MATERIALS
AND
METHODS
STUDY PLAN:
Initiatives were taken to develop a drug of herbal origin with better efficacy and safety for the treatment of psoriasis. One already identified herbal formulation of *Argimone maxicana* that has exhibited fast, painless and long-lasting remission in proof of concept studies was chosen for further development and characterization.
Following are the details of test article:

**Description** Greenish brown hygroscopic Powder

**Identification** Complies

(By HPLC)

**pH** 5.10 (1% aqueous solution)

**Water** 3.21 (%w/w) by KF

**Ash** 26.19 (Total)

01.16 (Acid Insoluble ash)

**Heavy Metals**
- **Lead** <0.5 ppm
- **Cadmium** 0.90 ppm
- **Mercury** 245.75 ppb
- **Arsenic** <1 ppm

**Total Amino acids** 10.37 (%w/w)

**Total Neutral Sugars** 8.28(%w/w)

**Berberine Content** 0.038(%w/w); anhydrous basis, by LC-MS

**Finger Printing: By HPLC**
- **Method-A (Alkaloids)** Complies
- **Method-B (Amino acid & related compounds)** Complies
- **Method-C** Complies
DESCRIPTION
Leaves of *Argemone mexicana* were fresh, dull green and spiny.

SPECIFICATIONS AND TESTS FOR BOTANICAL RAW MATERIAL

<table>
<thead>
<tr>
<th>Specification</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identification</td>
<td>TLC should comply.</td>
</tr>
<tr>
<td>Foreign Matter</td>
<td>NMT 2.0% w/w</td>
</tr>
<tr>
<td>Extractive Value in Water</td>
<td>NLT 4.0% w/w</td>
</tr>
<tr>
<td>Total Ash</td>
<td>NMT 5.0% w/w</td>
</tr>
<tr>
<td>Acid Insoluble Ash</td>
<td>NMT 0.5% w/w</td>
</tr>
<tr>
<td>Alkaloid Content (By Gravimetry)</td>
<td>NLT 0.07% w/w</td>
</tr>
</tbody>
</table>

RATIONALE FOR SPECIFICATIONS

Description:
Visual inspection provides the simplest and quickest means by which to establish identity, purity and quality. Medicinal plant materials should be entirely free from visible signs of contamination by moulds or insects and other animal contamination, including animal excreta. No discoloration, slime or signs of deterioration should be detected.

Identification by TLC:
Macroscopic identity of medicinal plant materials is based on shape, size, color, surface characteristics, texture, fracture characteristics and appearance of the cut surface. However, since these characteristics are judged subjectively and substitutes or adulterants may closely resemble the genuine material, it is necessary to substantiate the findings by physicochemical analysis like TLC.

Foreign Matter:
No foreign matter should be present including parts of the medicinal plant material other than leaves with the limits specified for the plant material. Any organism, part or product of an organism, mineral admixtures not adhering to the medicinal plant materials, such as soil, stones, sand and dust should not be present.
**Extractive Value:**
This method determines the amount of active constituents extracted with solvents from a given amount of medicinal plant material.

**Total Ash and Acid Insoluble Ash:**
This method is designed to measure the total amount of material remaining after ignition. This includes both ‘physiological ash’, which is derived from the plant tissue itself, and ‘non-physiological’ ash, which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plant surface. Acid insoluble ash measures the amount of silica present especially as sand and siliceous earth.

**Alkaloid Content:**
Alkaloids are responsible for a large number of activities as reported in literature hence, total alkaloid content has been chosen as a quality assurance parameter.

**Stability Information for the Botanical Raw Material:**
Fresh green leaves are used for preparation of plant extract. The deterioration of fresh leaves is fast, hence, most of the quality parameters have been set for lyophilized aqueous extract. The fresh leaves can only be used until 72 hrs, therefore, stability studies cannot be done.

**BOTANICAL DRUG SUBSTANCE**

**Description:** (Physical, Chemical or Biological Characteristics)
The aqueous lyophilized extract was obtained as greenish-brown hygroscopic powder with aggregates.

**The extract was fingerprinted by four different HPLC methods:**
(i) Alkaloids after solid phase extraction through drug test I cartridge
(ii) Amino acids (and related compounds) after solid phase extraction through cation exchange resin.
(iii) Entire profiling of the extract including the detection of UV inactive compound by evaporative light scattering detector (ELSD)
(iv) Organic acids and related compounds after solid phase extraction through anion exchange resin.
The extract was assayed by four different methods for:

(i) Total neutral sugars as glucose by colorimetry
(ii) Total amino acids (and related compounds) as valine by colorimetry
(iii) Content of phenylalanine by HPLC
(iv) Content of berberine by LC-MS

Berberine was identified as the marker compound:
The standard batch showed 7.27% of total neutral sugars, 13.83% of total amino acids (and related compounds), 0.010% of berberine, 31.64% of total ash, 0.42% of acid insoluble ash, 1.64% of moisture and pH (1% aqueous solution) was 6.25. Subsequent batches were compared with the standard batch in order to ensure batch consistency.

Therapeutic category: Anti-psoriasis

Name and Address of the Botanical Drug Substance Manufacturer:

a. Lupin Limited (Research Park)  
   46A/47A, Village Nande  
   Taluka Mulshi  
   Pune 411042, Maharashtra, India

b. Lupin Limited  
   198-202, New Industrial Area No. 2  
   Mandideep 462046  
   Raisen, Madhya Pradesh, India

c. Lupin Limited  
   A-28/1, M.I.D.C. Industrial Area, Chikalthana  
   Aurangabad 431 210, Maharashtra, India
PREPARATION OF THE BOTANICAL DRUG SUBSTANCE:
The leaves of the plant were taken and sorted thoroughly in order to
remove any foreign material, especially fruits and seeds. The sorted leaves
(227.5 Kg) were washed and ground in to a coarse paste. 42 liters of
demineralised water was added during grinding. This paste was soaked in
water (1.5 times; 405 liters) at room temperature for sixteen hours. The
mixture was stirred for 5 minutes after every two hour. The mixture was
centrifuged to yield first percolation (535 liters) of aqueous extract. The
residue (125 Kg) was soaked again in water (1.5 times; 188 liters) and left for
sixteen hrs. This was again centrifuged and residue washed with 20 liters of
water to yield a second percolation (206 liters). The two percolations were
combined and concentrated *in vacuo* below 35 °C to a thick viscous liquid
(110 liters) with a total solid content of 16.56%. This was further lyophilized
to give 17.5 Kg of a greenish brown powder 0. Yield: 7.69%.

ISOLATION AND CHARACTERIZATION OF MARKER COMPOUND

Isolation
Aqueous extract of *Argemone mexicana* (500 g) was dissolved in 2.5 litres of
water and partitioned with *n*-butanol (3 X 500 mL) to obtain butanol extract.
This butanol extract was dried to yield 15.8 g of viscous mass. This was then
adsorbed on silica gel to prepare a slurry. This was subjected to flash
chromatography and elution was carried out with chloroform with
increasing proportions of methanol. Berberine rich fractions were pooled
(0.84 g) on the basis of TLC with authentic sample. The pooled fraction was
purified over a silica gel column using ethyl acetate : methanol : water
(7:1.5:1.5) as eluent to yield berberine (8.1 mg). A chloride salt was prepared
(8.4 mg; LL-3799).

Characterization

Structure

![Chemical Structure](Image)
Test for Purity and Identity

The purity and identity of berberine chloride was established by m.p., HPLC, UV, IR, MS, NMR and by comparison with commercially available sample (Sigma Lot no. 119HO687).

**Melting Point:** The compound melted in the range of 188-189 °C.

**HPLC:** This was used to determine the purity, which was found to be 85.66% pure.

**UV $\lambda_{\text{max}}$ (MeOH):** 350.1, 266 and 229nm

**IR:** The IR spectrum was taken as a KBr dispersion and recorded in the range 450-4000 cm. The compound showed the following bands $\nu_{\text{max}}$: 3344.1, 1508.0, 1330.8, 1388.6, 1363.5, 1271.6, 1228.6, 1105.1, 1064.6, 1035.2 cm.

**MS:** The molecular weight of the compound as determined by +ve ion mode was found to be m/z 336.3 (M$^+$) corresponding to [C$_{20}$H$_{18}$NO$_4$]$^+$.  

**$^1$H NMR:** 7.78(s,1H, H-1), 7.07(s,1H, H-4), 3.39(t,2H, H-5), 4.93(t,2H,H-6), 9.90(s,1H,H-8), 8.16(d,1H,H-11), 8.01(d,1H,H-12), 8.97(s,1H,H-13), 6.15(s,2H,O-CH$_2$O), 4.05(s,3H,O-CH$_3$), 4.08(s,3H,O-CH$_3$). (DMSO-d$_6$, 200MHz) $\delta$

**$^{13}$C NMR:** 106.34(C-1), 148.53(C-2), 150.65(C-3), 109.30(C-4), 131.53(C-4a), 27.21(C-5), 56.05(C-6), 146.36(C-8), 121.32(C-8a), 144.50(C-9), 151.26(C-10), 127.57(C-11), 124.44(C-12), 133.84(C-12a), 122.27(C-13), 138.30(C-13a), 121.12(C-13b), 102.98(O-CH$_2$-O), 57.96(O-CH$_3$), 62.85(O-CH$_3$). (DMSO-d$_6$, 50MHz) $\delta$

Berberine chloride (Sigma Lot no. 119HO687)
Melting Point: The compound melted in the range of 197-198 °C.

UV $\lambda_{\text{max}}$ (MeOH): 350.1, 266 and 228.1 nm

IR: The IR spectrum was taken as a KBr dispersion and recorded in the range 450-4000 cm$^{-1}$. The compound showed the following bands $\nu_{\text{max}}$: 3344, 1506.9, 1392.0, 1365.0, 1276.0, 1333.9, 1276.6, 1230.9, 1104.4, 1036.7, 1059.9 cm$^{-1}$.

MS: The molecular weight of the compound as determined by +ve ion mode was found to be m/z 336.3 (M$^+$) corresponding to [C$_{20}$H$_{18}$NO$_4$]$^+$.

$^1$H NMR: 7.78(s, 1H, H-1), 7.07(s, 1H, H-4), 3.27(t, 2H, H-5), 4.93(t, 2H, H-6), 9.89(s, 1H, H-8), 8.19(d, 1H, 1H-11), 7.99(d, 1H, H-12), 8.94(s, 1H, H-13), 6.16(s, 2H, O-CH$_2$-O), 4.05(s, 3H, O-CH$_3$), 4.08(s, 3H, O-CH$_3$) (DMSO-d$_6$, 200MHz) $\delta$

$^{13}$C NMR: 106.32(C-1), 148.55(C-2), 150.69(C-3), 109.30(C-4), 131.56(C-4a), 27.20(C-6), 56.05(C-6), 146.34(C-8), 121.32(C-8a), 44.53(C-9), 151.27(C-10), 127.60(C-11), 124.42(C-12), 133.85(C-12a), 122.28(C-13), 138.34(C-13a), 121.08(C-13b), 102.96(O-CH$_2$-O), 57.93(O-CH$_3$), 62.81(O-CH$_3$) (DMSO-d$_6$, 50MHz) $\delta$

The Acceptable Limits and Analytical Methods Used to Assure the Identity, Strength, Quality and Purity of the Botanical Drug Substance:
To check the authenticity and quality of the botanical drug substance, specifications were issued (Spec. No. HDEL002-03). These specifications evolved over a period of time and various tests were modified and new tests included. The analysis was carried out as per in-house Standard Test Procedure (HDEL002-03)
Initial specifications included description, identification (TLC), total and acid insoluble ash, pH, loss on drying, limit test of heavy metals, alkaloid content by gravimetry and fingerprinting by HPLC (Tent. Spec. No. HDEL002). The specifications were improved by addition of assay by titrimetry (acidimetry) and fingerprinting by HPTLC (Spec. No. HDEL002-00). Further specifications were improved by addition of fingerprinting of amino acids (and related compounds) and alkaloids by HPLC and by ELSD detection (Spec. No. HDEL002-01). Historical batch data report along with specifications is given in Table 2 (Annexure B). Limit test of heavy metals was replaced with the content of individual metals, hence, revised specifications were issued (Spec. No. HDEL002-02). Specifications were further improved by addition of fingerprinting of organic acids (and related compounds) and content of phenylalanine by HPLC (Spec. No. HDEL002-03). Historical batch data of three batches is given in Table 3 along with the latest specifications.

**Description**
The aqueous lyophilized extract was obtained as a greenish-brown hygroscopic powder with aggregates.

**Specifications and Tests for Botanical Drug Substance:**

**Identification:**
HPLC: Should comply

**pH (1.0% aqueous solution)**
Should be between 4.5-6.5

**Water Content**
MT 6.0% w/w

**Ash**
- Total ash: NMT 35.0% w/w
- Acid Insoluble ash: NMT 2.5% w/w

**Heavy Metals**
- Lead: < 5.0 ppm
- Cadmium: < 2.0 ppm
- Mercury: < 2.0 ppm
- Arsenic: < 2.0 ppm
**Assay**

Total amino acids (and related compounds) as valine (colorimetry)  
NLT 7.0% w/w.

Total neutral sugars as glucose (colorimetry)  
NLT 4.0% w/w.

Content of berberine by LC-MS  
NLT 0.005% w/w.

Content of phenylalanine by HPLC  
NLT 0.2% w/w.

**Fingerprinting (by HPLC)**

- Alkaloids  
  To comply

- Amino acids (and related compounds)  
  To comply

- By using evaporative light scattering detector  
  To comply

- Organic acids (and related compounds)  
  To comply

**Microbial Limits (CFU/g)**

- Total aerobic bacterial count  
  NMT $10^5$

- Total yeast and mould count  
  NMT $10^3$

- Other enterobacterial count  
  NMT $10^3$

**Detection of pathogens/g**

- *Escherichia coli*  
  NMT 10

- Salmonella  
  Absent

**Bioassay**

- % Induction of IL-10 (20 μg/mL)  
  NLT 150

- EC$_{50}$ (μg/mL)  
  NMT 10
RATIONALE FOR SPECIFICATIONS

Identification by HPLC:
Must comply as it gives an actual assessment of chemical components present.

Water Content:
An excess of water in extracts will enhance microbial growth, presence of fungi and hence, deterioration. Limit for water content should therefore be set for every given material. It is especially important for hygroscopic materials like test article.

Total Ash and Acid Insoluble Ash: As in Raw Material

Heavy Metals:
Medicinal plant extracts may be contaminated with heavy metals because of environmental pollution. So tests should be performed.

Assay:
This is included to estimate the quantity of specific group of constituent/s present in the extract in order to ensure batch to batch consistency.

Fingerprint:
This parameter is included to compare the test raw material with the standard batch of extract and to ensure batch to batch consistency.

Microbial Limits:
Medicinal plants normally carry a great number of bacteria and moulds, after originating in soil. While a large range of bacteria and fungi form the naturally occurring microflora of herbs, aerobic spore forming bacteria frequently predominate. Therefore, even after processing the extract under controlled conditions it is necessary to monitor and control microbial limits.

Bioassay:
This parameter is necessary, as each batch should be tested for efficacy. In cases where the active principle is not identified, inspite of the fact that various finger-printings and assays may fall within range, actual efficacy pattern can only be assessed by bioassay.
ANIMAL TOXICOLOGY

Safety evaluation studies of a new herbal /botanical product are conducted to generate data to give information about safety index (LD_{50} / ED_{50}) and doses, which produce lethality and/or organ toxicity in experimental animals with a view to provide guidance to clinical investigators. The toxicology studies are divided into single dose (acute), repeated dose (long term), local toxicity, phototoxicity, immunotoxicity and genotoxicity.

Experimental Animals

Healthy young adult animals of approximately 5-6 weeks of age were obtained from Research Animal Facility (RAF), Lupin Limited (Research Park) Pune. At the start of the study after acclimation and randomization individual body weights did not exceed +20 % of mean body weights/sex.

Husbandry

Animals were housed @ 5/cage/sex/group in polypropylene cages with rice husk bedding. All the animals were having free access to potable water and standard pellet diet. The animal room temperature and humidity were maintained at approximately 22°C ±3 and 30-70% respectively. 100% fresh airflow within the animal testing unit was maintained. Illumination was controlled to give 12 hours light and 12 hours dark cycles.

1. ACUTE TOXICITY STUDIES IN WISTAR RATS AND SWISS MICE

Duration

After receipt, the animals were kept for minimum 5 days of acclimation period in the experimental room. Randomisation (if required) was carried out on day 5. On day 6 of acclimation dosing was started. The observation period was 14 days. Formulation of test article was prepared in distilled water and used for dose administration.

Dosing

Doses were administered once by oral route with a suitable feeding cannula.
OBSERVATIONS

Body weights
Body weights of individual animals were recorded immediately prior to dosing on day 0 and further on days 1, 4, 7, 10 and 14 following the dosing.

Clinical signs
Clinical signs were recorded in terms of time of onset, duration and severity. All treated animals were observed continuously for initial 6 hours after dosing on day 0, and twice daily thereafter till termination.

Necropsy
All animals dying during the study and those sacrificed at the end of observation period were subjected to necropsy. Gross pathological (macroscopic) changes were also recorded. Organs/tissues with gross pathological changes were preserved and processed for microscopic examination, if it is considered that such examination would yield additional useful information.

RESULTS
All findings such as clinical observations, body weight changes, mortality, gross and histopathological changes (if any) were tabulated, evaluated and correlated accordingly. The median lethal dose (LD₅₀) values calculated if required, from the observed mortality data by probit analysis using Finney's method (Finney D.G., Probit Analysis 3rd Edition Combridge University Press, 1971).

2. REPEATED DOSE TOXICITY STUDIES IN WISTAR RATS:
Repeated oral dose toxicity studies were carries out in wistar rats for 28 day and further for 180 days with the objectives to characterize the profile of test article following repeated exposure and to determine the dose response relationship. The information derived from this study would also serve to indicate the possible toxicity including neurological, physiological, biochemical, hematological and exposure-related morphological (pathological) effects likely to arise from repeated exposure of the test article and establish NOEL (No Observable Effect Level) dose and MTD (Maximum Tolerated Dose), safety criteria for human exposure/administration.
STUDY DESIGN
Appropriate no of animals were Animals were acclimatized for minimum five days and randomly allocated to the treatment and control groups. Before final assignment to the study and initiation of dosing, animals were subjected to a veterinary clinical examination to ensure that the selected animals were in a good state of health. During acclimation period, animals showing signs of ill health were subjected to replacement.

FORMULATION AND ADMINISTRATION OF TEST ARTICLE
Test article was administered orally by using appropriate feeding cannula. Precisely measured volume was administered to the rats as per weekly body weights. Treatment in this manner was continued once daily, seven days a week, for a period of 28 days.

OBSERVATIONS
The following observations were made during the course of study.

Mortality
Throughout the study, all animals were checked every day for mortality or moribund condition.

Clinical signs
All clinical signs of ill health, together with any behavioral change or reaction to treatment were recorded for individual animal. These examinations were carried out twice daily at suitable intervals after dosing to observe treatment-related changes. Dated and signed records of clinical signs were maintained on clinical observation data sheets for individual animal.

Body weights
The body weight of each animal was recorded on the day of commencement of treatment (Day-0) and at weekly intervals thereafter till termination. Body weight gain was calculated on weekly basis.
Feed consumption
The quantity of feed consumed by individual animal was calculated in such a manner that quantity of total feed consumed in each cage in a given period was divided by the population of animals in that particular cage to get the individual animal feed consumption. Intake was calculated/measured using the amount of feed offered and left after consumption during one day.

Ophthalmoscopy
In the last week of treatment, the eyes of all animals in control and high dose groups were examined by means of indirect all pupil ophthalmoscope (Keeler Limited, Clewer Hill Road, Windsor, Berkshire SL4 4AA, England) for any drug-related effects. Ophthalmoscopy was not extended to lower dose groups as no abnormality was detected in high dose group animals.

PATHOLOGY
At termination, blood was withdrawn, from orbital sinuses of individual animal. Potassium salt of EDTA and Heparin was used as anticoagulants for hematology and biochemistry respectively. Plasma was separated by centrifuging blood at 3000 rpm for 15 minutes. Urine sample from each animal was collected by using metabolic cage and subjected to routine analysis. The estimations performed on blood and urine samples are listed below, with an abbreviated title (for use in Appendices and Tables).

(A) Hematology
The following estimations were performed using Beckman Coulter Haematology (Ac T Diff), Coulter Corporation U.S.A., analyzer using Veterinary Software.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Abbreviation</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood corpuscles</td>
<td>WBC</td>
<td>(1000/μl)</td>
</tr>
<tr>
<td>Red blood corpuscles</td>
<td>RBC</td>
<td>(million/μl)</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>HGB</td>
<td>(g/dl)</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>HCT</td>
<td>(%)</td>
</tr>
<tr>
<td>Mean corpuscular volume</td>
<td>MCV</td>
<td>(fl)</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin</td>
<td>MCH</td>
<td>(pg)</td>
</tr>
<tr>
<td>Mean corpuscular hgb concentration</td>
<td>MCHC</td>
<td>(g/dl)</td>
</tr>
<tr>
<td>Platelets</td>
<td>PLT</td>
<td>(1000/μl)</td>
</tr>
</tbody>
</table>
**Differential WBC counts:**
Standard microscopy of blood smear stained with Leishman’s stain. 100 cells were counted and expressed as follows:

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>(%)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>(%)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>(%)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>(%)</td>
</tr>
<tr>
<td>Basophiles</td>
<td>(%)</td>
</tr>
</tbody>
</table>

**(B) Clinical Biochemistry**
The following electrolytes using Synchron EL-ISE Beckman, USA and biochemical parameters using Vet-Ex, Schiaparelli Biosystems Inc, U.S.A. were estimated.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (ALB)</td>
<td>mg/dl</td>
</tr>
<tr>
<td>Alanine amino transaminase (ALT)</td>
<td>IU/L</td>
</tr>
<tr>
<td>Aspartate amino transaminase (AST)</td>
<td>IU/L</td>
</tr>
<tr>
<td>Alkaline Phosphatase (ALP)</td>
<td>U/L</td>
</tr>
<tr>
<td>Cholesterol (CHOL)</td>
<td>mg/dl</td>
</tr>
<tr>
<td>Creatinine Kinase (CK)</td>
<td>U/L</td>
</tr>
<tr>
<td>Creatinine (CREAT)</td>
<td>mg/dl</td>
</tr>
<tr>
<td>Gamma Glutamyl Transpeptidase (GCT)</td>
<td>U/L</td>
</tr>
<tr>
<td>Glucose (GLU)</td>
<td>mg/dl</td>
</tr>
<tr>
<td>Total Protein (TP)</td>
<td>g/dl</td>
</tr>
<tr>
<td>Triglycerides (TRIG)</td>
<td>mg/dl</td>
</tr>
<tr>
<td>Total Bilirubin (TBILI)</td>
<td>g/dl</td>
</tr>
<tr>
<td>Uric Acid (UA)</td>
<td>mg/dl</td>
</tr>
<tr>
<td>Urea (UREA)</td>
<td>mg/dl</td>
</tr>
<tr>
<td>Sodium (Na)</td>
<td>mmol/L</td>
</tr>
<tr>
<td>Potassium (K)</td>
<td>mmol/L</td>
</tr>
<tr>
<td>Chloride (Cl)</td>
<td>mmol/L</td>
</tr>
</tbody>
</table>
(C) Urinalysis

Qualitative Tests:
pH, Glucose, Specific gravity, Ketones, Protein, Bilirubin, Occult Blood, Urobilinogen.
Tests were performed using Ames Multiple Reagent Diagnostic Strips obtained from Bayer Diagnostics India Ltd., Baroda, and were used as qualitative indicators of analyte concentration. Results were reported according to the following convention.

0 = Negative
Tr = Trace of analyte
+ = Small amount of analyte
++ = Moderate amount of analyte
+++ = Large amount of analyte

Microscopy
For microscopic examination, an aliquot of the urine sample was centrifuged at approximately 1500 rpm (revolutions per minute) for 10 minutes and the resulting deposit was spread on a glass slide. The deposits were examined for the presence of the following.

Epithelial cells (E)
Pus cells (P)
Erythrocytes (R)
Crystals (Cr)
Casts (C)

The grading of centrifuged deposit was done as follows.
0 = none found in any field examined
1 = few in some fields examined
2 = few in all fields examined
3 = many in all fields examined

(D) Necropsy
After completion of the treatment regime and reversal period (if any), all surviving animals were ethically sacrificed. Complete necropsy was carried out. The tissues collected from these animals were preserved in 10% neutral buffered formalin.
(E) Organ Weights
The following organs from animals dissected free of fat and weighed as soon as possible for absolute and relative organ weights during the course of necropsy.
Adrenals
Heart
Kidneys
Liver
Spleen
Testes (Male)

(F) Histopathological Examination
Tissues collected for microscopic examination were embedded in paraffin wax, sectioned at 5 micrometers and stained with hematoxylin and eosin. Histopathological examination was restricted to the tissues of animals from control and high dose groups.

List of Tissue collected for Histopathological Examination

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Tissue</th>
<th>Tissue</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal</td>
<td>Aorta</td>
<td>Brain</td>
<td>Caecum</td>
</tr>
<tr>
<td>Colon</td>
<td>Duodenum</td>
<td>Epididymis</td>
<td>Eyes</td>
</tr>
<tr>
<td>Femur</td>
<td>Heart</td>
<td>Jejunum</td>
<td>Kidney</td>
</tr>
<tr>
<td>Liver</td>
<td>Lung</td>
<td>Lymph nodes</td>
<td>Oesophagus</td>
</tr>
<tr>
<td>Ovaries</td>
<td>Pancreas</td>
<td>Parathyroid</td>
<td>Pituitary</td>
</tr>
<tr>
<td>Prostate gland</td>
<td>Salivary gland</td>
<td>Seminal Vesicle</td>
<td>Skeletal muscle</td>
</tr>
<tr>
<td>Skin</td>
<td>Spinal cord</td>
<td>Spleen</td>
<td>Stomach</td>
</tr>
<tr>
<td>Testes</td>
<td>Thymus</td>
<td>Thyroid</td>
<td>Trachea</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>Uterus</td>
<td>Any tissue showing gross lesions</td>
<td></td>
</tr>
</tbody>
</table>

STATISTICAL ANALYSIS
Dunnett's 't' test was employed to assess the significance levels of inter group differences between the control group and treated group. (Snedocor & Cochran. "Statistical Methods" 8th edition, Iowa state University Press, 1989). Graph pad Prism Software (10855, Sorrento Valley Road #203 San Diego CA 92121 USA) was used for statistical analysis.
3. ACUTE DERMAL IRRITATION STUDIES IN NZW RABBITS:

EXPERIMENTAL DESIGN
Three healthy adult albino male rabbits were procured from Research Animal Facility-NCER Lupin Limited (Research Park), Pune. The animals were housed individually in stainless steel cages and had free access to filtered drinking water and standard laboratory pellet diet. The housing temperature and humidity was maintained at 22\(^\circ\)C ± 3\(^\circ\)C and RH 30% - 70% respectively. An acclimation period of minimum five days after receipt of animal was observed.

APPLICATION OF TEST ARTICLE:
Test article was finely pulverized and moistened in the small quantity of sterilized & distilled water (commercially available water for injection) @ 0.5g/application site and such freshly made preparation was applied to the designated site of the dorsal area (right side) of the trunk of each animal and covered with a gauze patch, which is held in place with non irritant tape. Left flank of the trunk was used as control site. At the end of the exposure period (4hrs. post application) the test article was removed using distilled water without altering the existing response or the integrity of the dermis.

CLINICAL OBSERVATION
Animals will be examined for signs of erythema, oedema and the responses scored at 1, 4 post application and then 24, 48, 72 hours after patch removal. Dermal irritation is scored and recorded according to the grades in the table-1. In addition to the observation of the irritation, all the lesions and other toxic effects will be recorded and described.
# TABLE-1

## GRADING OF SKIN REACTION

### ERYTHEMA AND ESCHAR FORMATION

<table>
<thead>
<tr>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Erythema</td>
<td>0</td>
</tr>
<tr>
<td>Very Slight Erythema (Barely Perceptible)</td>
<td>1</td>
</tr>
<tr>
<td>Well Defined Erythema</td>
<td>2</td>
</tr>
<tr>
<td>Moderate to Severe Erythema</td>
<td>3</td>
</tr>
<tr>
<td>Severe Erythema (Beet Redness) to Eschar Formation</td>
<td>4</td>
</tr>
</tbody>
</table>

Maximum Possible: 4

### ODEMA FORMATION*

<table>
<thead>
<tr>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Oedema</td>
<td>0</td>
</tr>
<tr>
<td>Very Slight Oedema (Barely Perceptible)</td>
<td>1</td>
</tr>
<tr>
<td>Slight Oedema (Edges of Area Well Defined)</td>
<td>2</td>
</tr>
<tr>
<td>Moderate Oedema (Raised Approx. 1 millimeter)</td>
<td>3</td>
</tr>
<tr>
<td>Severe Oedema (Extending Beyond Area of Exposure)</td>
<td>4</td>
</tr>
</tbody>
</table>

Maximum Possible: 4

*Histopathological examination was on the discretion of pathologist to clarify doubtful reactions
4. ACUTE EYE IRRITATION STUDIES IN NZW RABBITS:

EXPERIMENTAL DESIGN
Three healthy adult albino male rabbits were procured from Research Animal Facility-NCER Lupin Limited (Research Park), Pune. Animals were housed individually in stainless steel cages and had free access to filtered drinking water and standard laboratory pellet diet. The housing temperature and humidity were maintained at 22°C ± 3°C and RH 30% - 70% respectively.

APPLICATION OF TEST ARTICLE:
A 0.1 ml of freshly prepared solution dissolved in sterilized water @ 250 mg/ml was instilled in the conjunctival sac of right eye of each animal after gently pulling the lower lid away from the eyeball. The lids are then gently held together for about two seconds in order to prevent the loss of the test compound. The left eye, which remains untreated, served as control.

DOSE LEVEL
A 0.1 ml of freshly prepared solution was instilled in to the conjunctival sac of right eye of each animal.

OBSERVATION PERIOD
The duration of observation period was not fixed rigidly but sufficient to fully evaluate, reversibility or irreversibility of effects observed if any.

CLINICAL OBSERVATION
Eyes were examined at 1, 24, 48, 72 hours and further if required, once daily up to day 14 or more. The grades of ocular reaction was recorded at each examination. Irritation was scored and recorded according to the grades in the table-2 In addition to the observation of the irritation, all the lesions and other toxic effects were recorded.
### TABLE-2

**GRADING FOR OCULAR LESIONS**

**CORNEA**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ulceration or opacity</td>
<td>0</td>
</tr>
<tr>
<td>Scattered or diffused area of opacity (other than slight dulling of normal luster)</td>
<td>1</td>
</tr>
<tr>
<td>Easily discernible translucent area, details of iris slightly obscured</td>
<td>2</td>
</tr>
<tr>
<td>Nacreous area, no details of iris visible, size of pupil barely discernible</td>
<td>3</td>
</tr>
<tr>
<td>Opaque cornea, iris not discernible through the opacity</td>
<td>4</td>
</tr>
</tbody>
</table>

- Area of corneal opacity will be noted

**IRIS**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia, or injection, any of these or combination of any of these, iris still reacting to light (Sluggish Reaction is positive)</td>
<td>1</td>
</tr>
<tr>
<td>No reaction to light, hemorrhage, gross destruction (Any or all of these)</td>
<td>2</td>
</tr>
</tbody>
</table>

**CONJUNCTIVAE**

[Redness (Palpebral and bulbar conjunctivae excluding cornea and iris)]

<table>
<thead>
<tr>
<th>Condition</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood vessels normal</td>
<td>0</td>
</tr>
<tr>
<td>Some blood vessels definitely hyperaemic (Injected)</td>
<td>1</td>
</tr>
<tr>
<td>Diffused, crimson color, individual vessels not easily discernible</td>
<td>2</td>
</tr>
<tr>
<td>Diffused, beefy red</td>
<td>3</td>
</tr>
</tbody>
</table>

**CHEMOSIS: Lids and/or Nictitating membranes**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Swelling</td>
<td>0</td>
</tr>
<tr>
<td>Any Swelling above normal (Including Nictitating membrane)</td>
<td>1</td>
</tr>
<tr>
<td>Obvious swelling with partial aversion of lids</td>
<td>2</td>
</tr>
<tr>
<td>Swelling with lid about half closed</td>
<td>3</td>
</tr>
<tr>
<td>Swelling with lids more than half closed</td>
<td>4</td>
</tr>
</tbody>
</table>
5. **IN-VITRO** CHROMOSOMAL ABERRATION INDUCTION POTENTIAL IN HUMAN LYMPHOCYTES:

**TEST SYSTEM AND MANAGEMENT**

**Cells, Media And Culture Conditions:**
Human peripheral blood lymphocytes were used. Whole blood treated with heparin as an anti-coagulant was obtained from healthy subjects and added to culture media RPMI 1640 with 10% fetal calf serum containing mitogen (phytohemagglutinin). The cultures were incubated at 37°C for minimum period of 48 h prior to treatment.

**Metabolic Activation:**
Cells were exposed to the test article both in the presence and absence of metabolic activation system. The metabolic activation system was co-factor supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme inducing agent in combination of phenobarbitone and β-naphthoflavone.

**EXPERIMENTAL PROCEDURE**

**Test Article Preparation**
Test article was dissolved in distilled water at limit concentration of 5mg/ml. Serial dilutions will be made for lower concentrations.

**Exposure Concentrations**
A dose range finding study for cytotoxicity was performed using concentrations of 5, 2, 0.8 and 0.32 mg/ml (i.e. 25, 10, 4, 1.6 μg/ml of culture). Mitotic Index (MI) was considered as indication for cytotoxicity. Based on the MI, concentrations for main study were selected. At least three analyzable concentrations were used.

**Controls**
Concurrent positive and negative (solvent or vehicle) controls both with and without metabolic activation were included in each experiment. Positive controls were as follows:
<table>
<thead>
<tr>
<th>Metabolic activation condition</th>
<th>Positive control chemical</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without metabolic activation</td>
<td>Mitomycin C</td>
<td>0.4 μg/ml</td>
</tr>
<tr>
<td>With metabolic activation</td>
<td>Cyclophosphamide (monohydrate)</td>
<td>15 μg/ml</td>
</tr>
</tbody>
</table>

**Treatment With Test Article**

Proliferating cells were treated with test article in the presence and absence of a metabolic activation system. Treatment of lymphocytes was commenced at about 48 h after mitogenic stimulation (PHA). Duplicate cultures were used at each concentration including concurrent negative and positive controls.

**Culture Harvest Time**

**Phase I**

Treatment with and without metabolic activation for 3-6 hours and harvesting of cells at about 1.5 normal cell cycle length (22 h).

**Phase II**

On the basis of Phase I negative results both with and without activation, an additional experiment without metabolic activation until cells harvesting at about 1.5 normal cell cycle length was performed.

**Chromosome Preparation**

Cell cultures were treated with colchicine (metaphase arresting agent) for two-three hours prior to harvesting. Then the cultures were treated hypotonically (0.075M KCl) and fixed with Carnoy’s fixative and the chromosome preparation from the cells was made. Two slides were prepared from each culture with proper identification. Such preparation were stained with 5% Giemsa stain.
ANALYSIS
The slides were coded before microscopic analysis. At least 1000 cells were counted in different fields of slides per culture to determine the mitotic index. A minimum of 100 consecutive metaphases scored under 100x oil immersion objective for aberrations.

6. MAMMALIAN BONE MARROW MICRONUCLEUS TEST IN SWISS MICE
   BY ORAL ROUTE:

STUDY DESIGN
The study design was as follows:

<table>
<thead>
<tr>
<th>Dose Groups</th>
<th>Dose levels (mg/kg)</th>
<th>Number of Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Males</td>
</tr>
<tr>
<td>Control</td>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>Low</td>
<td>II</td>
<td>500</td>
</tr>
<tr>
<td>Intermediate</td>
<td>III</td>
<td>1000</td>
</tr>
<tr>
<td>High</td>
<td>IV</td>
<td>2000</td>
</tr>
<tr>
<td>Positive control</td>
<td>V</td>
<td>4</td>
</tr>
<tr>
<td>(Mitomycin C)</td>
<td>(Intra-peritoneal)</td>
<td></td>
</tr>
</tbody>
</table>

The animals were housed sex wise in groups of five. A label, uniquely numbered along with project number identified each cage. Before final assignment to the study, animals were subjected to a veterinary clinical examination to ensure that the selected animals are in a good state of health.

ADMINISTRATION OF TEST ARTICLE
The test article was administered orally by using one ml syringe fitted with 18 G intubation needle. The animals were treated with test article for two consecutive days. However, The Positive control group was treated only once by intraperitoneal route with Mitomycin C at 4mg/kg body weight.
OBSERVATIONS
Following observations were made during the course of study.

MORTALITY
Throughout the study, all animals were checked early in each working day and again in the afternoon to observe for dead or moribund animals. At weekends and public holidays a similar procedure was followed except that the final check was carried out at approximately midday (one hour post administration).

CLINICAL SIGNS
All signs of ill health, together with any behavioral change or reaction to treatment were recorded for individual animal. These examinations were carried out daily at suitable intervals after dosing to observe treatment-related changes. Dated and signed records of clinical signs were maintained on clinical data sheets for individual animal.

BODY WEIGHT
Body weights of each animal were recorded at the time of allocation of animals to groups, on the day of commencement of each treatment and before sacrifice.

SACRIFICE AND CELL PROCESSING
All the animals were sacrificed by cervical dislocation at about 24 h after the last treatment. Both the femur bones dissected out and bone marrow cells were flushed with the help of fetal calf serum using 1 ml syringe fitted with 24 gauge needle. The cell suspension was centrifuged at 2000 rpm for 10 min. and smears were prepared on clean microslides. Two slides were prepared from each animal with proper identification. The preparation was stained with 5% Giemsa stain.

ANALYSIS
Slides were coded before microscopic analysis. At least 2000 polychromatic erythrocytes per animal were scored for incidence of micronuclei. The ratio of polychromatic to normochromatic erythrocytes was determined for each animal.
7. EVALUATION OF PHOTOTOXICITY POTENTIAL IN MICE:

STUDY DESIGN

<table>
<thead>
<tr>
<th>Dose Groups</th>
<th>Doses (mg/kg)</th>
<th>Number of Animals</th>
<th>Observation Period (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Control)</td>
<td>00 (Vehicle)</td>
<td>Male 5 Female 5</td>
<td>5</td>
</tr>
<tr>
<td>II (High)</td>
<td>1000</td>
<td>Male 5 Female 5</td>
<td>5</td>
</tr>
<tr>
<td>III (Positive control)</td>
<td>400</td>
<td>Male 5 Female 5</td>
<td>5</td>
</tr>
</tbody>
</table>

DURATION
After 4 days of drug administration, on day-5, all the animals were sacrificed.

PREPARATION OF ANIMALS
Animals with healthy intact skin were used for experiment. Twenty-four hours before test, fur was removed by close clipping the dorsal trunk area. Care was taken to avoid injury to skin.

DOsing
Doses were administered by oral route with a sterile syringe and 20G feeding cannula. Test article was dissolved in distilled water for attaining desired dose levels. Treatment was continued for a period of 4 days. Dose volume administered to individual animal was adjusted according to the most recent recorded body weight.

DOSES LEVELS
Doses of 0 and 1000 mg /kg of test article were administered by oral route. Vehicle equivalent to highest dose volume was administered to the control group animals. Sparfloxacin, a positive control was administered orally at 400 mg/kg as a suspension in 5% Tween-80 and normal saline.
ULTRAVIOLET IRRADIATION
One hour after administration, animals were irradiated (365 nm) for 3 hours daily for a period of 4 days. Cages containing animal were placed on platform 6 inches below UVA light source in a cabinet. Mean light intensity was controlled between 0.30 to 0.40 mw/cm². Radiometer (Cole-Parmer, VLX 3W) was used to measure light intensity. Cabinet temperature was maintained between 25-30°C.

OBSERVATIONS

Body Weights
Body weights of individual animal were recorded immediately prior to dosing on day 1 and on every day thereafter.

Clinical Signs
All signs of ill health, together with any behavioral changes or reaction to treatment were recorded for individual animal everyday. Dated and signed records of clinical signs were maintained on clinical data sheets for individual animal. Animals were observed 1h post irradiation and on next day before dosing, for the signs of erythema and oedema. Grading of skin reactions was scored and recorded according to grades shown in Table (Ref: OECD guidelines number 404 updated in1981 and adopted in July 1992). In addition to the observation of irritation, all other lesions and toxic effects, if exist were recorded.

<table>
<thead>
<tr>
<th>TABLE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Erythema and Eschar Formation</strong></td>
</tr>
<tr>
<td>No erythema.................................</td>
</tr>
<tr>
<td>Very slight erythema........................</td>
</tr>
<tr>
<td>Well defined erythema........................</td>
</tr>
<tr>
<td>Moderate to severe erythema...................</td>
</tr>
<tr>
<td>Severe erythema to Eschar......................</td>
</tr>
<tr>
<td><strong>Oedema Formation</strong></td>
</tr>
<tr>
<td>No oedema...................................</td>
</tr>
<tr>
<td>Very slight oedema..........................</td>
</tr>
<tr>
<td>Slight oedema..................................</td>
</tr>
<tr>
<td>Moderate oedema................................</td>
</tr>
<tr>
<td>Severe oedema..................................</td>
</tr>
</tbody>
</table>
**Mortality**
Throughout the study, all animals were checked every day periodically. Any animal showing signs of severe debility or intoxication, particularly if death appears imminent will be sacrificed to prevent loss of tissues through autolytic degeneration or cannibalism. Animals killed in extremis, or found dead in the cage will be subjected to detail microscopic examination. Skin and ear samples will be preserved in 10 % neutral buffered formalin for histopathological examination at termination on day five.

**Necropsy**
Animals in moribund condition during the study and those sacrificed at the end of observation period will be subjected to necropsy. Gross pathological changes will be recorded. Organs/tissues with gross pathological changes will only be preserved and processed for microscopic examination.

**Results**
Findings such as clinical observations, body weight changes, mortality, gross and histopathological changes will be tabulated, evaluated and correlated accordingly.

**8. MOUSE EAR SWELLING TEST:**

To obtain the information regarding delayed dermal contact sensitization potential of test article after 4 days repeated exposure to Balb-C mice and also to find out any other adverse effect likely to occur due to dermal exposure.

**Justification Of Dose:**
Dose was selected on the basis of results of pre-test. A maximum concentration of test article that produces minimal irritation (or a maximum possible concentration) to the belly region after a single topical application for 4 days and a concentration of test article that is non-irritating to the ear after a single topical application. 0.25% DNFB (Positive control article and application volume) in the vehicle of olive oil and acetone was selected as per "Methods in Immunotoxicology", Vol. - 2 edited by Gary Burleson. et. al., 1995, Willy - Liss Publication, Inc, 605 Third Avenue, New York, Pages 357-372.
Test System And Management

A total of 84 Balb-C mice of 4-6 Weeks old at receipt were obtained and on the basis of veterinary health check, animals were selected and randomly allocated to the treatment and control groups as shown in Study Design. Following materials and equipment were used during the study.

1. Digital vernier caliper (Mitutoyo Corporation Tokoyo, Japan) for the measurement of ear thickness.
2. Latex gloves
3. Small animal hair clipper with No. 40 blade
4. Vehicle - Distilled water
5. Microliter pipette.
6. Tuberculin syringe.
7. 26 gauge needles.
8. Freunds complete adjuvant (Sigma Chemical Company, U. S.)
10. DNFB (Di-nitro fluoro benzene, a known sensitizing agent)
11. Acetone and olive oil as a vehicle for positive control article.

Experimental Procedure

One week prior to initiation of study, animals were fed Vitamin "A" supplemented diet @250 IU/g of feed. A Pretest was performed to find out maximum concentration of test article that produces minimal irritation to the belly region after a single topical application of each for 4 days and to establish a concentration of test article that is non-irritant to the ear after a single topical application. This Pre-test for dermal irritation and toxicity study was conducted one week prior to the main study.

Dose Formulation

Test article was dissolved in distilled water (1 g in 2 ml) to achieve 50% concentration. Stock solution of 50% concentration was serially diluted for lower concentrations of 25 and 12.5%. Olive oil + Acetone mixture (1:4) as vehicle control and 0.25% DNFB in olive oil + Acetone (5 mg in 2 ml) as positive control for the pre-test was also prepared.
## STUDY DESIGN

<table>
<thead>
<tr>
<th>Groups</th>
<th>Different concentrations tested (%)</th>
<th>Application of the test article (μl)</th>
<th>No of mice Per group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Belly (4 days)</td>
<td>Ear (1 day)</td>
</tr>
<tr>
<td>Pre-Test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test Article Vehicle</td>
<td>0</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>Positive Control Olive oil and Acetone (1:4)</td>
<td>As such</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>Positive control DNFB with vehicle</td>
<td>0.25</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>Test Article</td>
<td>12.5</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>Main Test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>Test article</td>
<td>50</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>Positive Control Olive oil and Acetone (1:4)</td>
<td>As such</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>Positive control DNFB</td>
<td>0.25</td>
<td>50</td>
<td>20</td>
</tr>
</tbody>
</table>

### PRE-TEST

Six males and six females from the pretest group were used to test each concentration of test article. A total 4 concentrations i.e., 0, 12.5, 25, and 50% of test article and 0.25% DNFB as positive control and positive control vehicle olive oil + Acetone mixture (1:4), were evaluated.
On day-0, the first day of pre-test, each animal was prepared by clipping the hair from the belly region using a small animal hair clipper. After clipping the belly, the outer layers of epidermis of each mouse were removed from the clipped region with the help of rough strip. This procedure will be referred as a "stripping" hereafter. Skin was stripped until application region appears shiny. The belly region was stripped before application of the article everyday.

After stripping the belly, a volume of 100 μl test article was applied to the belly region using microliter pipette once daily for 4 days. At the same time, 20 μl test article was applied only once on day-1 to the ventral and dorsal surface of left ear and vehicle was applied in similar manner to the right ear. Before starting the experiment and 24 hours after first topical application of the ears (day-1), the thickness of all pre-test animal ears were measured using thickness measuring instrument (Digital Vernier caliper). While supporting with one hand, one ear of mouse was inserted between contacts of the instruments until its position with approximately 1-2 mm of the outer edge of the ear was covered. Once the reading was obtained, contralateral ear was measured in the same manner. Based on pretest data, a judgment was made for the level of concentration to be used for main study.

**MAIN STUDY**

**Induction Stage**

On day-0, clipping of belly region of all (36 M + 36 F) mice were carried out. Immediately after clipping, two intradermal injections of 20 μl Freund’s Complete Adjuvant (FCA) emulsion were given at separate sites in the skin of the shaved belly (each site flanks of the ventral midline) by using tuberculin syringe and 26 gauge needle.

Following intradermal injections, the belly skin of test and control group animals were stripped. A volume of 100 μl of test article at concentration determined by pretest i.e., 50% was topically applied to the belly skin of test group animals. Positive control group animals were treated with 50 μl of 0.25% DNFB in olive oil and acetone. Concurrent control group animals were treated with same volume of the vehicle.
Further applications were carried out on days-1, 3, and 5 after stripping the belly skin in all treated and control groups. Concurrent control group animals were treated with the vehicle on subsequent days.

**Challenge stage**

On day-10, each test article and vehicle treated mouse received next application and 24 contemporary control (DNFB and Positive Control Vehicle) mice out of 48 had received first application of 20 $\mu$l on the ventral and dorsal surface of left ear. The contralateral right ear was kept untreated. The thickness of both ears was measured on day-10 before topical application and also 24 and 48 hours after challenge application.

**Re-challenge**

As the test article did not show sensitizing potential after first challenge application, the second and final re-challenge application was performed on each animal on day-17. Remaining 24 positive control vehicle and 0.25% DNFB in olive oil and acetone mice were challenged on the ventral and dorsal surface of left ear (20 $\mu$l). The contralateral right ear was treated with the vehicle.

The procedure used for the first challenge application was applied for re-challenge application with the same concentration of test article.

The thickness measurement of both ears was carried out on day-17 before topical application and also 24 and 48 hours after re-challenge application.

**OBSERVATIONS**

**Clinical signs and mortality**

All signs of ill health, together with any behavioral changes or reaction to treatment were recorded for individual mouse and mortality, if any. These detailed examinations were carried out everyday during entire study period. Dated and signed records of clinical signs were maintained on clinical history sheets for individual animal.

Thickness of all pretest animal ears was measured before application and 24 hours thereafter, using digital vernier caliper. Everyday at the time of application the belly region skin was observed for dermal irritation. Dermal
irritation was also recorded 24 h after last application and dose was finalized. If any signs of systemic toxicity observed on any day was also recorded. Judgment concerning the presence or absence of sensitization was made for each animal.

The final judgment was based on the calculation of the potency index in challenge and re-challenge phase. The control group ear thickness measurement was used to identify any possible dermal irritation reaction, to avoid misinterpretation as a positive dermal sensitization response. Second challenge (Re-challenge) application was performed and the data from both challenge groups was compared.

**STATISTICAL ANALYSIS**

Percent ear swelling was calculated. Judgment concerning the presence or absence of sensitization was made for each animal. The judgment was based on the percent difference between the test and control ears. A "Positive " sensitization response was considered if the test ear of one or more animals shown at least 20% thicker than the control ear.

**9. PHARMACOKINETIC STUDIES IN RATS:**

**(A) Oral Route:**

The objectives of this study were to determine pharmacokinetic parameters and dose proportionality / linearity with test article treatment in young healthy Wistar rats under fasting conditions.

**TEST ARTICLE**

The test article an aqueous extract obtained from the plant *Argemone mexicana* has undergone extensive *in-vitro* and *in-vivo* pharmacological screening for its anti-psoriatic activity. One of the efficacy mechanisms of the extract is IL-10 induction from lymphocytes, found to be useful in the treatment of psoriasis. The extract does not have a chromophore and poses difficulty in estimation of the compound directly from the plasma. Hence, the IL-10 induction property was used as biomarker to evaluate the Pharmacokinetic parameters.
EXPERIMENTAL PROCEDURE

Dosing and Dose Levels
As per protocol, the rats received dose, by gavage equivalent to 75 mg/kg, 150 mg/kg and 300 mg/kg. All rats were fasted for 10-12 hours before dosing. Food was given 2 hours post drug administration. Water was allowed *ad libitum*.

Sample Collection & Storage
This study was a parallel design, with three treatments, three periods study as per sampling schedule. The three treatments were: 75, 150 and 300 mg/kg. Sixty rats (30 male + 30 female) comprising one group received one of the treatments in each period. Each group was further divided into 9 sub-groups of 3 rats each. The treatment schedule is outlined below:

<table>
<thead>
<tr>
<th>Period</th>
<th>Group</th>
<th>No of Animals</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>30 male + 30 female</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>30 male + 30 female</td>
<td>150</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>30 male + 30 female</td>
<td>300</td>
</tr>
</tbody>
</table>

Blood samples (1.5ml each) were collected from retro orbital plexus of eye at, pre-dose, 0.5,1,1.5, 2, 3, 4, 6 and 8 hours post p.o. dose of the study. A total 3x9=27 samples of 1.5 ml, from both males and females, using sodium citrate as an anticoagulant were collected at three dose levels of the study. Plasma samples were separated by centrifugation at 4600 R.P.M. for 15 minutes and stored below -70°C until analyzed. The time of sampling was recorded and any sample collected beyond 1 minute was marked as a deviation from schedule time and actual time was used for pharmacokinetic analysis in such a case.
ANALYSIS OF PLASMA SAMPLES

Sample Processing
Test article in plasma samples were measured by an indirect method for the % change in IL-10 induction from the baseline level. Briefly, plasma samples were incubated at 37°C in CO₂ incubator with human blood containing 10 μg/ml of Phyto-hemagglutinin (PHA) in ratio of 1:5. The samples were then centrifuged at 4600 R.P.M. for 15 minutes and produced IL-10 was measured with ELISA kit from R&D Systems. Method of IL-10 estimation was followed as per manufacturer’s instructions. Percent change in IL-10 induction was calculated with respect to the basal sample (at zero hour).

Pharmacokinetic Analysis
The following mean Pharmacokinetic parameters of test article in rats plasma were calculated using mean plasma levels with NCA module of WinNonlin Pro, Version 4 Pharsight, Inc, USA

$E_{max}$: Maximum measured biological response over the time span specified for oral doses. (Effect Maximum)

$T_{max}$: Time to reach $E_{max}$.

$AUC_{0-t}$: The area under the magnitude of biological response versus time curve, from time zero to the last measurable response, as calculated by the linear trapezoidal method.

(B) INTRAVENOUS ROUTE:
The main objectives of this study were to determine the pharmacokinetic parameters and dose proportionality / linearity with test article treatment in young healthy Wistar rats by intravenous (i.v.) route.

EXPERIMENTAL PROCEDURE

Dosing and Dose Levels
As per protocol, the rats received dose, by intravenous route equivalent to 25, 50 and 75 mg/kg. Food and drinking water was allowed *ad libitum.*
Sample Collection & Storage
This study was a parallel design, with three treatments, three periods study as per sampling schedule. The three treatments were: 25, 50 and 75 mg/kg. Thirty rats (15 male + 15 female) comprising one group received one of the treatments in each period. Each group was further divided into 3 sub-groups of 5 rats each. Male and female rats of a group received each treatment on the day of a period. The treatment schedule is outlined below:

<table>
<thead>
<tr>
<th>Period</th>
<th>Group</th>
<th>No of Animals</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>15 male + 15 female</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>15 male + 15 female</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>15 male + 15 female</td>
<td>75</td>
</tr>
</tbody>
</table>

Blood samples (1.5ml each) were collected from retro orbital plexus of eye at, pre-dose, 0.08, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6 and 8 hours post i.v. dose of the study. A total 11x3=33 samples of 1.5 ml, from both males and females, using sodium citrate as an anticoagulant were collected at three dose levels of the study. Plasma samples were separated by centrifugation at 4600 R.P.M. for 15 minutes and stored below -70°C until analyzed. The time of sampling was recorded and any sample collected beyond 1 minute was marked as a deviation from schedule time and actual time was used for pharmacokinetic analysis in such a case.

ANALYSIS OF PLASMA SAMPLES

Sample Processing
Test article in plasma samples were measured by an indirect method for the % change in IL-10 induction from the baseline level. Briefly, plasma samples were incubated at 37°C in CO₂ incubator with human blood containing 10 μg/ml of Phytohemagglutinin (PHA) in ratio of 1:5. The samples were then centrifuged at 4600g for 15 minutes and produced IL-10 was measured with ELISA kit from R&D Systems. Method of IL-10 estimation was followed as per manufacturer’s instructions. Percent change in IL-10 induction was calculated with respect to the basal sample (at zero hour).
Pharmacokinetic Analysis

The following mean Pharmacokinetic parameters of test article in rats plasma were calculated using mean plasma levels with NCA module of WinNonlin Pro, Version 4 Pharsight, Inc, USA

$E_{\text{max}}$: Maximum measured biological response over the time span specified for oral doses.

$T_{\text{max}}$: Time to reach $E_{\text{max}}$.

$\text{AUC}_{0-t}$: The area under the magnitude of biological response verses time curve, from time zero to the last measurable response, as calculated by the linear trapezoidal method.

RESULTS

The pharmacokinetic parameters were estimated using an indirect measurement (induction of IL-10) of test article at three dose levels in rats with single oral /intravenous dose. The data was also analyzed for gender differences at various dose levels.