



## International Journal of Current Research in Medical Sciences

ISSN: 2454-5716

P-ISJN: A4372-3064, E-ISJN: A4372-3061

www.ijcrims.com



Original Research Article

Volume 6, Issue 10 -2020

DOI: <http://dx.doi.org/10.22192/ijcrms.2020.06.10.002>

### Acute and Sub-acute toxicity study in Wister rats to evaluate toxicity profile of Seenthil Chooranam

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#### Abstract

The drug Seenthil Chooranam was prepared with care as per the literature evidence. Siddhaviathiya thirattu. To evaluate the acute toxicity study 15 rats were selected and divided into 5 groups (group I,II,III,IV,V VI) and they were administered with the drug with different graded doses ranging from 5mg/kg, 50mg/kg, 300mg/kg 1000mg/kg and 2000mg/kg body weight animal orally. Daily the animal were observed for clinical signs and mortality. The drug did not produced any mortality rate and is safe upto 2000mg/kg body weight. Subacute toxicity was conducted for about 28 days duration. No signs of toxicity was observed in animals from different dose groups during the dosing period of 28 days. No significant haematological changes occurred. The weights of the animal were recorded during the drug administration. There is increase in the body weight throughout the dosing period. There is no remarkable histopathological changes in lungs, spleen, kidney except that minor changes in liver only. By acute and subacute toxicity studies, the drug Seenthil Chooranam is found to be safe upto 300 to 900 mg/kg body weight.

**Keywords:** Navanatha Chenduram, Toxicity, Haematological analysis.

#### Introduction

Siddha system is one of the oldest system of medicine in India. The word siddha comes from the word 'siddha'. Which means an object to attain perfection or heavenly bliss. Siddha generally refers to 'Attama Siddhi' that is the eight super natural powers those who attained or achieved the above said powers are know as 'siddhars' siddhars were saintly figures. There were eighteen important siddhars in older days and they developed this system of medicine.

#### Origin

The origin of the system and the used of medicinal plants belong to the age of the sangam literature as early as 3000 B.C 'Tholkappiam' and 'Thirumanthiram' stands as a proof to this.

#### Basic principles

Siddha system of medicine is based on "Saiva siddhantha"

“அண்டத்தில் உள்ளதே பிண்டம்  
பிண்டத்தில் உள்ளதே அண்டம்  
அண்டமும் பிண்டமும் ஒன்றே  
அறிந்துதான் பார்க்கும் போதே”

According to siddha medical science the universe originally consisted of atoms which contributed to the five basic elements.

Prithivi	-	Earth
Appu	-	Water
Theyu	-	Fire
Vayu	-	Air
Agayam	-	Sky

These three elements are primarily responsible for the formation of three humours.

- Vatham
- Pitham
- Kabam

### The fundamental subjects of siddha methodology

1. Vadham (Alchemy)
2. Vaithiyam (Medicine)
3. Yogam (Yoga)
4. Gyanam (or) thathuvam (Philosophy)

### Chemistry in siddha system

In siddha system, chemistry had been found well developed into science auxiliary to medicine and alchemy.

The siddhars were aware of several chemical operation divided into several process such as

1. Calcinations
2. Sublimation
3. Distillation
4. Fusion
5. Separation
6. Fermentation
7. Exhalation etc.

### Toxicology in siddha system

Too much of anything is good for nothing.  
Which is the actual meaning of Tamil proverb.

அளவுக்கு மீறினால் அமிர்தமும் நஞ்சாகும்

This is also explained by Thiruvalluvar as

“பீலிபெய் சாகாடும் அச்சிறும் அப்பண்டம்  
சால மிகுத்துப் பெயின்”

In this materialistic world each and every object has two characters good and bad, which are lying invariable among them. So, whenever we go to prepare a medicine. We should remove the toxin and unwanted materials from them

In our country, toxic plants and materials were identified more than 5000 years ago, itself. In that time poisons are used with food stuffs to kill the enemies or to steal the property of our kingdom. Poisons are also used in the weapons for hunting.

Siddhars like Agasthiyar and Bogar were knew the usage of toxic drugs. They were well versed about the purification, lethal dose, therapeutic dose and usage, of the toxic drugs. “Seevaka chinthamani” in a samana literature gives details about the usage of toxic materials to kill or mesmerise the enemies. It also gives idea to identify those toxic materials from food, cloth, beverages etc.

By keeping all these facts in mind the author had selected Seenthil Chooranam for this dissertation study. This study makes a detailed idea about the toxicity of the drug.

### Objective

To collect raw materials and purification of the raw materials based on the relevant literature.

To evaluate the acute and subacute toxicity of Seenthil Chooranam in different dose levels.

## Materials and Methods

### Acute toxicity study in female wistar rats to evaluate toxicity profile of *Seenthil chooranam*

#### Objectives

The aim of this Study is to evaluate the toxicity of the test substance *Seenthil Chooranam*, when administered orally to Female Wistar Rats with different doses, so as to provide a rational base for the evaluation of the toxicological risk to man and indicate potential target organs.

#### Guidelines followed:

(a) OECD Guidelines No. 423,

#### Study Design and Controls:

Female Wistar Rats in controlled age and body weight were selected. *Seenthil chooranam* was administered at **5 mg/kg, 50 mg/kg, 300 mg/kg, 1000 mg/kg, and mg/kg** body weight of animal as suspension along with water.

➤ The results were recorded on that day of drug administration approximately 1<sup>st</sup>, 3<sup>rd</sup>, 4<sup>th</sup> hour in post dosing and further made into observation upto 14 days.

#### Experimental procedure

##### Animals

##### Supply

A total of 15 Female Wistar Rats with an approximate age of 6 weeks and purchased from M/s.Venkateshwara Enterprises Pvt. Ltd, Bangalore. On their arrival a sample of animals was chosen at random and weighed to ensure compliance with the age requested. The mean weights of Female Wistar Rats were 100-150 g respectively. The animals were housed in metabolic cages (55 x 32.7 x 19 cm), with sawdust litter, in such a way that each cage contained a maximum of 3 animals of the same sex.

All animals underwent a period of 20 days of observation and acclimatization between the date of arrival and the start of treatment. During the course of this period, the animals were inspected by a veterinary surgeon to ensure that they fulfilled the health requirements necessary for initiation of the Study.

##### Housing

The Female Wistar Rats were housed in metabolic cages (55 x 32.7 x 19 cm), placed on racks. From the week before initiation of the treatment, each cage contained a maximum of 6 rats of the same sex and treatment group.

Each cage was identified by a card, color coded according to the dose level. This card stated the cage number, number and sex of the animals it contained, Study number, test substance code, administration route, dose level and Study drug name, date of the arrival of the animals and initiation of treatment.

The temperature and relative humidity were continuously monitored. Lighting was controlled to supply 12 hours of light (7:00 to 19:00 hours) and 12 hours of dark for each 24-hour period.

The cages corresponding to each experimental group were distributed on racks in such a manner that external factors, such as environmental conditions, were balanced as far as possible.

##### Diet

All the rats had free access to a pelleted rat diet. The diet was analyzed by the manufacturer to check its composition and to detect possible contaminants.

##### Water

The water was offered ad libitum in bottles.

##### Administration route and procedure

The test substance was administered orally. The Female Wistar Rats belonging to the control group were treated with the vehicle (Water) at the same administration volume as the rest of the treatment groups.

## Numbering and Identification

The animals were marked on body with picric acid solution prepared in water. The marking within the cage was as below.

**Table-1 Numbering and Identification**

Group No	Animal Marking
1	Head
2	Body
3	Tail

The group no., cage no., sex of the animal and animal no. were identified as indicated below using cage label and body marking on the animals

**Table-2 Numbering and Identification**

Cage No	Group No	Animal Marking	Sex
1	I	H,B,T	Female
2	II	H,B,T	Female
3	III	H,B,T	Female
4	IV	H,B,T	Female
5	V	H,B,T	Female

## Doses

The doses for the study were selected based on literature search and range finding study. Following the period of fasting, the animals were

weighed and then drug was administered orally as single dose using a needle fitted onto a disposable syringe of approximate size at the following different doses.

**Table-3 Doses**

Group	Dose
Group-I	5 mg/kg
Group-II	50 mg/kg
Group-III	300 mg/kg
Group-IV	1000 mg/kg
Group-V	2000 mg/kg

The test item was administered as single dose. After single dose administration period, all animals were observed for 14 days.

## Dose Preparation

*Seenthil chooranam* was added in distilled water and completely dissolved to form for oral administration. The dose was prepared of a required concentration before dosing by dissolving, in distilled water. It was mixed well. The preparation for different doses was vary in concentrations to allow a constant dosage volume.

## Administration

The test item was administered orally to each Female Wistar rats as single dose using a needle fitted onto a disposable syringe of appropriate size at the following different doses. The concentration was adjusted according to its body weight. The volume was not exceeding 10 ml/kg bodyweight. Variability in test volume was minimized by adjusting the concentration to ensure a constant volume at all dose levels.

## Observation period

All animals were observed for any abnormal clinical signs and behavioral changes. The appearance, change and disappearance of these clinical signs, if any, were recorded for approximately 1.0, 3.0 and 4.0 hours post-dose on day of dosing and once daily thereafter for 14 days. Animals in pain or showing severe signs of distress were humanely killed. The cageside observation was included changes in skin, fur, eyes and mucous membranes, occurrence of secretions and excretions. Autonomic activity like lacrimation, piloerection, pupil size and unusual respiratory pattern, changes in gait, posture, response to handling, presence of clonic or tonic movements, stereotypes like excessive grooming and repetitive circling or bizarre behavior like self-mutilation, walking backwards etc were observed. At the 14th day, sensory reactivity to stimuli of different types (e.g. auditory, visual and proprioceptive stimuli) was conducted. Auditory stimuli responses were measured by clicker sound from approximately 30 cm to the rats; visual stimuli response were measured with the help of shining pen light in the eye of rats and placing a blunt object near to the eye of rats. Response to proprioceptive stimuli was measured by placing

anterior/dorsal surface of animals paw to the table edge. The responses of reactions for these three exercises were normal in animals belonging to both the controls as well as drug treatment dose groups.

## Mortality and Morbidity

All animals were observed daily once for mortality and morbidity at approximately 1.0, 3.0 and 4.0 hours post dose on day of dosing and twice daily (morning and afternoon) thereafter for 14 days.

### Sub-acute toxicity study in wistar rats to evaluate toxicity profile of Seenthil chooranam

#### 1. Objective

The objective of this 'Sub-Acute Toxicity Study of Seenthil Chooranam on Wistar Rats' was to assess the toxicological profile of the test item when treated as a single dose. Animals should be observed for 28 days after the drug administration. This study provides information on the possible health hazards likely to arise from exposure over a relatively limited period of time.

#### 2. Test Guideline Followed

**OECD 407 Method - Sub-Acute Toxic Class Method (Repeated Dose 28-Day Oral Toxicity Study in Rodents)**

#### 3. Test Item Detail

Name: **Seenthil Chooranam**

#### 4. Test System Detail

The study was conducted on 5 male and 5 female Wistar rats. These animals were selected because of the recommended rodent species for oral studies as per followed guideline and availability of Animals 8-12 weeks old male and female rats were selected after physical and behavioral examination. The body weight range was fallen within  $\pm 20\%$  of the mean body weight at the time of Randomization and grouping. The rats were housed in standard laboratory condition in Polypropylene cages, provided with food and

water *adlibitum* in the Animal at M/s. Sree Venkateshwara Enterprises Pvt. Ltd, Bangalore. The experimental protocol was approved by Institutional Animal Ethical Committee as per the guidance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, government of India.

### 5. Acclimatization

The animals were selected after veterinary examination by the veterinarian. All the selected animals were kept under acclimatization for a week.

### 6. Randomization & grouping

One day before the initiation of treatment (days 0-last day of acclimatization), the selected animals were randomly grouped into three different groups containing minimum 10 male animals per group both sex.

### 7. Numbering and Identification

The animals were marked on body with picric acid solution prepared in water. The marking within the cage was as below.

**Table-1 Numbering and Identification**

Group No	Animal Marking
1. Control	H,B,T,HB,NM (Male) H,B,T,HB,NM (Female)
2. Low dose of seenthil chooranam 300mg/kg	H,B,T,HB,NM (Male) H,B,T,HB,NM (Female)
3. Middledose of seenthil chooranam 600mg/kg	H,B,T,HB,NM (Male) H,B,T,HB,NM (Female)
4. High dose of seenthil chooranam 900mg/kg	H,B,T,HB,NM (Male) H,B,T,HB,NM (Female)

The group no., cage no., sex of the animal and animal no. were identified as indicated below

using cage label and body marking on the animals:

Cage No	Group No	Animal Marking	Sex
1	1. Control	H,B,T,HB,NM H,B,T,HB, NM	Male Female
2	2. Low dose of seenthil chooranam 300mg/kg	H,B,T,HB,NM H,B,T,HB, NM	Male Female
3	3. Middledose of seenthil chooranam 600mg/kg	H,B,T,HB,NM H,B,T,HB, NM	Male Female
4	4. High dose of seenthil chooranam 900mg/kg	H,B,T,HB,NM H,B,T,HB, NM	Male Female



## Husbandry

### Housing

The Wistar rats were housed in standard polypropylene cages with stainless steel top grill. Paddy husk was used as bedding. The paddy husk was changed at least twice in a week. From the week before initiation of the treatment, each cage contained a maximum of 6 rats of the same sex and treatment group.

### Environmental conditions

The animals were kept in a clean environment with 12 hour light and 12 hour dark cycles. The air was conditioned at  $22\pm 3^{\circ}\text{C}$  and the relative humidity was maintained between 30-70% with 100% exhaust facility. The cages corresponding to each experimental group were distributed on racks in such a manner that external factors, such as environmental conditions, were balanced as far as possible.

## Feed & feeding schedule

'Sai Durga Animal Feed, Bangalore. Feed was provided *adlibitum* throughout the study period, except over night fasting (18-20 hours) prior to dose administration. After the substance has been administered, food was withheld for a further 3-4 hours.

### Water

The water was offered *adlibitum* in bottles. There was periodically analyzed to detect the presence of possible contaminants

### Doses

The doses for the study were selected based on literature search and range finding study. Following the period of fasting, the animals were weighed and then extract was administered orally as single dose using a needle fitted on to a disposable syringe of approximate size at the following different doses.

**Table-2 Dose level**

Test group	Dose to animals (mg/kg body-weight/day)	Number of animals
Group-I	<b>1. Control</b>	10 (5Male and 5 Female)
Group-II	<b>2. Low dose of seenthil chooranam 300mg/kg</b>	10(5Male and 5 Female)
Group-III	<b>3. Middledose of seenthil chooranam 600mg/kg</b>	10(5Male and 5 Female)
Group-IV	<b>4. High dose of seenthil chooranam 900mg/kg</b>	10(5Male and 5 Female)

The test item was administered as single dose. After single dose administration period, all animals were observed for 28 days.

### Dose Preparation

**Seenthil chooranam** was added in distilled water and completely dissolved to form oral administration. The dose was prepared of a required concentration before dosing by dissolving **Seenthil Chooranam** in distilled water. It was mixed well. The preparation for

different doses was vary in concentrations to allow a constant dosage volume.

### Administration

The test item was administered orally to each rat as single dose using a needle fitted on to a disposable syringe of appropriate size at the following different doses. The concentration was adjusted according to its body weight. The volume was not exceeding 10 ml/kg body weight. Variability in test volume was minimized by adjusting the concentration to ensure a constant volume at all dose levels.

## Observations

These observations were also performed on weekends. The observations included but were not limited to changes in skin and fur, in the eyes and mucous membranes, in the respiratory, circulatory, central nervous and autonomous systems, somatomotor activity and behavior.

## Clinical signs of toxicity

All the rats were observed at least twice daily with the purpose of recording any symptoms of ill-health or behavioral changes. Clinical signs of toxicity daily for 28 days.

## Food intake

Prior to the beginning of treatment, and daily, the food intake of each cage was recorded for period of 28 days and the mean weekly intake per rats was calculated.

## Water intake

Water intake was checked by visual observation during the Study. In addition, the water consumption in each cage was measured daily for a period of 28 days.

## Body weight:

The body weight of each rat was recorded one week before the start of treatment, and during the course of the treatment on the day of initial, 3rd, 7th, 10th, 14th, 17th, 20th, 24th and 28th days (day of sacrifice). The mean weights for the different groups and sexes were calculated from the individual weights.

## Blood Collection

Blood was collected through retro-orbital sinus from all the animals of different groups on 28th day. The blood was collected in tubes containing Heparin/EDTA as an anticoagulant. Animals were fasted over night prior to the blood collection.

## Laboratory studies

During the 4th week of treatment, samples of blood were withdrawn from the orbital sinus from each group, under light ether anesthesia after fasting for 16 hours. The blood samples are used to evaluate Hematological parameters like RBC, WBC, and PLATELETS etc..... The collected blood samples also centrifuged 10000 rpm in 10 minutes to separate the serum. The separated serum used to evaluate biochemical parameters like SGOT, SGPT, ALP and BILIRUBIN ect.....

## Hematology

The following hematological parameters were analysed using Autoanalyser

Hb	:	Haemoglobin (g %)
PCV	:	Packed Cell Volume
WBC	:	White Blood Corpuscles (x103/cmm)
RBC	:	Red Blood Corpuscles (x106/cmm)
Blood Platelet count		(x103/cmm)

## Differential WBC count:

N	:	Neutrophils (%)
L	:	Lymphocytes (%)
M	:	Monocytes (%)
E	:	Eosinophils (%)
RDW	:	Red Cell Distribution Width.
MPV	:	Mean Platelet Volume

## Clinical Biochemistry:

The following clinical Bio parameters were analysed using Auto analyser

Total serum protein (g/dl)

ALT/SGPT (U/L)	:	Alanine amino transferase
AST/SGOT (U/L)	:	Aspartate amino transferase (U/L)
ALP (U/L)	:	Alkaline serum phosphatase (U/L)
CHL	:	Cholesterol (mg/dL)
HDL	:	High density lipoprotein
TG	:	Triglyceride



## Estimation of hematological parameters: <sup>1</sup>

### Collection of blood for hematological studies

After the treatment period the animals were anaesthetized by ketamine hydrochloride and the blood was collected from Retro-orbital sinus by using capillary into a centrifugation tube which contains EDTA for haematological parameters. The haematological parameters like RBC, WBC and Hb percentage, Differential cell count, MCV, MCHC, Hematocrit, MCH, platelet count were estimated by the following procedures.

### Enumeration of Red Blood Cells: <sup>1</sup>

Ramnic 2007)

Reagents : RBC diluting fluid

#### Procedure:

Using a red blood cell pipette of haemocytometer, well mixed blood was drawn up to 0.5 mark and RBC diluting fluid was taken up to mark II. The fluid blood mixture was shaken and transferred onto the counting chamber. The cells were allowed to settle to the bottom of the chamber for 2 min. See the fluid does not get dried. Using 45X or high power objective the RBC's were counted uniformly in the larger corner squares.

The cells were expressed as number of cells  $\times 10^{12}/l$

### Enumeration of WBC: <sup>2</sup> John 1972)

#### Reagents:

Turk's fluid: Turk's fluid was prepared by mixing 2ml of acetic acid with 100 ml of distilled water. To this 10 drop of aqueous methylene blue 3 % w/v) was added. This solution haemolysis the red cells due to acidity so that counting of white cells becomes easy.

#### Procedure:

Using a white blood cell pipette of haemocytometer, well mixed blood was drawn up to 0.5 mark and WBC diluting fluid was taken up to mark II. The fluid blood mixture was shaken and transferred onto the counting chamber. The cells were allowed to settle to the bottom of the chamber for 2 min. See the fluid does not get dried. Using 10X or low power objective the WBC's were counted uniformly in the larger corner squares.

The cells were expressed as number of cells/10mm.

### Differential Leucocyte count: <sup>3</sup> John 1972)

#### Reagent:

Leishmann's stain: 150mg of powdered leishmann's stain was dissolved in 133ml of acetone free methanol.

#### Procedure:

A blood film stained with leishmann's stain was examined under oil immersion and the different types of WBCs were identified. The percentage distribution of these cells was then determined. Smears were made from anticoagulant blood specimens and stained with leishmann's stain. The slides were preserved for counting the number of lymphocytes and neutrophils, per 100 cells were noted.

From the different Leukocyte count and WBC count, absolute lymphocyte and neutrophil count were calculated.

Absolute neutrophil count =

$$\frac{\text{Number of neutrophils}}{100} \times \text{TWBC}$$

Absolute lymphocyte count =

$$\frac{\text{Number of lymphocytes}}{100} \times \text{TWBC}$$

J. C. Dacie and S. M. Lewis, Practical haematology, London: Churchill Livingstone, 1984, pp. 5.

### Measurement of biochemical parameters estimation

Haemoglobin (Hb), was estimated using whole blood. Remaining parameters were measured in serum. All of the above biochemical parameters were estimated using semi-autoanalyzer (Photometer 5010 v<sub>5+</sub>, Germany) with enzymatic kits procured from Piramal Healthcare limited, Lab Diagnostic Division, Mumbai, India.

### Determination of aspartate aminotransferase (AST)

Aspartate aminotransferase, also known as Glutamate Oxaloacetate Transaminase (GOT) catalyses the transamination of L-aspartate and keto glutarate to form oxaloacetate and L-glutamate. Oxaloacetate formed is coupled with 2,4- Dinitrophenyl hydrazine to form hydrazone, a brown coloured complex in alkaline medium which can be measured colorimetrically.

### Reagents

Buffered aspartate (pH 7.4); 2,4- DNPH reagent; 4N sodium hydroxide; working pyruvate standard; solution I (prepared by diluting 1 ml of reagent 3 to 10 ml with purified water).

### Procedure

Rietman and Frankle method was adopted for the estimation of SGOT. (Reitmann S, Frankel S, 1957. A colorimetric method for the determination of serum oxaloacetic and glutamic pyruvate transminases. American Journal of Clinical Pathology, 28: 56-63. The reaction systems used for this study included blank, standard, test (for each serum sample) and control

(for each serum sample). 0.25 ml of buffered aspartate was added into all the test tubes. Then 0.05 ml of serum was added to the test group tubes and 0.05 ml of working pyruvate standard into the standard tubes. After proper mixing, all the tubes were kept for incubation at 37°C for 60 min, after which 0.25 ml each of 2,4- DNPH reagent was added into all the tubes. Then, 0.05 ml of distilled water and 0.05 ml of each serum sample was added to the blank and the serum control tubes respectively. The mixture was allowed to stand at room temperature for 20 min. After incubation, 2.5 ml of solution I was added to all test tubes. Mixed properly and optical density was measured in a spectrophotometer at 505 nm within 15 min.

The enzyme activity was calculated as

AST (GOT) activity in IU/L) = [(Absorbance of test - Absorbance of control)/ (Absorbance of standard - Absorbance of blank)] x concentration of the standard

## Results

### Determination of alanine aminotransferase (ALT)

Alanine aminotransferase, also known as Glutathione Peroxidase (GPT) catalyses the transamination of L-alanine and keto glutarate to form pyruvate and L- Glutamate. Pyruvate so formed is coupled with 2,4 – Dinitrophenyl hydrazine to form a corresponding hydrazone, a brown coloured complex in alkaline medium which can be measured colorimetrically.

### Reagents

Buffered alanine (pH 7.4), 2,4–DNPH, 4N sodium hydroxide, working pyruvate standard, solution I (prepared by diluting 1 ml of reagent 3 to 10 ml with purified water).

### Procedure

Rietman and Frankle method was dopted for the estimation of SGPT. The reaction systems used for this study included blank, standard, test (for each serum sample) and control (for each serum

sample). 0.25 ml of buffered alanine was added into all the test tubes. This was followed by the addition of 0.05 ml of serum into the test group tubes and 0.05 ml of working pyruvate standard into the standard tubes. After proper mixing, all the tubes were kept for incubation at 37°C for 60 minutes, after which 0.25 ml each of 2,4- DNPH reagent was added into all the tubes. Then, 0.05 ml of distilled water and 0.05 ml of each serum sample was added to the blank and the serum control tubes respectively. The mixture was allowed to stand at room temperature for 20 min. After incubation, 2.5 ml of solution I was added to all test tubes. Mixed properly and optical density was read against purified water in a spectrophotometer at 505 nm within 15 min.

The enzyme activity was calculated as ALT (GPT) activity in IU/L = [(Absorbance of test - Absorbance of control)/ (Absorbance of standard - Absorbance of blank)] x concentration of the standard.

#### Determination of alkaline phosphatase (ALP)

Alkaline phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4-aminoantipyrine in presence of the oxidising agent potassium ferricyanide and forms an orange-red coloured complex, which can be measured spectrometrically. The color intensity is proportional to the enzyme activity.

#### Reagents:

Buffered substrate  
Chromogen Reagent  
Phenol Standard, 10 mg%

#### Procedure:

ALP was determined using the method of Kind (Kind PRM, King EJ, 1972. *In-vitro* determination of serum alkaline phosphatase. *Journal of Clinical Pathology* 7: 321-22\). The working solution was prepared by reconstituting one vial of buffered substrate with 2.2 ml of water. 0.5 ml of working buffered substrate and 1.5 ml of purified water was dispensed to blank,

standard, control and test. Mixed well and incubated at 37°C for 3 min. 0.05 ml each of serum and phenol standard were added to test and standard test tubes respectively. Mixed well and incubated for 15 min at 37°C. Thereafter, 1 ml of chromogen reagent was added to all the test tubes. Then, added 0.05 ml of serum to control. Mixed well after addition of each reagent and the O.D of blank, standard, control and test were read against purified water at 510 nm.

Serum alkaline phosphatase activity in KA units was calculated as follows

$$[(O.D. \text{ Test} - O.D. \text{ Control}) / (O.D. \text{ Standard} - O.D. \text{ Blank})] \times 10$$

#### Determination of bilirubin

In toxic liver, bilirubin levels are elevated. Hyperbilirubinemia can result from impaired hepatic uptake of unconjugated bilirubin, such a situation can occur in generalized liver cell injury, certain drugs (e.g Rifampin and probenecid) interfere with the rat uptake of bilirubin by the liver cell and may produce a mild unconjugated hyperbilirubinemia. Bilirubin level rises in diseases of hepatocytes, obstruction to bilirubin excretion into duodenum, in haemolysis and defects of hepatic uptake and conjugation of Bilirubin pigment such as Gilbert's disease. Elevation of total serum bilirubin may occur due to:

1. Excessive haemolysis or destruction of the red blood cells. Eg: Haemolytic disease of the new born.
2. Liver diseases. Eg. Hepatitis and cirrhosis.
3. Obstruction of the biliary tract. Eg. Gall stones.

The method is based on the reaction of Sulfonilic acid with sodium nitrite to form azobilirubin which has maximum absorbance at 546nm in the aqueous solution. The intensity of the color Produced is directly proportional to the amount of direct or total bilirubin concentration present in the sample.

## Reagents

1. Diazo A-(Reagent-R1) :Ready to use
2. Diazo B-(Reagent-R2):Ready to use
3. Bilirubin Activater :Ready to use

## Procedure

Kind & King's method was followed for the estimation of Bilirubin. Five hundred  $\mu\text{l}$  of working reagent was added to 50  $\mu\text{l}$  of rat serum & incubated for 5 min at 37°C. Absorbance was measured AT 546 NM in semi auto analyzer against the standard.

The Bilirubin content was calculated using the following equation:

Total bilirubin (mg/dt) = Abs of the sample blank x 15.

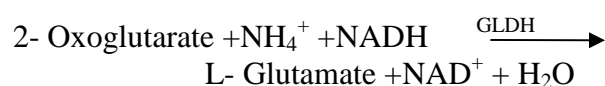
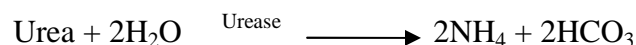
## Estimation of Urea

Urea is the nitrogen-containing end product of protein catabolism. States associated with elevated levels of urea in blood are referred to as hyper uremia or azotemia.

## Method

Estimation of urea was done by Urease-GLDH: enzymatic UV test.

## Principle



**Table Reagents**

R 1	TRIS pH 7.8	120 mmol/l
	2-Oxoglutarate	7 mmol/l
	ADP	0.6 mmol/l
	Urease	6 KU/l
	GLDH	1 KU/l
R 2	NADH	0.25 mmol
R 3	Standard	40 mg/dl

## Procedure

- a. Take 1000  $\mu\text{l}$  of reagent-1 and 250  $\mu\text{l}$  of reagent-2 in 5 ml test tube.
- b. To this, add 10  $\mu\text{l}$  of serum.
- c. Mix well and immediately read the test sample at 340 nm Hg 334 nm Hg 365 nm optical path 1 cm against reagent blank (2-point kinetic).
- d. And note down the value.

**Normal range:** 10 – 50 mg/dl.

## Estimation of uric acid

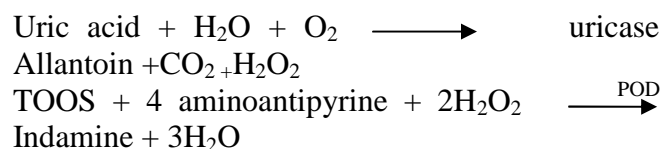
Uric acid and its salts are end products of the purine metabolism. In gout the most common

complication of hyperuricemia, ie. Increased serum levels of uric acid lead to formation of monosodium urate crystal around the joints.

## Method

Enzymatic photometric test using TOOS (N ethyl- N (hydroxyl -3- sulfopropyl)-m- toluidin)

## Principle



**Table Reagents**

R1	Phosphate buffer pH 7.0	100mmol/l
	TOOS	1mmol/l
	Ascorbate oxidase	1 KU/l
R2	Phosphate buffer pH 7.0	100mmol/l
	4- amino antipyrine	0.3mmol/l
	K <sub>4</sub> (Fe(CN) <sub>6</sub> )	10µmol/l
	Peroxidase	1KU/l
	Uricase	50U/l

**Procedure**

- Take 800µl of reagents -1 in a 2ml centrifuge tube.
- To this add 20µl of serum.
- Mix well and incubate at 30°C for 5 minutes.
- Then add 200µl of reagent 2
- Mix well incubate for 5min at 37°C
- Measure the not down the values.

**Normal range:** 1.9-8.2mg/dl

**Estimation of creatinine:**

Estimation of Creatinine by Jaffe Method (modified)

**Principle:**

Creatinine forms a coloured complex with picrate in alkaline medium.

The rate of formation of the complex is measured.

**Reagents:**

Reagent 1	Standard Creatinine
	(2mg/100ml)
Reagent 2	Picric acid solution
Reagent 3	sodium hydroxide solution

**Procedure:**

Take 500 µl of reagent -2 and 500 µl of reagent -3 in a 5ml test tube. To this add 100 µl of serum. Mix well and immediately read the test sample at Hg 492 nm 1cm light path and note down the values.

Normal range is 0.6 -1.1 mg/dl.

**Terminal studies****Sacrifice and macroscopic examination**

On completion of the 4 weeks of treatment, All Wistar rats were sacrificed by ether inhalation. A full autopsy was performed on all animals which included examination of the external surface of the body, all orifices, cranial, thoracic and abdominal cavities and their contents both *in situ* and after evisceration. As the number of animals exceeded the number that could be sacrificed in one day, the autopsies were carried out over three consecutive days at the end of the treatment period.

**Organ weights:**

After the macroscopic examination the following organs were weighed after separating the superficial fat: Brain, Heart, Spleen Kidneys, Testes, Liver, Lungs, pancreas and stomach.

**Histopathological studies**

Anatomy of the liver was studied immediately after sacrificing the animals. A small portion was fixed in 10% neutral buffered formalin as described by Luna 14 . Thin sections of 4-5 µm were taken, stained with Hematoxylin and Eosin and histology was studied<sup>7</sup>

Luna, LG. Manual of histology, staining methods of Armed Forces Institute of Pathology. 3 rd Edn., New York, Mc Graw Hill, 1968

**Effect of Acute Toxicity (14 Days) of *Seenthil Chooranam*****Table: Shows Physical and behavioral examinations.****Table –1 Physical and behavioral examinations.**

Group no.	Dose(mg/kg)	Observation sign	No. of animal affected.
Group-I	5mg/kg	Normal	0 of 3
Group- II	50mg/kg	Normal	0 of 3
Group-III	300mg/kg	Normal	0 of 3
Group-IV	1000mg/kg	Normal	0 of 3
Group-V	2000mg/kg	Normal	0 of 3

**Table- 2 Home cage activity**

Functional and Behavioural observation	Observation	5mg/kg Group (G-I)	50mg/kg (G-II)	300mg/kg (G-III)	1000mg/kg (G-IV)	2000mg/kg (G-V)
		Female n=3	Female n=3	Female n=3	Female n=3	Female n=3
Body position	Normal	3	3	3	3	3
Respiration	Normal	3	3	3	3	3
Clonic involuntary Movement	Normal	3	3	3	3	3
Tonic involuntary Movement	Normal	3	3	3	3	3
Palpebral closure	Normal	3	3	3	3	3
Approach response	Normal	3	3	3	3	3
Touch response	Normal	3	3	3	3	3
Pinna reflex	Normal	3	3	3	3	3
Tail pinch response	Normal	3	3	3	3	3

**Table-3 Hand held observation**

Functional and Behavioral observation	Observation	Control	5 mg/ kg (G-I)	50 mg/kg (G-II)	300mg/kg (G-III)	1000mg/kg (G-IV)	2000mg/kg (G-V)
		Female n=3	Female n=3	Female n=3	Female n=3	Female n=3	Female n=3
Reactivity	Normal	3	3	3	3	3	3
Handling	Normal	3	3	3	3	3	3
Palpebral closure	Normal	3	3	3	3	3	3
Lacrimation	Normal	3	3	3	3	3	3
Salivation	Normal	3	3	3	3	3	3
Piloerection	Normal	3	3	3	3	3	3
Pupillary reflex	Normal	3	3	3	3	3	3
Abdominal tone	Normal	3	3	3	3	3	3
Limb tone	Normal	3	3	3	3	3	3



**Table-4 Mortality**

Group no	Dose no(mg/kg)	Mortality
<b>Group-I</b>	<b>5(mg/kg)</b>	<b>0 of 3</b>
<b>Group-II</b>	<b>50(mg/kg)</b>	<b>0 of 3</b>
<b>Group-III</b>	<b>300(mg/kg)</b>	<b>0 of 3</b>
<b>Group-IV</b>	<b>1000(mg/kg)</b>	<b>0 of 3</b>
<b>Group-V</b>	<b>2000(mg/kg)</b>	<b>0 of 3</b>

From acute toxicity study it was observed that the administration of *Seenthil Chooranam* upto the dose of 2000 mg/kg to the rats do not produce drug-related toxicity and mortality. So No-Observed-Adverse-Effect- Level (NOAEL) of *Seenthil Chooranam* is 2000 mg/kg.

*Seenthil Chooranam* was administered single time at the dose of 5mg/kg, 50mg/kg, 300mg/kg, 1000mg/kg and 2000mg/kg to rats and observed for consecutive 14 days after administration. Doses were selected based on the pilot study and literature review. All animals were observed daily once for any abnormal clinical signs. Weekly body weight and food consumption were recorded. No mortality was observed during the entire period of the study. Data obtained in this study indicated no significance physical and behavioural signs of any toxicity due to administration of *Seenthil Chooranam* at the doses of 5mg/kg, 50mg/kg, 300mg/kg, 1000mg/kg and 2000mg/kg to rats.

At the 14th day, all animals were observed for functional and behavioral examination. In functional and behavioral examination, home cage activity, hand held activity were observed. Home cage activities like Body position, Respiration, Clonic involuntary movement, Tonic involuntary movement, Palpebral closure, Approach response, Touch response, Pinna reflex, Sound responses, Tail pinch response were observed. Handheld activities like Reactivity, Handling, Palpebral closure, Lacrimation, Salivation, Piloerection, Papillary reflex, abdominal tone, Limb tone were observed. Functional and behavioral examination was normal in all treated groups. Food consumption of all treated animals was found normal as compared to normal group.

Body weight at weekly interval was measured to find out the effect of *Seenthil Chooranam* on the growth rate. Body weight change in drug treated animals was found normal.

The present study was conducted to know single dose toxicity of *Seenthil Chooranam* on female wistar rats. The study was conducted using 15 female Wistar rats. The female animals were selected for study of 8- 12 weeks old with weight range of within  $\pm 20$  % of mean body weight at the time of randomisation. The groups were numbered as group I, II, III, IV and V and dose with 5mg/kg, 50mg/kg, 300mg/kg, 1000mg/kg and 2000mg/kg of *Seenthil Chooranam*. The drug was administered by oral route single time and observed for 14 days. Daily the animals were observed for clinical signs and mortality. Body weight of all animals was recorded once in a week.

There were no physical and behavioral changes observed in wistar rats of 5mg/kg, 50mg/kg, 300mg/kg, 1000mg/kg and 2000mg/kg to rats during 14 days.

Body weight of all animals did not reveal any significant change as compared to vehicle control group.

Food consumption of all group animals was normal.

Mortality was not observed in any treatment groups.

The study shows that *Seenthil chooranam* did not produce any toxic effect at dose of 5mg/kg, 50mg/kg, 300mg/kg, 1000mg/kg and 2000mg/kg to rats. So No-Observed-Adverse-Effect-Level (NOAEL) of *Seenthil Chooranam* is 2000 mg/kg.

**Effect of sub-acute doses (28 days) of Seenthil chooranam on body weight (physical parameter)**

Group	Control	S C.300mg/kg	S C.600mg/kg	S C.900mg/kg
1 <sup>st</sup> day	121.333±0.802773	119.333±2.52543	119.5±2.06155	116.167±1.37639
7 <sup>th</sup> day	127.667±0.918937	123.5±2.17179	124.5±2.12525	121.5±1.20416
14 <sup>th</sup> day	133.667±0.918937	130.167±2.12001	130.833±1.95647	129.5±2.26201
21 <sup>st</sup> day	140.667±1.14504	137.333±2.30459	139.5±1.82117	137.833±2.24227
28 <sup>th</sup> day	146.833±1.24944	145.833±2.21234	148±1.87972	144.333±1.83787

Values are expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's (n=6); <sup>ns</sup>p>0.05, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, calculated by comparing treated groups with control group.

**Effect of sub-acute doses (28 days) of Seenthil chooranam on food intake in gms**

Group	Control	S C.L.D	S C.M.D	S C.H.D
1 <sup>st</sup> day	39.1667±2.60021	44.6667±2.72845	43±1.78885	42.8333±1.7591
7 <sup>th</sup> day	39±1.67332	44.3333±2.71621	49.8333±1.85143	46±2.74469
14 <sup>th</sup> day	48.1667±2.15123	49.8333±1.24944	47.6667±2.44495	45.5±3.39362
21 <sup>st</sup> day	41±7.93305	57±2.68328	51.3333±3.29309	50±5.8023
28 <sup>th</sup> day	54±2.0166	50.8333±2.32976	50.1667±3.84202	48.8333±3.34083

Values are expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's (n=6); <sup>ns</sup>p>0.05, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, calculated by comparing treated groups with control group.

**Effect of sub-acute doses (28 days) of Senthil chooranam on water intake in ml**

Group	Control	S C.L.D	S C.M.D	S C.H.D
1 <sup>st</sup> day	92.6667±6.06447	86.1667±6.17207	86.1667±4.18264	84.5±6.14682
7 <sup>th</sup> day	88.6667±3.46089	78±5.40987	82.6667±5.46301	93.1667±3.40995
14 <sup>th</sup> day	78.3333±7.07421	68.1667±4.88137	85±4.19524	81.6667±4.97103
21 <sup>st</sup> day	97.3333±3.61171	100.333±4.66667	79.6667±5.72519	92±5.43446
28 <sup>th</sup> day	105.167±3.65529	85.8333±7.7821	95.6667±6.96499	82.1667±9.36453

Values are expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's (n=6); <sup>ns</sup>p>0.05, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, calculated by comparing treated groups with control group.

**Effect of sub-acute doses (28 days) Of senthil chooranam on organ weight in gms**

Group	Control	S C.L.D	S C.M.D	S C.H.D
Brain	1.429±0.05659	1.382±0.04868	1.56±0.05891	1.526±0.03254
Heart	0.7453±0.05658	0.7157±0.03437	0.6607±0.01903	0.79±0.05168
Liver	7.472±0.3561	6.595±0.4149	7.678±0.7887	6.721±0.2416
Lungs	1.728±0.1224	1.333±0.05962	1.663±0.1328	1.627±0.03301
Testis	2.477±0.04468	2.497±0.1368	1.66±0.06429	1.963±0.2917
Utres	0.6517±0.03308	2.14±1.49	2.088±1.467	0.6003±0.01868
Kidney	L	0.7043±0.01146	0.5747±0.01954	0.5957±0.001453
	R	0.666±0.01808	0.6073±0.03545	0.5607±0.007881

Values are expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's (n=6); <sup>ns</sup>p>0.05, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, calculated by comparing treated groups with control group.

**Effect of sub-acute doses (28 days) of Seenthil chooranam on Haematological parameters**

Group	Control	S C.L.D	S C.M.D	S C.H.D
RBC (X10 <sup>6</sup> /μL)	3.467±0.4256	4.387±0.3014	4.563±0.6697	4.593±0.4317
WBC(X10 <sup>3</sup> /μL)	11.3±1.716	10.77±0.8413	12.9±1.531	10.5±0.5132
HB (g/dl)	10.17±0.9838	11.47±0.6386	12±0.9452	10.73±0.8511
Polymorphs (%)	6±1	9.667±2.028	8.667±0.8819	8±1.155
Lymphocytes (%)	84±1.155	81±1.528	81.67±2.963	88±2.309
Monocytes (%)	5.667±1.202	5.333±0.8819	6±0.5774	4.667±1.202

Values are expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's (n=6); <sup>ns</sup>p>0.05, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, calculated by comparing treated groups with control group.

**Effect of sub-acute doses (28 days) of Seenthil chooranam on Biochemical parameters**

Group	Control	S C.L.D	S C.M.D	S C.H.D
SGOT (U/L)	103.8±10.43	114.5±14.07	99.18±5.671	86.08±2.707
SGPT (U/L)	47.4±4.711	104.9±19.86	63.2±3.427	46.97±3.444
ALP (U/L)	136.967±4.36666	148.733±9.75728	131±2.64575	162.333±27.3882

Values are expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's (n=6); <sup>ns</sup>p>0.05, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, calculated by comparing treated groups with control group.

Group	Control	S C.L.D	S C.M.D	S C.H.D
Total Bilirubin (g/ml)	1.333±0.05364	1.553±0.07535	1.393±0.1325	1.683±0.1255

Values are expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's (n=6); <sup>ns</sup>p>0.05, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, calculated by comparing treated groups with control group.

**Effect of sub-acute doses (28 days) of Seenthil chooranam on Biochemical parameters**

Group	Control	S C.L.D	S C.M.D	S C.H.D
Urea (mg/dl)	22.63±2.258	31.2±1.301	28.27±3.299	21.87±4.332
Uric acid (mg/dl)	2.767±0.2541	1.86±0.9016	1.633±0.1598	2±0.2948
Creatinine (mg/dl)	0.4433±0.04978	0.52±0.02309	0.6567±0.0491	0.7067±0.09333

Values are expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's (n=6); <sup>ns</sup>p>0.05, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, calculated by comparing treated groups with control group.

**Effect of sub-acute doses (28 days) of Seenthil chooranam on Electrolytes**

Group	CONTROL	S C.L.D	S C.M.D	S C.H.D
Sodium (mg/ml)	4.7±0.05774	6.8±0.05774***	6.2±0.2***	5.4±0.1155**
Calcium (mg/ml)	1.593±0.07753	4.633±0.2186***	3.3±0.1528***	5.79±0.06658***
Phosphorus (mg/ml)	0.38±0.02309	0.46±0.06429 <sup>ns</sup>	0.5467±0.01764*	0.6533±0.01764**

Values are expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's (n=6); <sup>ns</sup>p>0.05, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, calculated by comparing treated groups with control group.

**Clinical signs:**

All animals in this study were free of toxic clinical signs throughout the dosing period of 28 days.

**Mortality:**

All animals in control and in all the treated dose groups survived throughout the dosing period of 28 days.

**Body weight:**

Results of body weight determination of animals Table-1 from control and different dose groups

exhibited comparable body weight gain throughout the dosing period of 28 days.

**Food consumption:**

During dosing and the post-dosing recovery period, the quantity of food consumed by animals from different dose groups was found to be comparable with that by control animals.

**Organ Weight:**

Group Mean Relative Organ Weights (% of body weight) are recorded in Table No.4 Comparison of organ weights of treated animals with respective control animals on day 28 was found to be comparable similarly.

### Hematological investigations:

The results of hematological investigations (Table 4) conducted on day 28 revealed following significant changes in the values of different parameters investigated when compared with those of respective controls; however, the increase or decrease in the values obtained was within normal biological and laboratory limits or the effect was not dose dependent.

### Biochemical Investigations:

Results of Biochemical investigations conducted on days 28 and recorded in Table 2 revealed the following significant changes in the values of hepatic serum enzymes studied. When compared with those of respective control. However, the increase or decrease in the values obtained was within normal biological and laboratory limits.

### Histopathology:

In histopathological examination, revealed normal architecture in comparison with control and treated animal.

- 1) All the animals from control and all the treated dose groups up to 900 mg/kg survived throughout the dosing period of 28 days.
- 2) No signs of toxicity were observed in animals from different dose groups during the dosing period of 28 days.
- 3) Animals from all the treated dose groups exhibited comparable body weight gain with that of controls throughout the dosing period of 28 days.
- 4) Food consumption of control and treated animals was found to be comparable throughout the dosing period of 28 days
- 5) Haematological analysis conducted at the end of the dosing period on day 28, revealed no abnormalities attributable to the treatment.
- 6) Biochemical analysis conducted at the end of the dosing period on day 28 no abnormalities attributable to the treatment.
- 7) Organ weight data of animals sacrificed at the end of the dosing period was found to be comparable with that of respective controls.

8) Histopathological examination revealed normal architecture in comparison with control and treated animal.

In conclusion *Seenthil Chooranam* can be considered safe, as it did not cause either any lethality or adverse changes with general behavior of rats and also there were no observable detrimental effects (**300 to 900 mg/kg body weight**) over a period of 28 days. Our results have demonstrated that the *Seenthil Chooranam* is relatively safe when administered orally in rats.

### Discussion

The present study is aimed to find out whether the drug "Seenthil Chooranam" has any adverse effect in acute and subacute administration

The ICP-OES analysis revealed that heavy metals like arsenic, cadmium, mercury and lead are in below detection limit.

By scanning electron microscope (SEM) the sizes of the particles were found to be in the range of 1 to 3 MICRON.

Biochemical analysis shows the presence of calcium, starch, ferrous iron, tannic acid, unsaturated compound, reducing sugar, amino acid.

Phytochemical evaluation of the drug reveals the presence of alcohols, phenols, Alkenes, Nitroes, Aromatics, Alkanes, Aromatic amines, Alkyl halides, Aliphatic amines, Alkynes Carboxylic acid.

On the basis of acute toxicity result the study shows that Seenthil Chooranam did not produce any toxic effect at the dose of 2000mg/kg to rats. On the basis of sub acute toxicity result, the study reveals that Seenthil Chooranam did not cause either any lethality or adverse changes with general behaviour of rats and also there were no observable detrimental effects (300 to 900 mg/kg body weight) over a period of 28 days.

The hematological analysis revealed no abnormalities attributable to the treatment.



In histopathological examination, revealed normal architecture in comparison with control and treated animal.

These results indicate that Seenthil Chooranam upto 300-900 mg/kg body showed minor changes in liver only.

## Conclusion

The study shows that *Seenthil chooranam* did not produce any toxic effect at dose of 5mg/kg, 50mg/kg, 300mg/kg, 1000mg/kg and 2000mg/kg to rats. So No-Observed-Adverse-Effect-Level (NOAEL) of *Seenthil chooranam* is 2000 mg/kg.

In conclusion Seenthil Chooranam can be considered safe, as it did not cause either any lethality or adverse changes with general behavior of rats and also there were no observable detrimental effects (300 to 900 mg/kg body weight) over a period of 28 days. Our results have demonstrated that the Seenthil Chooranam is relatively safe when administered orally in rats.

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How to cite this article:

D. Logadevi, R. Suguna. (2020). Acute and Sub-acute toxicity study in Wister rats to evaluate toxicity profile of Seenthil Chooranam. Int. J. Curr. Res. Med. Sci. 6(10): 9-30.  
DOI: <http://dx.doi.org/10.22192/ijcrms.2020.06.10.002>