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PRECLINICAL EVALUATION OF SAMUTHARA CHOORANAM – TOXICOLOGICAL AND PHARMACOLOGICAL STUDY (IMMUNOMODULATOR AND ANTIOXIDANT ACTIVITY)

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ABSTRACT

Siddha system of medicine is the oldest documented medical system which deals with the physical, psychological, social and spiritual well being of an individual. Here the drug "Samuthara chooranam" taken from the siddha text will expect to give good results on managing the autoimmune disorders. The primary purpose of this preclinical study is to evaluate the efficacy and safety of the drug which will help the medicine for widespread global acceptance. An acute and sub-acute toxicity study was performed as per OECD guideline-423 and no other significant changes were observed in animal models. The immunomodulator study was conducted in raw 264.7 cell line and proven to be a potent immunomodulator drug. The antioxidant study was determined by DPPH assay and hence proven to be a potent radical scavenging drug. The overall study results that the drug "Samuthara chooranam (SC)" has high efficacy and safety in preclinical assessment and expected to give best results on the management of autoimmune disorders in further clinical assessment.

KEYWORDS: Samuthara chooranam, Immunomodulator, Antioxidant, Autoimmune disorders, Raw 264.7 cell line.

INTRODUCTION

The immune system is the capacity to mount an inflammatory response to non-self while avoiding harm to self-tissues. The essential feature of an autoimmune disease is that tissue

injury is caused by the immunologic reaction of the organism against its own tissues. Autoimmunity, on the other hand, refers merely to the presence of antibodies or T lymphocytes that react with self-antigens and does not necessarily imply that the self-reactivity has pathogenic consequences. The recent researches say that Immunomodulatory drugs modify the response of the immune system either increasing (Immunostimulators) or decreasing (Immunosuppressive) the immune response cells like lymphocytes, macrophages, neutrophils, natural killer (NK) cells and cytotoxic T lymphocytes i.e., either for tissue regeneration or by targeting two or more pathways to synergistically repair the different components of autoimmune imbalance.

Antioxidant drugs have the ability to demolish free radicals, protects the structural integrity of cells and tissues. Immunomodulatory drugs along with supplementation of antioxidant drugs preserve an ample function of immune cells against homeostatic disturbances caused by oxidative stress.

The herbal-mineral formulation drug "Samuthara chooranam" taken from the siddha text 'Pranarashamirtha Sindhu' may have high efficacy and safety on managing the signs and symptoms of autoimmune disorders. Many review articles show that the ingredients of the "Samuthara chooranam" having both Immunomodulator and Antioxidant activity which is the reason behind medicine to take up for the study on the management of autoimmune disorders.

Preclinical assessment of Pharmacology and Toxicology profile of the drug "Samuthara chooranam" provides the Efficacy and Safety of the drug and also plays an inevitable role in deciding and designing further clinical studies in future.

MATERIALS AND METHODS

Selection of Drugs

Samuthara chooranam consists of Kadukkai (Terminalia chebula), Omam (Trachyspermum ammi), Inji (zingiber officinale), Thippili (Piper longum), Perungayam (Ferula asafoetida), Vaividangam (Embelia ribes), Induppu (Rock salt), Kaluppu (Sodium chloride impura), Yavacharam (Potassium carbonate) were purchased from the raw drug shop R.N.RAJAN & CO, Paris. After getting proper authentication from the Head of the Department of Medicinal Botany and Pharmacology (Gunapadam), GSMC, Chennai-106 the medicines were prepared.

Purification of Raw Drugs

The herbal and mineral drugs were purified under the basis of Gunapadam Mooligai and Thaathu- Jeevam textbook.

Method of Preparation

The purified drugs were grinded separately and mixed well together. Then filter them as a fine powder and weighed. Atlast the powder was stored in the air tight container.

Administration: Two grams (twice daily) for 48 days with ghee as the adjuvant.

1. Toxicological Study

After getting proper permission from the **Institutional Animal Ethics Committee (IAEC No: XLVIII/23/CLBMCP/2016),** Acute and Sub-acute Toxicity for the trial drug **Samuthara Chooranam** was carried out in **Wistar albino rats.**

A. Acute Oral Toxicity Study of *SC* (OECD Guideline – 423)

Introduction

- The acute toxic class method is a stepwise procedure with the use of 3 animals of a single sex per step.
- Depending on the mortality and/or the moribund status of the animals, on average 2-4 steps may be necessary to allow judgement on the acute toxicity of the test substance.
- This procedure is reproducible, uses very few animals and is able to rank substances in a similar manner to the other acute toxicity testing methods.
- The acute toxic class method is based on biometric evaluations with fixed doses, adequately separated to enable a substance to be ranked for classification purposes and hazard assessment.
- In principle, the method is not intended to allow the calculation of a precise LD50, but does allow for the determination of defined exposure ranges where lethality is expected since death of a proportion of the animals is still the major endpoint of this test.
- The method allows for the determination of an LD50 value only when at least two doses result in mortality higher than 0% and lower than 100%.
- The use of a selection of pre-defined doses, regardless of test substance, with classification explicitly tied to number of animals observed in different states improves the opportunity for laboratory to laboratory reporting consistency and repeatability.

Principle of the Test

It is the principle of the test 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.

a. No further testing is needed
b. Dosing of three additional animals, with the same dose
c. 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.

Methodology

Selection of Animal Species

The preferred rodent species is the wistar albino rat, although other rodent species may be used. Healthy young adult animals are commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant. Each animal, at the commencement of its dosing, should be between 6 to 8 weeks old and the weight (150-200gm) should fall in an interval within±20% of the mean weight of any previously dosed animals.

Housing and Feeding Conditions

The temperature in the experimental animal room should be 22ºC $\pm$ 3ºC. Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be group-caged by dose, but the number of animals per cage must not interfere with clear observations of each animal.

Preparation of animals

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 7 days prior to dosing to allow for acclimatization to the laboratory conditions.

Test Animals and Test Conditions

Sexually mature Female Wistar albino rats (150-200gm) were obtained from TANUVAS, Madhavaram, Chennai. All the animals were kept under standard environmental condition (22±3°C). The animals had free access to water and standard pellet diet (Sai meera foods, Bangalore).

Preparation for Acute Toxicity Studies

Rats were deprived of food overnight (but not water 16-18 h) prior to administration of the, ***Samuthara chooranam (SC)***

The principles of laboratory animal care were followed and the Institutional Animal Ethical Committee approved the use of the animals and the study design

IAEC approved Number: 1248/AC/09/CPCSEA-9/DEC-2013/12

Test Substance	**: Samuthara Chooranam (SC)**
Animal Source	**:** Tanuvas, Madhavaram, Chennai.
Animals	**:** Wister Albino Rats (Female-3+3)
Age	**:** 6-8 weeks
Body Weight on Day 0	**:** 150-200gm.
Acclimatization	**:** Seven days prior to dosing.
Veterinary examination	**:** Prior and at the end of the acclimatization period.
Identification of animals	**:** By cage number, animal number and individual marking by using Picric acid.
Number of animals	**:** 3 Female/group,
Route of administration	**:** Oral
Diet	**:** Pellet feed supplied by Sai meera foods Pvt Ltd, Bangalore
Water	**:** Aqua guard portable water in polypropylene bottles.
Housing & Environment	**:** The animals were housed in Polypropylene cages provided with bedding of husk.
Housing temperature	**:** between 22ºC $\pm$ 3ºC.
Relative humidity	**:** between 30% and 70%,
Air changes	**:** 10 to 15 per hour and
Dark and light cycle	**:** 12:12 hours.
Duration of the study	**:** 14 Days

Administration of Doses

Samuthara chooranam was suspended in water and administered to the groups of wistar albino rats in a single oral dose by gavage using a feeding needle. The control group received an equal volume of the vehicle. Animals were fasted 12 hours prior to dosing. Following the period of fasting, the animals were weighed and then the test substance was administered. Three Female animals are used for each group. The dose level of 5, 50, 300 and 2000 mg/kg body weight was administered stepwise. 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 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. Finally, the number of survivors was noted after 24 hrs and these animals were then monitered for a further 14 days and observations made daily. The toxicological effect was assessed on the basis of mortality.

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. It should be determined by the toxic reactions, time of onset and length of recovery period, and may thus be extended when considered necessary. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed. 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 humanly killed. When animals are killed for human reasons or found dead, the time of death was recorded.

B. Repeated Dose 28-Day Oral Toxicity Study of SC

Test Substance : **Samuthara chooranam (SC)**

Animal Source : Tanuvas, Madhavaram, Chennai.

Animals : Wister Albino Rats (Male -24, and Female-24)

Age : 6-8 weeks

Body Weight : 150-200gm.

Acclimatization : Seven days prior to dose.

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

Identification of animals : By cage number, animal number and individual marking by using Picric acid

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

Water : Aqua guard portable water in polypropylene bottles.

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

Housing temperature : between 22ºC $\pm$ 3ºC.

Relative humidity : between 30% and 70%,

Air changes : 10 to 15 per hour

Dark and light cycle : 12:12 hours.

Duration of the study : 28 Days.

Table 1

Groups	No of Rats
Group I Vehicle control (Water)	12 (6male,6 female)
Group II low dose X (20mg)	12 (6male,6 female)
Group III STR- Mid dose 5X (100mg)	12 (6male,6female)
Group IV STR- High dose 10X(200mg)	12 (6male,6female)

METHODOLOGY

Randomization, Numbering and Grouping of Animals

48 Wistar Albino Rats (24M + 24F) were selected and divided into 4 groups. Each group consist of 12 animals (Male -6, and Female-6). First group treated as a control and other three group were treated with test drug (low, mid, high) for 28 days. Animals were allowed acclimatization period of 7 days to laboratory conditions prior to the initiation of treatment. Each animal was marked with picric acid. The females were nulliparous and non-pregnant.

Justification for Dose Selection

As per OECD guideline three dose levels were selected for the study. They are low dose (X), mid dose (5X), high dose (10X). X is calculated from the dose (2000 mg) and the X dose is 20mg/animal, 5X dose is 100mg/animal, 10X dose is 200mg/animal.

Preparation and Administration of Dose

Samuthara chooranam suspended in with water, it was administered to animals at the dose levels of X, 3X, 6X. The test substance suspensions were freshly prepared every two days once for 28 days. The control animals were administered vehicle only. The drug was administered orally by using oral gavage once daily for 28 consecutive days.

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.

Food and water Consumption

Food and water 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.

Necropsy

All the animals were sacrificed by excessive anesthesia on day 29. Necropsy of all animals was carried out.

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 Bio

chemistry and potassium EDTA (1.5 mg/ml) for Haematology as anticoagulant. Blood samples were centrifuged at 3000 rpm for 10 minutes.

Haematological Investigations

Haematological parameters were determined using Haematology analyzer.

Biochemical Investigations

Biochemical parameters were determined using auto-analyzer.

Histopathology

Control and highest dose group animals will be initially subjected to histopathological investigations. If any abnormality found in the highest dose group than the low, then the mid dose group will also be examined. Organs will be collected from all animals and preserved in 10% buffered neutral formalin for 24 h and washed in running water for 24 h. The organ sliced 5 or 6µm sections and were dehydrated in an auto technicon and then cleared in benzene to remove absolute alcohol. Embedding was done by passing the cleared samples through three cups containing molten paraffin at 50°C and then in a cubical block of paraffin made by the "L" moulds. It was followed by microtome and the slides were stained with Haematoxylin-eosin red.

Statistical analysis

Findings such as body weight changes, water and food consumption, haematology and blood chemistry were subjected to One-way ANOVA followed by dunnett test using a computer software programme – Graph pad version 7. All data were summarized in tabular form, (Table-6 to 12).

2. Pharmacological Study

A. Immunomodulator Activity - Cell Line Study

The evaluation of the immunomodulatory activity of Samuthara chooranam was carried out in cultured raw 264.7 cell line in Biogenix Research Center.

Determination of In-vitro Immunomodulatory Effect of Extracts on Cultured Macrophage Raw Cell Lines

RAW 264.7 cells will be grown to 60% confluence followed by activation with 1 µL lipopolysaccharide (LPS) (1µg/mL). LPS stimulated RAW cells were exposed with different concentration (25, 50, 100 µg/mL) of sample and incubated for 24 hours. After 24 hours of

incubation the cells were digested and centrifugation was done at 6000 rpm for 10 minutes. Supernatant was discarded and cells were then resuspented in 200µl of cell lysis buffer (0.1M Tris HCl, 0.25M EDTA, 2M Nacl, 0.5% Triton x-100). The samples were then kept at 4^0C for 20 minutes. After incubation, the immunomodulatory response was performed by estimating nitrite levels in the cell lysate.

Estimation of Cellular Nitrite Levels

The level of nitrite was estimated by the method of Lepoivre et al. (Lepoivre et. al. 1990) To 0.5 mL of cell lysate, 0.1 mL of sulphosalicylic acid was added and vortexed well for 30 minutes. The samples were then centrifuged at 5,000 rpm for 15 minutes. The protein-free supernatant was used for the estimation of nitrite levels. To 200 µL of the supernatant, 30 µL of 10% NaOH was added, followed by 300 µL of Tris-HCl buffer and mixed well. To this, 530 µL of Griess reagent was added and incubated in the dark for 10–15 minutes, and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained.

B. Antioxidant Study

DPPH Radical Scavenging Assay

The radical scavenging activity of different extracts was determined by using DPPH assay according to Chang et al [2001]. The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517 nm. Ascorbic acid (10mg/ml DMSO) was used as reference.

Principle

1, 1-diphenyl-2-picryl hydrazyl is a stable free radical with pink colour which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as,

$$DPPH + [H\text{-}A] \rightarrow DPPH\text{-}H + (A)$$

Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

Reagent Preparation

0.1ml DPPH solution was prepared by dissolving 4mg of DPPH in 100ml of ethanol.

Procedure

Different volumes of extracts 1.25µl - 20µl (12.5 - 200µg/ml) from a stock concentration 10mg/ml were made up to a final volume of 20µl with DMSO and 1.48ml DPPH (0.1mM) solution was added. A control without the test compound, but an equivalent amount of distilled water was taken. The reaction mixture incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm. 3ml of DPPH was taken as control.

Calculation

$$\% \text{ inhibition} = \frac{control - test}{control} X100$$

RESULTS AND DISCUSSIONS

1. Toxicological Study

A. Acute oral toxicity study of SC

Observation done

Table 2: Dose finding experiment and its behavioral Signs of acute oral Toxicity (SC).

S.L.	Group Control	Observation	S.L.	Test Group	Observation
1	Body weight	Normal	1	Body weight	Normally increased
2	Assessments of posture	Normal	2	Assessments of posture	Normal
3	Signs of Convulsion Limb paralysis	Normal	3	Signs of Convulsion Limb paralysis	Absence of sign (-)
4	Body tone	Normal	4	Body tone	Normal
5	Lacrimation	Normal	5	Lacrimation	Absence
6	Salivation	Normal	6	Salivation	Absence
7	Change in skin color	No significant color change	7	Change in skin color	No significant color change
8	Piloerection	Normal	8	Piloerection	Normal
9	Defecation	Normal	9	Defecation	Normal
10	Sensitivity response	Normal	10	Sensitivity response	Normal
11	Locomotion	Normal	11	Locomotion	Normal
12	Muscle gripness	Normal	12	Muscle gripness	Normal
13	Rearing	Mild	13	Rearing	Mild
14	Urination	Normal	14	Urination	Normal

Behaviour

The animals will be observed closely for behaviour in the first four hours which includes abnormal gait, aggressiveness, exophthalmos, ptosis, akinesia, catalepsy, convolusion, excitation, head twitches, lacrimation, loss of corneal reflex, loss of traction, piloerection reactivity of touch, salivation, scratching, sedation, chewing, head movements, sniffing, straub, tremor and writhes, diarrhea, leathery, sleep and coma.

Body Weight

Individual weight of animals was determined before the test substance was administered and weights will be recorded at day 1, 7, and 14 of the study. Weight changes were calculated and recorded. At the end of the test, surviving animals were weighed and humanly killed.

Food and water Consumption

Food and water consumed per animal was calculated for control and the treated dose groups.

Mortality

Animals were observed for mortality throughout the entire period.

Table 3: Observational study Results of SC.

No	Dose mg/kg	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.	Control	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.	2000mg	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

1.Alertness 2.Aggressiveness 3.Pile erection 4.Grooming 5.Gripping 6.Touch Response 7.Decreased Motor Activity 8.Tremors 9.Convulsions 10.Muscle Spasm 11.Catatonia 12.Muscle relaxant 13.Hypnosis 14.Analgesia 15.Lacrimation 16.Exophthalmos 17.Diarrhea 18.Writhing 19.Respiration 20.Mortality. (+ Present, - Absent)

Table 4: Body weight Observation of SC.

Dose	Days		
	1	7	14
Control	220.6±31.474	221.4 ± 34.324	224.2 ± 27.623
High Dose	210.5± 27.75	211.7 ± 31.67	213.4 ± 32.67
P value (p)*	Ns	Ns	Ns

Table 5: Water intake (ml/day) of Wistar albino rats group exposed to SC.

Dose	Days		
	1	6	14
Control	58.5 ± 6.74	60 ± 9.13	60.4 ± 4.13
High Dose	60.4 ± 2.33	60.6. ± 1.11	60.9 ± 6.19
P value (p)*	NS	NS	NS

N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test) .

Table 6: Food intake (gm/day) of Wistar albino rats group exposed to SC.

Dose	Days		
	1	7	14
Control	40.56 ± 9.36	42.6 ± 4.42	41.6 ± 7.46
Low Dose	39.4 ± 1.64	39.3 ± 1.22	39.2 ± 6.24
P value (p)*	NS	NS	NS

N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test) .

Inference

All data were summarized in tabular form, (Table 2 - 6) 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, description of toxic symptoms, weight changes, food and water intake. Hence no significant changes are observed in body weight, water and food intake and no mortality are exhibited in rats.

B. Sub Acute Toxicity(Repeated Dose 28- day oral toxic study)

Table 7: Body weight of wistar albino rats group exposed to SC.

Dose	Days				
	1	7	14	21	28
Control	220.6±33.673	221.4±40.114	221.7±39.661	222.6±39.73	222.7±41.311
Low Dose	180.2±21.124	180.7 ± 33.64	181.4±21.514	182 ± 21.66	182.42±12.76
Mid Dose	176.6 ± 10.64	176.3 ± 22.74	176.4 ± 38.12	178.1±33.36	179.7 ± 23.12
High Dose	187.4 ± 36.74	187.6 ± 32.72	187.6 ± 32.46	187 ± 22.78	186.92±26.49
P value (p)*	NS	NS	NS	NS	NS

NS- Not Significant, ** (p > 0.01),*(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table 8: Water intake (ml/day) of Wistar albino rats group exposed to SC.

Dose	Days				
	1	6	14	21	28
Control	61.5 ± 8.95	61 ± 6.23	58.5 ± 6.23	59 ± 8.196	61.5 ± 3.96
Low Dose	56.5 ± 3.31	56.4 ± 3.62	56.7 ± 3.26	56.2 ± 3.29	56.9 ± 3.13
Mid Dose	55.7 ± 4.33	56.3 ± 2.11	57.1 ± 2.43	58.4 ± 2.11	58.4 ± 2.34
High Dose	60.1 ± 1.32	60.2 ± 2.13	60.7 ± 2.13	65.2 ± 1.73	63.4 ± 2.65
P value (p)*	NS	NS	NS	NS	NS

N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table 9: Food intake (gm/day) of Wistar albino rats group exposed to SC.

Dose	Days				
	2	7	23	22	28
Control	37 ± 5.37	38.5 ± 3.22	39.5 ± 3.37	38.5 ± 3.37	37 ± 3.12
Low Dose	43.7 ± 2.98	45.3 ± 1.22	45.1 ± 1.18	45.4 ± 2.12	45.6 ± 2.42
Mid Dose	47.2 ± 3.75	47.2 ± 3.60	47.2 ± 4.25	47.4 ± 2.68	49.2 ± 2.44
High Dose	46.2 ± 2.34	46.2 ± 2.64	49.6 ± 2.66	48.2 ± 3.20	48.0 ± 3.62
P value (p)*	NS	NS	NS	NS	NS

N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table 10: Haematological parameters of Wistar albino rats group exposed to SC.

Category	Control	Low dose	Mid dose	High dose	P value (p)*
Haemoglobin (g/dl)	13.8±0.88	13.80±0.66	14.14±0.66	13.28±0.96	N.S
Total WBC ($\times 10^3$ l)	11.91±0.59	11.25±0.73	11.48±0.91	11.20±1.17	N.S
Neutrophils (%)	33.65±0.06	32.23±0.14	35.41±1.36	35.20±2.20	N.S
Lymphocyte (%)	70.24±1.48	70.12±3.12	70.20±2.66	70.10±2.16	N.S
Monocyte (%)	0.86±0.07	0.84±0.09	0.82±0.03	0.81±0.06	N.S
Eosinophil (%)	0.54±0.09	0.56±0.02	0.56±0.06	0.57±0.04	N.S
Platelets cells 10^3/µl	687.17±8.76	688.71±8.16	683.18±9.0	687.16±9.74	N.S
Total RBC (10^6/µl)	7.99±0.12	7.99±0.57	7.82±0.59	8.05±0.72	N.S
PCV%	37.79±0.6	41.35±1.13	43±1.68	45.82±2.54	N.S
MCHC g/Dl	33.6±2.23	35.09±1.29	36.98±1.22	34.03±1.24	N.S
MCV fL (µm^3)	49.07±3.64	50.20±1.22	51.20±1.24	52.24±1.44	N.S

N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table 11: Biochemical Parameters of Wistar albino rats group exposed to SC.

Biochemical Parameters	Control	Low Dose	Mid Dose	High Dose	P Value (p)*
Glucose (R) (mg/dl)	74.45±13.4	76.16±8.44	78.26±11.20	76.42±11.6	N.S
T.Cholesterol (mg/dl)	115.26±1.83	115.45±1.83	116.42±1.78	116.22±1.73	N.S
Trigly(mg/dl)	46.35±1.48	46.32±1.48	44.58±1.30	45.66±1.33*	N.S
LDL	73.8±2.43	73.24±2.54	73±2.44	73.64±24.32	NS
VLDL	15.2±2.44	15.42±4.64	15.44±6.64	15.64±34.36	NS
HDL	26.66±6.88	26.86±2.24	26.68±4.66	26.78±21.22	NS
Ratio 1(T.CHO/HDL)	4.42±2.44	4.46±3.14	4.44±8.44	4.46±22.22	NS
Ratio 2(LDL/HDL)	2.83±24.22	2.84±2.22	2.86±2.20	2.66±46.02	NS
Albumin (g/dL)	3.3±0.17	3.43±0.12	3.34±22.02	3.54±6.86	NS

NS- Not Significant, ** (p > 0.01), * (p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table 12: Renal function test of Wistar albino rats group exposed to SC.

Parameters	Control	Low dose	Mid Dose	High Dose	P Value (p)*
UREA (mg/dl)	13.35±0.99	14.31±0.46	14.06±1.38	14.48±1.42	N.S
Creatinine (mg/dl)	0.58±0.08	0.46±0.06	0.62±0.04	0.66±0.02	N.S
BUN (mg/dL)	15.12±0.10	15.10±0.60	16±0.44	16.10±2.12	NS
URIC ACID (mg/dl)	5.37±0.35	5.11±0.43	5.7±1.25*	5.48±0.23	N.S

NS- Not Significant, ** (p > 0.01), * (p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test)

Table 13: Liver Function Test of Wistar albino rats group exposed to SC.

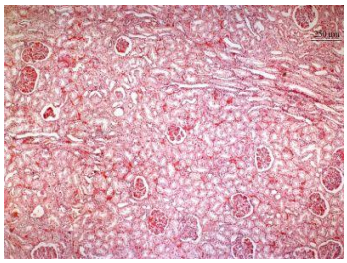
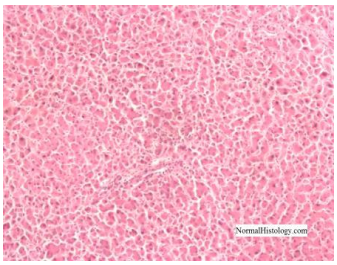
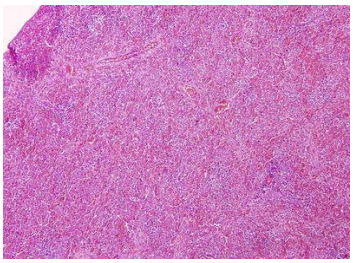
Parameters	Control	Low dose	Mid dose	High dose	P Value (p)*
T.Bilirubin (mg/dl)	0.50±0.07	0.55±0.06	0.59±0.08	0.56±0.05	N.S
SGOT/AST(U/L)	114.95±1.39	116.35±0.51	117.01±1.53	116.55±1.03	N.S
SGPT/ALT(U/L)	71.23±1.28	75.91±1.59	75.34±1.48	74.32±0.68	N.S
ALP(U/L)	146.25±8.77	141±16.17	148.16±24.07*	149.33±14.65*	N.S
T.Protein(g/dL)	6.32±0.38	7.48±0.34	7.016±0.23	6.53±0.46	N.S

NS- Not Significant, ** (p > 0.01), * (p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Histopathology

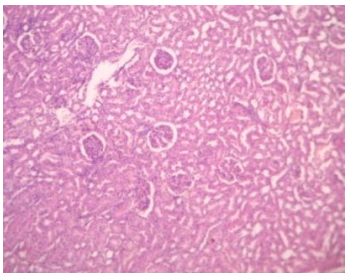
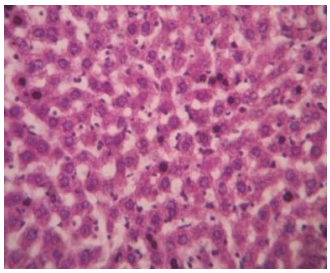
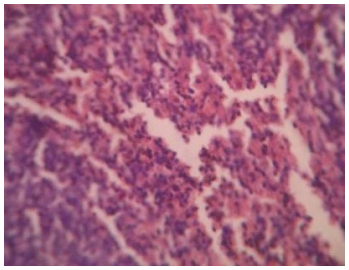
Control Group

Kidney **Liver** **Spleen**

Test Group (High Dose)

Kidney **Liver** **Spleen**

2. Pharmacological Study

A. Immunomodulator Study

Table 14: Immunomodulator activity of SC

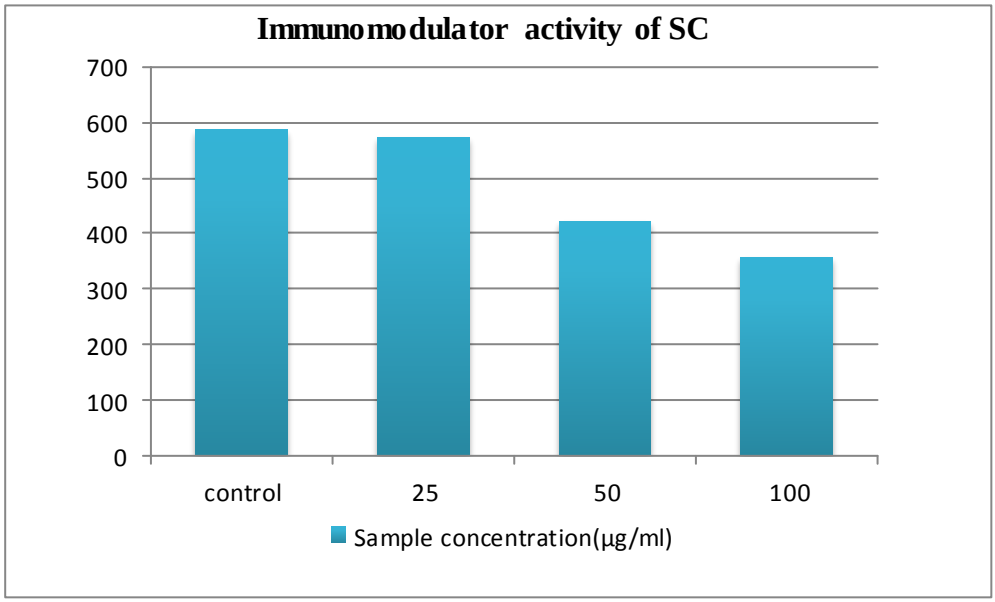
Sample Concentration(µg/ml)	OD at 540nm	Concentration(µg)
Control	0.1185	586.575
25	0.1158	573.21
50	0.0849	420.255
100	0.0723	357.885

Standard – Nitrite level

Table 15: Standard nitrite level for Immunomodulatory activity.

Concentration(µg)	OD(540 nm)
100	0.021
200	0.42
300	0.06
400	0.08
500	0.17

Chart I – Immunomodulator activity of SC



Inference

Lipopolysaccharide (LPS) was treated as control with 586.57µg concentration of nitrite level production in the raw 264.7 cell line. Here the formulation drug SC at the dose of 25, 50 µg/ml shows a significant decrease in the production of nitrite level of about 573.21 and 420.25 µg respectively. 100 µg/ml concentration of SC shown maximum level of decrease in nitrite concentration of about 357.88 µg. The decrease in nitrite concentration clearly proves that increase in phagocytosis in macrophage 264.7 cell line and hence proven to be a potent Immunomodulator drug.

B. Antioxidant Study

Table 16: Ascorbic acid standard for Antioxidant activity.

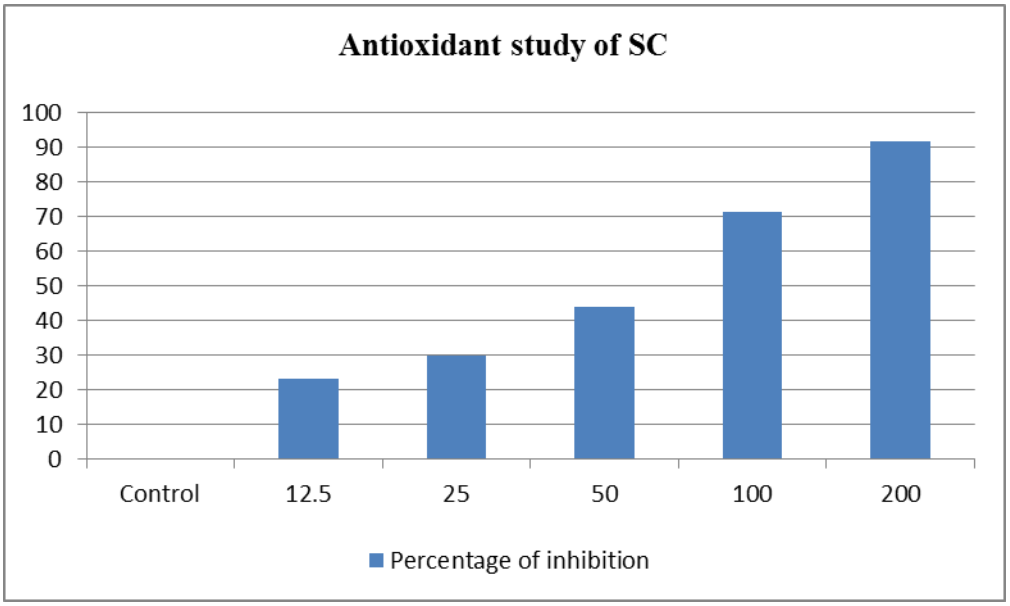
Concentrations (µg/ml)	Absorbance	Percentage of inhibition
Control	1.7983	
12.5	1.4044	21.90
25	1.0782	40.04
50	0.7121	60.40
100	0.2921	83.75
200	0.0692	96.15

Table 17: Antioxidant activity of Samuthara chooranam (SC).

Concentrations (µg/ml)	Absorbance	Percentage of inhibition
Control	0.8085	
12.5	0.6190	23.44
25	0.5677	29.78

50	0.4536	43.90
100	0.2312	71.40
200	0.0663	91.80

Chart II Antioxidant study of SC.



Inference

200µg/ml level of Samuthara chooranam has 91.80% of inhibiting the free radical production and oxidative degeneration and hence proven to be a rich Antioxidant drug.

CONCLUSION

Acute oral toxic study and Repeated dose 28-day oral toxic study was done in Wistar Albino Rats for the sample Samuthara chooranam and hence no other significant changes and mortality were observed in their behavior, body weight, water intake, food intake, LFT, RFT. It proves that the trial drug Samuthara chooranam is safe for animal models.

In the Pharmacological study, while the dose of SC is increased, the level of nitrite concentration is decreased from 586.57 to 357.88 µg which indicates the presence of an increase in Phagocytosis. Thus 100µg/ml concentration of Samuthara chooranam shows maximum level of decrease in nitrate level of about 357.88 µg and thus proven to be a potent **Immunomodulator** drug.

In Antioxidant study, 200µg/ml concentration of Samuthara chooranam shows 91.8% of free radical scavenging property and proven to be a rich **Antioxidant** drug.

Thus the overall study results that the drug **"Samuthara chooranam"** has **high efficacy and safety in preclinical assessment** and expected to give best results on the management of autoimmune disorders in further clinical assessment.

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