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Preclinical Toxicological Screening of Siddha Formulation Maampisin Chooranam by Acute and Sub-acute Toxicity Studies in Wistar Rats

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Abstract

Traditional drugs are popular, effective and generally safe remedies against variety of diseases and are being used widely the world over. This assumes significance since a World Health Organization survey has found that around 70%–80% of the world's populations rely on non-conventional medicine, mainly of herbal source, in the primary healthcare sector. The use of Siddha medicines as complements or alternatives medicines has been on the increase. The reasons, which have given rise to this trend, include the effectiveness, cheapness, availability and accessibility of these natural medicines.Maampisin Chooranam (MC) is novel Siddha formulation comprises of several bioactive components listed in the Siddha text for various ailments in humans. As per the safety regulations drug should prove its short and long term safety in rodents before extrapolating its clinical benefits in humans.Present investigation aimed at evaluating the safety profile of the test drug MC by acute and sub-acute oral toxicities in both male and female wistar rats in accordance with OECD regulatory guidelines.In the acute study, a single dose of 2000 mg/kg was orally administered and animals were monitored for 14 days. In the sub-acute study, repeated doses (20, 100 and 200 mg/kg/day) of the test drug MC were administered for 28 days and biochemical, hematological and histopathological parameters were evaluated.Results of the present investigation showed that there was no sign of toxicity and no mortality after single and repeated administration of *the test drug MC at varying doses in tested rats.* There was no significant difference in mean body weight, food/water intake, behavioral, C.N.S, C.V.S, A.N.S vitalsin control and test group rats.Further most of the biochemical, hematological and serological observation showed normal levels in both male and female rats. No significant pathological difference was observed in the histological examination of brain, heart, lungs, liver and kidney tissues of rats treated with highest dose of MC.Single and repeated oral administration of the siddha drug MCmay be safe and considered as relatively non-toxic at the varying doses of 20, 100 and 200 mg/kg/day dose level.It was concluded from the results of the present investigation that the acute or sub-acute oral administration of the test drug MC is considerably safe and doesn't alter any of the physiology in the rats.

Keywords: Siddha medicines, Maampisin Chooranam, OECD, Acute , Sub-acute, Biochemical, Hematological, Serological, Histological examination.

1. Introduction

It is a well-known fact that Traditional Systems of medicines always played important role in meeting the global health care needs. They are continuing to do so at present and shall play major role in future also. The system of medicines which are considered to be Indian in origin or the systems of medicine, which have come to India from outside and got assimilated in to Indian culture are known as Indian Systems of Medicine . India has the unique distinction of having six recognized systems of medicine in this category. They are-Ayurveda, Siddha, Unani and Yoga, Naturopathy and Homoeopathy. Though Homoeopathy came to India in 18th Century, it completely assimilated in to the Indian culture and got enriched like any other traditional system hence it is considered as part of Indian Systems of Medicine. Apart from these systems- there are large number of healers in the folklore stream who have not been organized under any category [1]. Siddha System of medicine is practiced in some parts of South India especially in the state of Tamilnadu. It has close affinity to Ayurveda yet it maintains a distinctive identity of its own. This System has come to be closely identified with Tamil civilization. The term '*Siddha*' has come from '*Siddhi*'- which means achievement. *Siddhars* were the men who achieved supreme knowledge in the field of medicine, yoga or *tapa* (meditation) [2].

Toxicology is the study of the adverse effects of chemical or physical agents on living organisms. A toxicologist is trained to examine and communicate the nature of those effects on human, animal, and environmental health. Toxicological research examines the cellular, biochemical, and molecular mechanisms of action as well as functional effects such as neurobehavioral and immunological, and assesses the probability of their occurrence. Fundamental to this process is characterizing the relation of exposure (or dose) to the response. Risk assessment is the quantitative estimate of the potential effects on human health and environmental significance of various types of chemical exposures (eg, pesticide residues in food, contaminants in drinking water). The variety of potential adverse effects and the diversity of chemicals in the environment make toxicology a broad science, which often demands specialization in one area of toxicology. Our society's dependence on chemicals and the need to assess potential hazards have made toxicologists an increasingly important part of the decision-making processes. Regulatory requirements have strongly recommending the need of toxicological profiling of medicinal preparations before intended to use in humans for clinical efficacy. Hence it becomes highly essential for a drug to prove its safety and efficacy before prescribing the same for clinical application. The main aim of the present research work is to evaluate the short and long term safety of the Siddha formulation Maampisin Chooranam by acute and sub-acute toxicity studies in rodent.

2. Materials and Methods

2.1. Animal

Healthy adult Wistar albino rats were used for the study. The animals were housed in poly propylene cages and were kept in well ventilated with 100% fresh air. A 12 light / dark cycle were maintained. Room temperature was maintained between 22 $\pm$ 2^{0}Cand relative humidity 50–65%. They were provided with food (Sai feeds, Bangalore, India) and water *ad libitum*. All the animals were acclimatized to the laboratory for 7 days prior to the start of the study. The experimental protocol was approved by The Institutional Animal Ethics Committee of C.L.Baid Metha College of Pharmacy, Chennai, Tamil Nadu, India with the IAEC approval number: LI/15/CLBMCP/2017

2.2.Acute toxicity Study

The animals were randomly divided into control group and treatment groups of 6 female wistar albino rats of 3 in each group .The animals were fasted overnight (12- 16 hrs) with free access to water. Group I served as control and the study was conducted with single oral administration of study drug *Maampisin Chooranam* (MC) 2000mg/kg (p.o) to group II rats. The animals were observed continuously for first 72 h and then 14 days for emerging signs of behavioral changes, body weight changes and for mortality.

Occurrence of toxicity in animals were observed continuously for the first 4 to 24 hr and observed periodically for the next 14 days. Observation includes the change in skin, fur, eyes and mucus membrane. Appearance of C.N.S,C.V.S and A.N.S related toxicity such as tremors, convulsions, sedation, steric behavior, respiratory distress, cardiovascular collapse, response to sensory stimuli, salivation, diarrhea, lethargy, sleep, coma and mortality were observed with special attention [3].

Body weight was recorded periodically. At the end of the experiment all animals were subjected for gross necropsy and observed for pathological changes.

2.3.Sub-Acute toxicity Study

The animals were randomly divided into control group and drug treated groups 48 wistar albino rats (24 male and 24 female) were selected and divided into 4 groups. Each group consist of 12 animals (6 Males and 6 Females). First group served as a control and other three group were treated with test drug MC (20, 100 and 200 mg/kg/day) for 28 days. Animals were allowed acclimatization period of 7 days to laboratory conditions prior to the initiation of treatment. The female rats were nulliparous and non-pregnant.

The rats were weighed periodically and observed for signs of toxicity pertains to C.N.S, C.V.S, A.N.S including behavioral changes, food - water intake and morphological changes. At the end of 28^{th} day, the animals were fasted for overnight with free access to water. On 29^{th} day the animals were sacrificed with excess anesthesia. Blood samples were collected from aorta and stored in EDTA (ethylenediamine –tetra acetate) for Hematological analysis and for serum generation for biochemical analysis. The vital organs were harvested and carefully examined for gross lesions. The organs were preserved in 10% formalin for histopathological assessment and interpretation [4].

2.4.Hematological analysis

Blood samples were analyzed using established procedures and automated Bayer Hematology analyzer. Parameters evaluated include Red Blood Cells (RBC) count, White blood cell count (WBC), Platelet Count, Hemoglobin (Hb), Packed Cell Volume (PCV), Mean cell Haemoglobin Concentration (MCHC), Mean Red Cell Volume (MCV), Mean Cell Hemoglobin (MCH), Mean platelet volume (MPV), Neutrophils, Eosinophil's, Basophils, Lymphocytes and Monocytes using hematological analyzer.

2.5. Biochemical analysis [9]

Serum samples were analyzed for Bilirubin, Aspartate Transaminase (AST), Alanine amino Transaminase (ALT) and Alkaline Phosphatase (ALP), High Density Lipoprotein (HDL), Low density Lipoprotein (LDL) , Very low density Lipoprotein (VLDL) , Triglycerides (TGL), Total Cholesterol ,Total protein, Urea, Blood urea nitrogen (BUN), Creatinine, Albumin, Total Protein, Glucose, Uric acid, using auto analyzer.

2.6.Histopathological evaluation [5]

Vital organs were harvested and the histological slides of organs were made and observed under the microscope. The pathological observations of cross section of these organs were performed on gross and microscopic bases. Histological examinations were performed on the preserved tissues with particular emphasis on those which showed gross pathological changes.

2.7.Statistical analysis[6]

The statistical analysis will be carried by one way ANOVA (GRAPH PAD PRISM 5 computer program). Results were expressed as mean ± standard error.A statistical comparison was carried out using the Dunnet's test for the control and treatment group. P-values less than 0.05 were set as the level of significance.

3. Results

3.1. Effect of MC on clinical signs of rats in Acute Oral Toxicity Study

The dose of MC used for acute toxicity study is 2000mg/kg is higher than the normal therapeutic dose. No mortality observed at this dose level, further no significant change with respect to clinical signs on acute toxicity observed for (24-48 h) and a long period (14 days). The results were tabulated in Table 1.

Table 1:Effect of Maampisin Chooranam on clinical signs in Acute Oral Toxicity Study

SN	Group Control	Observation	SN	Group 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

3.2.Effect of MC on Body weight of rats in acute toxicity study

No significant change was observed in body weight of female rats treated with MC at the dose of 2000mg/ kg. The results were tabulated in Table 2.

Table 2:Effect of Maampisin Chooranam on Body weight of rats in acute toxicity study

Dose	Days		
	1	7	14
Control	166.6± 1.95	168.2± 4.82	169.2 ± 3.12
MC 2000 mg/kg	172.3± 2.18	174.2± 1.26	177.2 ± 3.27
P value (p)*	NS	NS	NS

3.3.Effect of MC on Body weight rats in Sub-acute oral toxicity study.

No significant toxicity was observed in rats during the 28 consecutive days of treatment via oral route with MC at low, mid and high dose of 20, 100 and 200 mg/ kg b.w. The results were tabulated in Table 3.

Table 3:Effect of Maampisin Chooranam on Body weight of rats in Sub-acute toxicity study

Dose	Days				
	1	7	14	21	28
Control	172.0± 4.23	172.4 ± 3.42	174.7 ± 1.36	174.2 ± 1.33	175.7 ± 1.31
Low dose	171.2 ± 3.12	172.7 ± 4.64	175.4± 3.18	175.8 ± 1.86	176.12± 2.36
Mid dose	178.6± 1.34	179.3 ± 2.14	180.4 ± 6.32	182.1 ± 3.16	183.7 ± 3.12
High dose	187.4± 8.14	189.6 ± 3.12	189.6 ± 2.16	190.0± 6.21	191.92 ± 2.19
P value (p)*	NS	NS	NS	NS	NS

NS- Not Significant, **(p > 0.01),*(p >0.05), n = 12 values are mean ± S.D (One way ANOVA followed by Dunnett's test)

3.4.Effect of MC on food and water intake of rats in Sub-acute oral toxicity study.

No significant change was observed in body weight of both male and female rats treated with MC at low, mid and high dose of 20, 100 and 200 mg/ kg b.w. The results were tabulated in Table 4 and 5.

Table 4:Effect of Maampisin Chooranam on food intake of rats in Sub-acute toxicity study

Dose	Days				
	1	7	14	21	28
Control	29.12 ±5.37	28.5±4.22	29.5±4.27	32.5±3.87	33.12±6.32
Low dose	33.7±4.98	34.3±1.22	33.1±6.18	35.4±6.12	35.6±2.12
Mid dose	32.2±4.75	33.2±6.80	37.2±1.25	33.4±2.68	32.2±1.44
High dose	32.2±2.34	32.2±2.64	34.6±2.16	36.2±3.14	37.0±1.62
P value (p)*	NS	NS	NS	NS	NS

NS- Not Significant, **(p > 0.01),*(p >0.05), n = 12 values are mean ± S.D (One way ANOVA followed by Dunnett's test)

Table 5:Effect of Maampisin Chooranam on water intake of rats in Sub-acute toxicity study

Dose	Days				
	1	7	14	21	28
Control	51.5 ± 7.15	50.0 ± 8.23	58.5± 6.63	49.12±7.19	51.5±3.96
Low dose	38.5±3.41	39.4±3.62	39.27±4.12	38.2±3.29	39.9±3.13
Mid dose	36.7±4.13	36.3±2.21	37.1±4.13	38.4±6.31	38.4±3.34
High dose	32.1±1.32	33.2±4.13	34.7±3.13	32.2±1.73	30.4±2.65
P value (p)*	NS	NS	NS	NS	NS

NS- Not Significant, **(p > 0.01),*(p >0.05), n = 12 values are mean ± S.D (One way ANOVA followed by Dunnett's test)

3.5.Effect of MC on Hematological parameters of rats in Sub-acute oral toxicity study

No statistically significant differences were recorded in hematological parameters of rats treated with MC at low, mid and high dose of 20, 100 and 200 mg/ kg b.w.. The results were tabulated in Table 6.

Table 6: Haematological parameters of rats exposed to Maampisin Chooranam

Category	Control	Low dose	Mid dose	High dose	P value (p)*
Haemoglobin(g/dl)	14.8±1.88	13.1±3.16	13.64±3.66	14.28±0.96	N.S
Total WBC ($\times10^3$ l)	10.91±2.59	10.25±6.73	11.28±2.31	11.40±6.14	N.S
Neutrophils(%)	32.65±1.06	32.13±4.14	33.11±1.46	34.40±3.20	N.S
lymphocyte (%)	69.34±2.48	70.16±6.12	71.58±4.66	74.13±4.16	N.S
Monocyte (%)	0.78±0.17	0.76±0.04	0.80±0.13	0.83±0.36	N.S
Eosinohil(%)	0.64±0.09	0.64±0.16	0.75±0.43	0.73±0.14	N.S
Platelets cells10^3/µl	687.17±8.76	722.71± 2.16	705.18± 2.0	735.16± 3.14	N.S
Total RBC 10^6/µl	7.99±0.12	6.82±1.37	7.12±1.89	7.18±7.72	N.S
PCV%	37.79±0.6	41.35±8.13	42.18±1.68	43.82±2.54	N.S
MCHC g/dL	33.6±2.23	32.19±5.29	33.18±4.22	32.93±1.24	N.S
5MCV fL(µm^3)	49.17±3.64	48.29±1.22	50.18±1.24	50.94±1.44	N.S

N.S- Not Significant, **(p > 0.01), *(p >0.05), n = 12 values are mean ± S.D (One way ANOVA followed by Dunnett's test)

3.6.Effect of MC on Biochemical parameters of rats in Sub-acute oral toxicity study

No statistically significant differences were recorded in biochemical parameters of rats treated with MC at low, mid and high dose of 20, 100 and 200 mg/ kg b.w. The results were tabulated in Table 7 -9.

Table 7: Biochemical parameters of rats exposed to Maampisin Chooranam

Biochemical Parameters	Control	Low dose	Mid dose	High dose	P Value (p)*
Glucose (R) (mg/dl)	76.45±13.4	76.16±2.34	75.26±2.20	77.42±2.64	N.S
T.Cholosterol(mg/dl)	115.26±1.83	109.45±4.13	118.42±4.78	123.22±3.73	N.S
Trigly(mg/dl)	46.35±1.48	44.22±1.48	48.58±1.30	47.66±3.33	N.S
LDL	72.81±2.13	76.24±8.14	74.8±2.14	70.64±4.32	NS
VLDL	15.2±2.44	14.42±4.64	14.04±1.64	13.94± 1.46	NS
HDL	26.66±6.88	23.86±6.24	26.10±2.66	30.68±1.12	NS
Ratio 1(T.CHO/HDL)	4.42±2.44	4.46±3.14	4.64±2.14	4.18 ± 2.12	NS
Ratio 2(LDL/HDL)	2.83±4.22	2.14 ± 2.22	2.28± 2.20	2.16±6.22	NS
Albumin(g/dL)	3.63±0.17	3.18±0.42	3.16±2.62	2.94±4.16	NS

NS- Not Significant,**(p > 0.01), * (p >0.05), n = 12 values are mean ± S.D (One way ANOVA followed by Dunnett's test)

Table 8: Renal function test of rats group exposed to Maampisin Chooranam

Parameters	Control	Low dose	Mid dose	High dose	P Value (p)*
Urea (mg/dl)	13.35±0.99	13.14±0.16	12.96±1.98	12.28±3.62	N.S
Creatinine(mg/dl)	0.28±0.08	0.36±0.06	0.52±0.04	0.66±0.02	N.S
BUN(mg/dL)	15.02±0.10	14.28±1.92	14.09±1.34	14.02±4.32	NS
Uric acid(mg/dl)	5.17±0.35	5.01±1.03	5.12±3.15	4.58±1.33	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 9: Liver Function Test of rats exposed to Maampisin Chooranam

Parameters	Control	Low dose	Mid dose	High dose	P Value (p)*
T Bilirubin (mg/dl).	0.48±0.07	0.40±1.26	0.41±3.28	0.39±1.25	N.S
SGOT/AST(U/L)	79.95±1.39	76.15±1.31	77.31±3.03	79.25±4.03	N.S
SGPT/ALT(U/L)	31.23±1.28	32.91±3.59	36.24±7.48	34.12±1.68	N.S
ALP(U/L)	143.25±8.70	146.12±1.37	143.16±4.17	145.33±1.65	NS
T.Protein(g/dL)	5.32±0.38	5. 22±1.14	6.01±3.23	6.93±1.46	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)

3.7. Effect of MC on Histopathological changes of rats in Sub-acute oral toxicity study

No abnormality were detected in the histopathological analysis of organs (Brain, Lung, Heart, Kidney and Liver) retrieved from the rats treated with MC at high dose of 200 mg/ kg b.w. The results were illustrated in figure 1 and 2.

Figure 1: Histopathological representation organs of control group rats in Sub-acute oral toxicity study

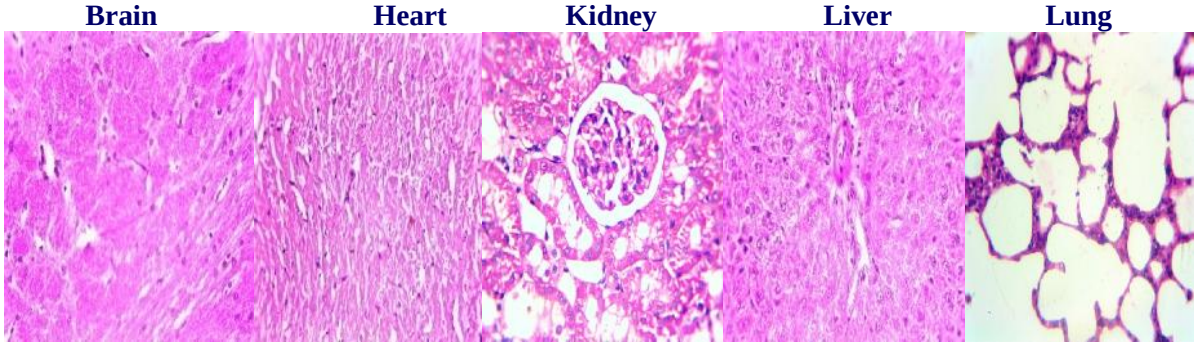
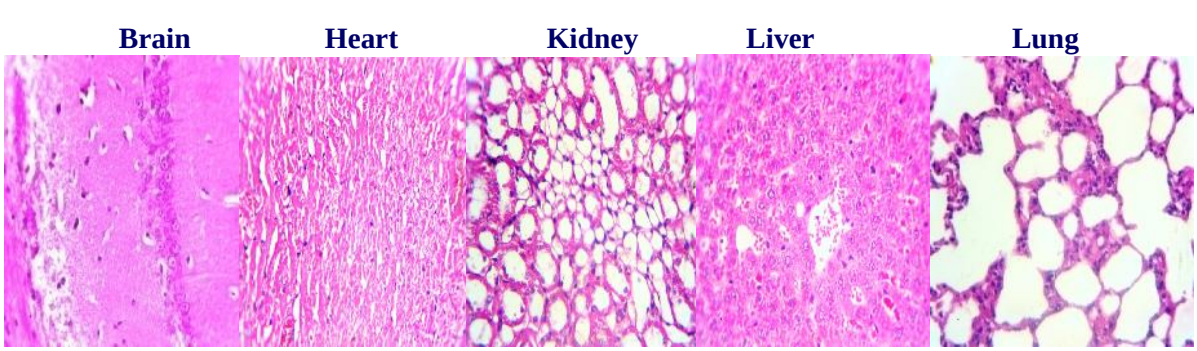


Figure 2: Histopathological representation organs of MC treated rats in Sub-acute oral toxicity study



4. Discussion

Safety is of the highest concern for a drug dealing with human lives, Even WHO also strongly insisting on safety rather than efficacy. It was roughly estimated that about 10,000 molecules have identified each year but of which only one come to the market after clearing preclinical and clinical evaluations. The reason why 99% of the drug fails is because of undesirable toxic effects encountered by the drug in preclinical and also in clinical level. The ultimate aim of the toxicity study is to establish the safety margin of the drugs in rodents as the siddha preparations being prescribed widely to the larger category of people since several years it become regulatory essential for the researcher to justify the safety in humans and animals as well.

Toxicity studies on Siddha formulations are commonly used to evaluate the possible health risk of the intrinsic chemical compounds in the preparation which could result in adverse effects from the plant [8]. Specifically, acute toxicity and LD_{50} determination have been described as initial steps in the toxicological evaluations of the herbal medicines, and data from such evaluations provide comprehensive information on the toxicological classification of such traditional medicines [9].

Acute toxicity study was carried out in accordance with OECD guideline 423 by which the test drug Maampisin Chooranam administered at the dose of 2000mg/kg. Results of the study revealed that there is no mortality in the treated rats after post administration period of 14 days. Further there is no significant change in any of the observed parameters like body weight, food intake, water intake, social behavior, sensory and motor coordination, muscle strength, exploratory behavior etc.Sub-acute studies provide information on dosage regimens, target organ toxicity, and identify observable adverse effect that may affect the average life span of experimental animals. Consequently, in this study, the Siddha drug MC was evaluated in rats at doses of 20,100, and 200 mg/kg for 28 days. A drastic change in body weight is a critical evaluator of toxicity and may serve as a sensitive indication of the general wellbeing of animals [10,11]. The mean body weight gained by the animals in all the treatment groups may be an indication that the test drug MC did not interfere with their normal metabolism as closely supported by the non-significant difference in this parameter when compared with the control group. The increase in body weight could be attributed to the nutritive components in their feed and the palatability of the test drug MC.
Investigation on the haematological parameters can be used to determine the extent of the deleterious effect of foreign compounds in plant extracts on the blood constituents of an animal [12,13]. RBC and HGB counts could be an indication of toxicity appears in the blood. This implies that the morphology and osmotic fragility of the RBC, as well as HGB incorporation into the RBC. This index suggest that the oxygen-carrying capacity of the blood and amount of oxygen delivered to the tissues following treatment with the test drug MC intact. In the present study treatment with MC at three dose level 20, 100 and 200 mg/kg reveals no significant change in any of the hematological parameters like Red Blood Cells (RBC) count, White blood cell count (WBC), Platelet Count, Hemoglobin (Hb), Packed Cell Volume (PCV), Mean cell Haemoglobin Concentration (MCHC), Mean Red Cell Volume (MCV), Mean Cell Hemoglobin (MCH), Mean platelet volume (MPV), Neutrophils, Eosinophil's, Basophils, Lymphocytes and Monocytes [14].

Impaired hepatocellular function may lead to a reduction in serum concentrations of albumin, total protein and bilirubin. There is no significant change in serum concentrations of total protein, albumin and bilirubin in the treated and control groups further suggests that the synthetic functions of the liver is not altered at any of the test doses of MC.Data analysis of animals' blood biochemistry parameters can be translated for risk evaluation in humans, since changes in serological system have a higher predictive value for human toxicity. Sub-acute toxicity studies also showed no significant changes in the serological parameters between control and treated groups. Transaminases (AST and ALT) and ALPs are generally used as indices for liver and kidney damage respectively. No significant change was found in serum levels of AST, ALT, and ALP enzymes level of rats treated with MC at the dose of 20, 100 and 200 mg/kg. Test drug MC therefore did not provoke any detrimental effect on liver and kidney.

Besides complementing biochemical investigations, histological examination of organs following exposure to pharmacological agents is an important consideration in assessing the safety of such agents on organ injury [15]. Hence, the apparently preserved histoarchitectural features as evident from microscopic examinations. Arrangement of the neurons appears intact with no signs of degeneration was observed in sample. Myocardial fiber mass appears denser with no

signs of degeneration or fibrosis were observed in samples. Appearance of portal triad was normal with no signs of inflammatory cell infiltration. Liver parenchyma appears normal. No evidence of necrosis was observed in samples. Appearance of proximal and distal convolutes tubules was normal with no evidence of atrophy. Microscopic examination of lung revealed normal alveoli and alveolar sac with no signs of infiltration. Hence, the apparently preserved histoarchitectural features as evident from microscopic examinations of the brain, heart, lung, kidneys and liver sections of the MC drug treated animals in this study are another supportive fact that the organs were void of injuries and further indicate that *test drug MC*was not toxic to them at the tested doses. Furthermore, the no treatment-induced infiltration and inflammation as shown in the microscopic examination of the organs from MC treated groups are also supportive of its capability to maintain and sustain histoarchitectural integrity of the organs.

5. Conclusion

Overall, it is evident from the present study that Siddha formulation Maampisin Chooranam is well tolerated in acute toxicity study at the dose of 2000 mg/kg b.w. in Wistar rats. Following its 28-day repeated daily oral dose administration in the animals, it may be concluded that test drug MC does not elicit any treatment-related adverse effect with respect to hematological, serological and histopathological observation at the doses investigated and thus may be classified to be relatively safe and practically nontoxic for consumption.

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