	12.088	12.868
		33	14.493	14.079	9.783	8.303
		34	14.498	14.286	11.896	10.526
		35	15.530	14.885	12.500	11.450
G5	25% HCA (v/v) in DMF	41	16.917	16.858	14.662	15.326
		42	18.868	18.657	15.849	14.925
		43	17.329	17.754	14.440	13.768
		44	18.352	18.631	14.981	14.829
		45	17.375	18.182	13.514	13.258

Key: HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide

Appendix 3.4 (Continued)

Sex: Female

Group NO.	Dose Concentration (%)	Mouse NO.	Left Ear Thickness (% Change)		Right Ear Thickness (% Change)	
			Day 0 - 2	Day 0 - 5	Day 0 - 2	Day 0 - 5
G1	Control (DMF)	6	4.247	4.215	2.703	1.533
		7	3.717	3.704	2.230	2.593
		8	4.382	4.435	1.992	2.016
		9	4.365	5.200	2.778	2.000
		10	4.016	4.743	1.606	1.976
G2	10% Acephate 97% DF (w/v) in DMF	16	6.531	6.024	4.082	4.016
		17	7.917	8.230	4.583	4.115
		18	7.631	8.537	3.614	4.472
		19	7.115	7.200	3.557	4.400
		20	7.724	9.836	5.285	4.508
G3	25% Acephate 97% DF (w/v) in DMF	26	10.204	10.359	7.755	7.171
		27	10.843	7.813	8.835	7.813
		28	12.185	13.389	7.983	8.787
		29	11.789	12.346	8.537	8.642
		30	10.081	12.757	8.468	8.230
G4	50% Acephate 97% DF (w/v) in DMF	36	16.601	17.480	11.067	10.976
		37	14.453	14.449	11.719	9.886
		38	14.741	14.229	9.960	9.881
		39	15.226	15.702	10.700	10.331
		40	14.063	13.672	10.547	10.547
G5	25% HCA (v/v) in DMF	46	19.421	19.592	14.050	13.469
		47	17.814	17.213	14.170	14.344
		48	19.763	19.216	14.229	14.510
		49	18.391	18.774	13.793	14.559
		50	19.167	19.512	15.000	13.821

Key: HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide

Appendix 3.5

DPM VALUE FOR INDIVIDUAL MOUSE

Sex: Male

Group NO.	Dose Concentration (%)	Mouse NO.	Measured DPM	DPM*	DPM/ Node
G1	Control (DMF)	1	660	622	311.00
		2	1049	1011	505.50
		3	735	697	348.50
		4	889	851	425.50
		5	620	582	291.00
G2	10% Acephate 97% DF (w/v) in DMF	11	1063	1025	512.50
		12	536	498	249.00
		13	1094	1056	528.00
		14	783	745	372.50
		15	1066	1028	514.00
G3	25% Acephate 97% DF (w/v) in DMF	21	1004	966	483.00
		22	885	847	423.50
		23	1803	1765	882.50
		24	1362	1324	662.00
		25	1072	1034	517.00
G4	50% Acephate 97% DF (w/v) in DMF	31	1870	1832	916.00
		32	618	580	290.00
		33	870	832	416.00
		34	1297	1259	629.50
		35	1534	1496	748.00
G5	25% HCA (v/v) in DMF	41	3212	3174	1587.00
		42	3901	3863	1931.50
		43	4611	4573	2286.50
		44	2576	2538	1269.00
		45	2122	2084	1042.00

Key: DPM = Disintegration per minute, Measured Background DPM of 5% TCA = 38, Measured DPM = Value measured by Liquid Scintillation Analyser, DPM* = Measured DPM (Individual Animal) - Background DPM, DPM/Node = DPM*/2, HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide.

Appendix 3.5 **(Continued)**

Sex: Female

Group NO.	Dose Concentration (%)	Mouse NO.	Measured DPM	DPM*	DPM/ Node
G1	Control (DMF)	6	571	533	266.50
		7	1063	1025	512.50
		8	1209	1171	585.50
		9	871	833	416.50
		10	1407	1369	684.50
G2	10% Acephate 97% DF (w/v) in DMF	16	940	902	451.00
		17	992	954	477.00
		18	-	-	-
		19	1636	1598	799.00
		20	2051	2013	1006.50
G3	25% Acephate 97% DF (w/v) in DMF	26	1087	1049	524.50
		27	1287	1249	624.50
		28	2868	2830	1415.00
		29	1011	973	486.50
		30	1563	1525	762.50
G4	50% Acephate 97% DF (w/v) in DMF	36	2042	2004	1002.00
		37	2316	2278	1139.00
		38	1618	1580	790.00
		39	704	666	333.00
		40	1217	1179	589.50
G5	25% HCA (v/v) in DMF	46	5163	5125	2562.50
		47	5166	5128	2564.00
		48	6033	5995	2997.50
		49	6297	6259	3129.50
		50	4340	4302	2151.00

Key: DPM = Disintegration per minute, Measured Background DPM of 5% TCA = 38, Measured DPM = Value measured by Liquid Scintillation Analyser, DPM* = Measured DPM (Individual Animal) - Background DPM, DPM/Node = DPM*/2, HCA = α-Hexylcinnamaldehyde, DMF = Dimethylformamide.

Note: DPM value of Animal number 38 was 122 and it considered as outlier.