7. EVALUATION OF FORMULATION FOR ASTHMA

7.1. SAFETY PROFILE STUDY

Principle

The principle of the test study is that based on a stepwise procedure with the use of a minimum number of animals per step. Sufficient information is obtained on the acute toxicity of the test substance to enable its classification.

The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex. Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step, i.e.;

No further testing is needed,
Dosing of three additional animals, with the same dose
Dosing of three additional animals

At the next higher or the next lower dose level. The method will enable a judgment with respect to classifying the test substance to one of a series of toxicity classes.

Test animals and Test Conditions:

Sexually mature either sex albino mice (28-35g) were obtained from the animal laboratory of the School of Pharmaceutical sciences, Vels University. All the animals were kept under standard environmental condition (27±2°C). The animals had free access to water and standard pellet diet (Sai meera foods, Bangalore). Mice were deprived of food but not water (16-18 h) prior to administration of the formulation.

The principles of laboratory animal care were followed and the Department’s ethical committee approved the use of the animals and the study design IAEC PROTOCOL NO: XII/CPCSEA/IAEC/VELS/PCOL/08.08.2010.
**Preparation of Animals:**

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days prior to dosing to allow for acclimatization to the laboratory conditions.

**Preparation of Doses:**

In general test substances should be administered in a constant volume over the range of doses to be tested by varying the concentration of the dosing preparation. Where a liquid end product or mixture is to be tested however, the use of the undiluted test substance, i.e. at a constant concentration, may be more relevant to the subsequent risk assessment of that substance, and is a requirement of some regulatory authorities. In either case, the maximum dose volume for administration must not be exceeded. The maximum volume of liquid that can be administered at one time depends on the size of the test animal.

In rodents, the volume should not normally exceed 1mL / 100 g of body weight; however in the case of aqueous solutions 2 mL / 100 g body weight can be considered. With respect to the formulation of the dosing preparation, the use of an aqueous solution / suspension / emulsion is recommended wherever possible, followed in order of preference by a solution / suspension / emulsion in oil (e.g. corn oil) and then possibly solution in other vehicles. For vehicles other than water the toxicological characteristics of the vehicle should be known. Doses must be prepared shortly prior to administration unless the stability of the preparation over the period during which it will be used is known and shown to be acceptable.

**Number of Animals and Dose Levels:**

Three animals are used for each step. The dose level used as the starting dose was selected from one of four fixed levels 50, 300, 2000 and 4000 mg / kg body weight. The available information suggests that mortality is likely at the highest starting dose level 2000 mg / kg body weight, so the trial or limit test was conducted. Even though there is inadequate information on the test substance, hence for animal welfare reasons the starting dose of 300mg / kg body weight was selected. The time interval between treatment groups is determined by the onset, duration, and severity of toxic signs. The test
substance-related mortality was not produced in animals, so further testing at the next lower level need not be carried out.

**OBSERVATIONS:**

Animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead. All observations are systematically recorded with individual records being maintained for each animal. Observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern. Attention was directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The principles and criteria summarized in the Humane Endpoints Guidance Document taken into consideration. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress was humanely killed. When animals are killed for humane reasons or found dead, the time of death should was recorded.

**Body Weight:**

Individual weight of animals was determined before the test substance was administered and at least weekly thereafter. Weight changes was calculated and recorded. At the end of the test surviving animals were weighed and humanely killed.

**Pathology:**

All test animals were subjected to gross necropsy. All gross pathological changes were recorded for each animal. Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 or more hours also was considered.

**Data and Reporting:**

All data were summarized in tabular form, showing for each test group the number of animals used, the number of animals displaying signs of toxicity, the number of animals found dead during the test or killed for humane reasons, time of death of individual animals, a description and the time course of toxic effects and reversibility, and necropsy findings.
7.1.1. ACUTE TOXICITY STUDY

Administration of Doses:

Siddha formulation was suspended in 0.5% CMC with vigorous mixing and was administered to the groups of mice in a single oral dose by gavages using a feeding needle. The control group received an equal volume of the vehicle. Animals were fasted prior to dosing. Following the period of fasting, the animals were weighed and then the test substance was administered. After the substance has been administered, food was withheld for a further 3-4 hours. The principle of laboratory animal care was followed. Observations were made and recorded systematically and continuously observed as per the guideline after substance administration. The visual observations included skin changes, mobility, aggressively, sensitivity to sound and pain, as well as respiratory movements. They were deprived of food, but not water 16–18 h prior to the administration of the test suspension. Finally, the number of survivors was noted after 24 h and these animals were then maintained for a further 14 days and observations made daily. The toxicological effect was assessed on the basis of mortality. Acute oral toxicity study was performed as per guidelines 423 of organization for economic Co-operation and Development (OECD), acute toxic class method (Donald, 1997).

Siddha Formulation and Vehicle:

The formulation is partially soluble in water but the particle size is large and so the rapid settling was observed. Hence in order to ensure the uniformity in drug distribution in the medium the suspension was made with 0.5% CMC solution and it was found suitable for dose accuracy.

Test Animals and Test Conditions:

Sexually mature either sex albino mice (28-35g) were obtained from the animal laboratory of the School of Pharmaceutical sciences, Vels University. All the animals were kept under standard environmental condition (27±2°C). The animals had free access to water and standard pellet diet (Sai meera foods, Bangalore). Mice were deprived of food but not water (16-18 h) prior to administration of the formulation. Three animals are used for each step. The dose level used as the starting dose was selected from one of four fixed levels 50, 300, 2000 and 4000 mg / kg body weight. The available information
suggests that mortality is likely at the highest starting dose level 2000 mg / kg body weight, so the trial or limit test was conducted.

The results of acute toxicity study were expressed in the form of Tables No.16.

7.1.2. SUB ACUTE TOXICITY STUDY

28-Day Sub-acute Oral Toxicity Study

Test Substance: Siddha formulation (Chooranam)

Animal Source: Animal house of Vels University, Chennai.

Animals: Male and Female *wistar Rats*

Age: 6-8 weeks

Body Weight on Day 0: Males: Mean 114.25g Females: Mean 118.02g

Acclimatization: Seven days prior to dosing.

Veterinary examination: Prior to and at the end of the acclimatization period.

Identification of animals: By cage number, animal number and individual marking on fur.

Diet: Pelleted feed supplied by Sai meera foods Pvt Ltd, Bangalore

Water: Aqua guard portable water in polypropylene bottles *ad libitum*.

Housing & Environment: The animals were housed in Polypropylene cages provided with bedding of husk.

Housing temperature: Between 20° & 24°C,

Relative humidity: Between 30% and 70%,

Air changes: 10 to 15 per hour and

Dark and light cycle: Each of .12 hours.

Justification for Dose Selection:

As stated in results of acute toxicity studies in mice indicated that Siddha formulation was non toxic up to the dose level of 2000 mg / kg body weight observed after 48 hours of oral drug treatment. On the basis of these results, the doses selected for the study was 100mg / kg, 200 mg / kg and 400mg / kg body weight. The oral route was selected for use because oral route is considered to be a proposed therapeutic route.
Preparation and Administration of Dose:

Siddha formulation was suspended in 0.5% CMC in distilled water to obtain concentrations of 200mg / ml. It was administered to animals at the dose levels of 100mg / kg, 200mg / kg and 400mg / kg in the dose volume of 10mL / kg. The test substance suspensions were freshly prepared every two days once for 28 days. The control animals were administered vehicle only. Administration was by oral (gavage), once daily for 28 consecutive days.

Randomization, Numbering and Grouping of Animals:

Six rats were in each group randomly divided into four groups for dosing up to 28 days. Animals were allowed acclimatization period of 7 days to laboratory conditions prior to the initiation of treatment. Each animal was fur marked with picric acid. The females were nulliporous and non-pregnant.

OBSERVATIONS:

Experimental animals were kept under observation throughout the course of study for the following:

Body Weight:

Weight of each rat was recorded on day 0, at weekly intervals throughout the course of study and at termination to calculate relative organ weights. From the data, group mean body weights and percent body weight gain were calculated.

Food and water Consumption:

The quantity of food consumed by groups consisting of six animals of for different doses was recorded at weekly interval. Food consumed per animal was calculated for control and the treated dose groups.

Clinical Signs:

All animals were observed daily for clinical signs. The time of onset, intensity and duration of these symptoms, if any, were recorded.

Mortality:

All animals were observed twice daily for mortality during entire course of study.
**Ophthamoscopy:**

The eyes of experimental animals in control as well as treated groups given different dose levels were examined prior to the initiation of the dosing and in 4\(^{th}\) and the 6\(^{th}\) week of the study. Eye examination was carried out using a hand slit lamp after induction of mydriasis with Atropine sulphate solution.

**Functional Observations:**

At the end of the 4\(^{th}\) week exposure, ‘sensory reactivity’ to graded stimuli of different types (auditory, visual and proprioceptive stimuli), ‘motor reactivity’ and ‘grip strength’ were assessed.

**Laboratory Investigations:**

Following laboratory investigations were carried out on day 29 in animals’ fasted over-night. Blood samples were collected from orbital sinus using sodium heparin (200IU/ml) for Blood chemistry and Potassium EDTA (1.5 mg / ml) for Haematology as anticoagulant. Blood samples were centrifuged at 3000 r.p.m. for 10 minutes.

**Haematological Investigations:**

Haematological parameters were determined using Haematology analyzer.

**Biochemical Investigations:**

Biochemical parameters were determined using auto-analyzer.

**Urine Analysis:**

Urine samples were collected in week 4 and in week 6 and for estimation of normal parameters. The estimations were performed using appropriate methodology.

**Necropsy:**

All the animals were sacrificed on day 29. Necropsy of all animals was carried out and the weights of the organs including liver, kidneys, adrenals, spleen, brain, heart, uterus and testes/ovaries were recorded.
Histopathology:

Tissue samples of organs from control and treated animals at the lowest and highest dose level (100 mg/kg & 400 mg/kg) were preserved in 10% formalin. The organs included brain, heart, kidneys, liver, lungs, spleen, stomach, and uterus of the animals were preserved they were subjected to histopathological examination.

The results of histo-pathological study were shown in Figure.No.30

Statistical Analysis:

Findings such as clinical signs of intoxication, body weight changes, food consumption, haematology and blood chemistry were subjected to One-way Anova followed by dunnet’t’ test using a computer software programme -INSTAT-V3 version.

The results of sub acute toxicity study were expressed in the form Table Nos.16 to 25.

7.2. ANTI-ASTHMATIC ACTIVITY

Bronchial asthma is one of the most complex disorders of the airway characterized by reversible airway obstruction inflammation, bronchial hyper-responsiveness and excessive mucous production (Goswami et al., 2010). It is known that asthma can be triggered by various infections, dust, cold air, exercise, emotion, perfumes, chemical, environmental tobacco smoke and histamine (Kelly et al., 2005). In asthma some pathophysiological changes to the airways, thickening of the airways walls, which have been produced reduction of airflow and the development of hyper responsiveness in airway system (Beasley et al., 1993).

Asthma is a chronic condition. These symptoms may be due to liberation of endogenous and intrinsic mediators like histamine, leukotrienes (LTs), bradykinin, prostaglandins (PGs), nitric oxide, platelet activating factors (PAF), chemokines and endothelin from mast cells during the allergic reactions and inflammation of the air passages in the lungs. Nearly 7-10% of world population suffers from bronchial asthma. For management of asthma attacks adrenergic relief is most requirements (Rang et al., 2001).
7.2.1. HISTAMINE-INDUCED BRONCHOSPASM IN GUINEA PIGS

(Armitage et al., 1961)

Histamine induced bronchoconstriction is the traditional immunological model of antigen induced airway obstruction. Histamine when inhaled causes hypoxia and leads to convulsion in the guinea pigs and causes very strong smooth muscle contraction, profound hypotension and capillary dilation in the cardiovascular system. A prominent effect caused by histamine is severe bronchoconstriction in the guinea pigs that causes asphyxia and death (Vogel, 2002). Histamine was released after degranulation of mast cell by an antigen exposure by antigenic stimulation causing smooth muscle contraction, increased vascular permeability and mucus formation. Histamine is one of the important mediator of allergy, inflammation and bronchoconstriction. Targeting histamine, either prevention of its release from mast cell or use of histaminergic receptor antagonist becomes part of antihistaminic therapy in allergic diseases (Parmar et al., 2010).

The guinea pigs fasted for 24 hours were exposed to an atomised fine mist of 2% histamine dihydrochloride aerosol (dissolved in normal saline) using nebulizer at a pressure of 40 mm Hg in the histamine chamber (24 x 14 x 24 cm, made of perplex glass) (M/s Inco Ambala). Guinea pigs exposed to histamine aerosol showed progressive signs of difficulty in breathing leading to convulsions, asphyxia, and death. The time until signs of convulsion appeared is called pre-convulsion time (PCT/PCD). By observation experience was gained so that the preconvulsion time can be judged accurately. As soon as PCD commenced, animals were removed from the chamber and placed in fresh air to recover. In the present experiments the criterion used was time for onset of dyspnoea and percent protection was calculated. Those animals which developed typical histamine asthma within 3 min were selected out three days prior to the experiment and were given habituation practice to restrain them in the histamine chamber. They were divided in groups of ten animals each. Mepyramine 8.0 mg/kg, p.o., and the 200 mg/ kg, 400mg/ kg doses of Siddha formulations were administered orally 30 min prior to exposure. Animals, which did not develop typical asthma within 6 min, were taken as protected. PCD was determined on single administration of the dose, results obtained were considered as day ‘0’ value. Further the formulation was administered for seven days, on 7th day again same procedure was followed and results obtained were considered as day ‘7’ value. The percentage inhibition was determined by using the following formula.
% inhibition = \((1 - \frac{T1}{T2}) \times 100\).

Where T1 is Day ‘0’ value

T2 is Day ‘7’ value.

The results of histamine-induced bronchospasm in guinea pigs were given in Table no. 26 and illustrated in Figure. No.31

7.2.2. ACETYLCHOLINE-INDUCED BRONCHOSPASM IN GUINEA PIGS (Kumar & Ramu, 2002)

Similar procedure was repeated by exposure of aerosol of 0.5% acetylcholine in another set of animals (each group having ten animals) using Atropine sulphate (2 mg/kg) as a standard.

The results of acetylcholine-induced bronchospasm in guinea pigs were given in Tables no. 27 and illustrated in Figures. No. 32

7.2.3. MAST CELL DEGRANULATION BY COMPOUND 48/80

(Kaley & Weiner, 1971)

This was carried out as per the method described by Kaley and Weiner, (1971) with little modification. Male albino rats were sacrificed by cervical dislocation. The animals were immediately injected with 15 ml of pre-warmed (37°C) buffered salt solution (BSS; NaCl 137 mM; KCl 2.7 mM; MgCl\(_2\) 1.0 mM; CaCl\(_2\) 0.5 mM; NaH\(_2\)PO\(_4\) 0.4 mM; Glucose 5.6 mM; HEPES 10 mM) into the peritoneal cavity, and massaged gently in this region for 90 s, to facilitate cell recovery. A midline incision was made and the peritoneum was exposed. The pale fluid was aspirated using a blunted plastic Pasteur pipette, and collected in a plastic centrifuge tube. The fluid was then centrifuged at 1000 rpm for 5 min, and the supernatant discarded to reveal a pale cell pellet. The cell pellets were re-suspended in fresh buffer and re-centrifuged. Aliquots of the cell suspension were incubated with different doses of the test compounds or disodium cromoglycate, before challenge with compound 48/80. The aliquots were carefully spread over glass slides, and the mast cells were stained with 1% toluidine blue, and counterstained with 0.1% light green. The slides were dried in air and the mast cells counted from randomly selected high power objective fields (X450). The effect of Siddha formulation on mast cells was studied by incubating the mast cells for 10 min with the above compounds in a different concentration. In another set of experiments the mast cells which were pre-incubated
Siddha formulation were exposed to the mast cell degranulator, compound 48:80 (10 µg/ml), and the incubation continued for a further 10 min. Then, the mast cells were carefully spread over glass slides. The percent degranulation of the mast cells in each treatment was calculated. DSCG 20 µg/ml was also included in the study for comparison.

The results of mast cell degranulation were given in Table no.28 and illustrated in Figure. No.33.

7.3. ANTI INFLAMMATORY ACTIVITY

Inflammation or phlogosis is a pathophysiological response of living tissue to injuries that leads to the local accumulation of plasmonic fluid and blood cells. Although it is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can be induced, maintain or aggravate many diseases (Goswami et al., 2010). However, studies have been continuing on inflammatory diseases and the side effects of the currently available anti-inflammatory drugs pose a major problem during their clinical use (Kelly et al., 2005). Therefore, development of newer and more powerful anti-inflammatory drugs with lesser side effects is necessary.

Every day anti-inflammatory drugs are taken by more than 30 million people worldwide; of these, 40% of consumers are older than 60. Population studies have shown that 10–20% of all people who are 65 years or older either are currently receiving or have recently received a prescription for non-steroidal anti-rheumatic drugs. During the next 20 years the number of people over 65 is expected to increase from 380 million to 600 million. The very frequent use of NSAIDs is based on the fact that these agents have many indications for which a large number of patients exist. These indications include chronic polyarthritis, psoriatic arthritis, ankylosing spondylitis, osteoarthritis, gout, inflammatory soft tissue rheumatism, low back pain, postoperative and post-traumatic inflammation, thrombophlebitis and vasculitis. Over the past 140 years other substances have been introduced for therapy, collectively termed non-steroidal anti-inflammatory drugs (NSAIDs). In the past few years there have been significant advances in explaining the mechanism of action of NSAIDs.

7.3.1. CARRAGEENAN-INDUCED HIND PAW EDEMA IN RATS

The acute hind paw edema was produced by injecting 0.1 ml of carrageenan (freshly prepared as 1% suspension in 1% CMC) locally into the plantar aponeurosis of
the right hind paw of rats (Winter et al., 1962). Siddha formulation (200 & 400, mg/kg, p.o.) was administered to two different groups while the other two groups served as negative, and positive controls, and received vehicle (1 ml/kg, p.o.), and standard drug, acetylsalicylic acid (ASA, 300 mg/kg, p.o.), respectively. For each treatment group six animals were used. Different doses of the test sample, and ASA were administered 1 hour prior to the injection of carrageenan. A mark was made at the ankle joint of the paw of rat and pedal volume up to this point was measured using plethysmometer at 0 h (just before) and 1-, 2- and, 3-hour post carrageenan injections. Increase in the paw edema volume was considered as the difference between 0 and 1 hour, 2hour, or 3 hr. Percent inhibition of edema volume between treated and control groups was calculated as follows:

Percent inhibition = 1 – VT/VC × 100

Where, VC, and VT represent the mean increase in paw volume in control and treated groups, respectively. All the animals were fasted for 12 h and deprived of water only during the experiment. The deprivation of water was to ensure uniform hydration and to minimize variability in oedematous response.

The results of carrageenan-induced hind paw edema in rats were given in Table no.29 and illustrated in Figure. No.34

7.3.2. FORMALDEHYDE-INDUCED HIND PAW VOLUME

The test was performed according to the technique developed by Brownlee (1950). Pedal inflammation was induced by injecting 0.1 ml of 4% formaldehyde solution below the plantar aponeurosis of the right hind paw of the rats. The paw volume was recorded immediately prior to compound administration (0 h), and then at 1.5 h, and 24 h after formaldehyde injection. Vehicle (1 ml/kg, p.o.), and the Siddha formulation at the dose of 200 mg/kg and 400 mg/kg, p.o and standard drug, ASA (300 mg/kg, p.o.) were administered 1 hour prior to formaldehyde injection. Percent inhibition of edema volume between treated, and control groups were calculated as follows:

Percent inhibition = 1 – VT/VC× 100

Where, VC and VT represent the mean increase in paw volume in control and treated groups, respectively.
Twenty four rats were divided into 4 groups of 6 rats each for various treatments as shown in Table. No.27. Subsequently 30 min after above treatment, 0.1ml of 1% formalin was injected subcutaneously into the planter region of right hind paw to induce oedema. The paw volume was measured initially and at 1.5 hours and 24 hours after formalin injection using plythesmographic method. Percentage inflammation was calculated for comparison.

The results of percentage of inhibition and chooranam in histamine-induced inflammation was shown in Table no. 30 and illustrated in Figure. No.35.

**7.3.3. COTTON PELLET GRANULOMA IN RATS**

The effect of Siddha formulation at different concentration on chronic or proliferative phase of inflammation was assessed in cotton pellet granuloma rat model as described by (Winter and Porter, 1957). Autoclaved cotton pellets weighing 10 ± 1 mg each were implanted subcutaneously through small incision made along the axilla region of the rats anesthetized with sodium thiopental (45 mg/kg, i.p.). The different groups of rats were administered with 200 mg/kg and 400 mg/kg of chooranam in 0.5% CMC. Prednisolone (10 mg/kg, p.o.) once daily for 7 consecutive days from the day of cotton pellet insertion. The control group received vehicle (1 ml/kg, p.o.). On the eighth day, all the rats were anaesthetized and the cotton pellets covered by the granulomatous tissue were excised and dried in hot air oven at 60°C till a constant weight was achieved. Granuloma weight was obtained by subtracting the weight of dry cotton pellet on 0 day (before start of cotton pellet on eighth day. Percent inhibition of granuloma formation treated, and control group was calculated using following formula

\[
\text{Percent inhibition} = 1 - \frac{VT}{VC} \times 100
\]

Where, VC, and VT represent the mean increase in granuloma formation in control and treated groups, respectively.

The effect of chooranam on chronic inflammation by cotton – pellet granuloma was expressed in the Table No.31 and Figure No.36.