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Acute and Sub-Acute Toxicity Study of Siddha Polyherbal Formulation – Maha Manjishtathi Kashayam

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ABSTRACT

Medicines obtained from natural sources have become the basis for pharmaceutical drugs. Traditional herbal medicines are naturally occurring plant-derived substances, these have been used for treatment and cure of various diseases. Toxicological research and testing help to live safely and predict benefit from synthetic and natural substances while avoiding harm. The toxicity study is done for data profiling and safety of the herbal drugs, the toxicity study of various plant and herbal formulations are reported. Study aimed to assess the acute and sub-acute toxicity of the herbal formulation *Maha manjishtathi kashayam*. In the acute arm of the study, a single dose of 2000 mg/kg was administered to Wistar Albino mice which were observed for physical symptoms and behavioral changes for 24hrs. In a sub-acute toxicity study repeated doses of the herbal preparation was administered to Wistar rats of both genders, separately. The animals received three doses herbal product (50 mg/kg/day, 100 mg/kg/day and 200 mg/kg/day) for a period of 28 days. On 28th day of experiment, blood sampling of animals was done for hematological and biochemical analysis i.e. liver and renal function parameters, lipid profile and then sacrificed for histopathological examination of liver and kidney.

INTRODUCTION:

The natural wealth of India includes many herbs having medicinal property. There are several kinds of the literature shows that India has been using traditional medicines since ancient times. Three major traditional systems exist in India using medicinal plants are Ayurveda, Unani and Siddha. There is a common thread running through this system is their fundamental principal and practices. Indian materia medica includes about 2000 drugs of natural origin which are derived from different traditional systems and traditional practices [1]. Herbal medicines have attained widespread acceptability as natural therapeutic agents for various diseases like diabetes, arthritis, renal and liver diseases, obesity, and cardiovascular disorders [2].

The medicinal uses of *Maha manjishtathi kashayam* is in the Siddha literature (Agasthiyarpilaitamil). But to our knowledge, there is yet no record in the literature of the toxicity profile of this medicine preparation. Acute and sub-acute toxicity data may be required to predict the safety or otherwise of long-term low-dose exposure. The present investigations were therefore carried out in Baidmehtha college of Pharmacy laboratory to determine the acute and sub-acute toxicity profile of MMK in rodents.

METHODS:

SELECTION OF DRUGS:

I have selected the trail drug **"*MAHA MANJISHTATHI KASHAYAM*"**(Internal) for the study from classical Siddha literature **"*AGASTHIYARVAIDHIYA PILLAI TAMIL*"**

The raw drugs were procured from the raw drug shop R.N.RAJAN & CO, Chennai. After proper authentication by the Pharmacognosist, Siddha central research institute, Arumbakkam, Chennai-106.

My CTRI number is CTRI/2018/05/013686.

- Manjitti-*Rubiacordifolia*-35gms
- Kadukaithol-*Terminaliachebula*-35gms
- Thandrikai-*Terminaliabellarica*-35gms
- Nellimulli-*Phyllanthusemblica*-35gms

- Kadugurohini-*Picrorhizascrophuleriflora*– 35gms
- Vasambu-*Acoruscalamus*-35gms
- Maramanjal -*Cosciniumfenestratum*-35gms
- Veppammarapattai-*Azadirectaindica*-35gms
- Seenthilkodi -*Tinospora cordifolia*- 35gms

STANDARD OPERATING PROCEDURE FOR MAHA MANJISHTATHI KASHAYAM:

1. All ingredients are dried and coarsely powdered.
2. Powder is added to in3.5litres of boiling water and heated.
3. Reduced to1.5 liters and made askashayam.
4. After cooling, pour it into a sterilized container or bottle.

DOSAGE: 30ml, twiceadayfor48days.

INDICATION: All kinds of "KUTTAM".

EXPERIMENTAL ANIMALS:

Young healthy Wistar albino mice (both sexes), 6-8 weeks old, weighing about 150-200gm were used in this study. The animals were purchased from TANUVAS, Madhavaram, and Chennai. All the animals were kept under standard environmental condition (22±3°C). The animals had free access to water and a standard pellet diet (Sai meera foods, Bangalore).

Preparation of animal:

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. Animals were acclimatized to laboratory environment for a week before start study. The protocol used in this study was approved by the Animal Ethical Committee of C.L Baidmehtha College of pharmacy, Chennai.

(IAEC approved number is 1248/AC/09/CPCSEA-9/DEC-2013/12)

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 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 + 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, and 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 conditions (22±3°C). The animals had free access to water and a standard pellet diet (Sai meera foods, Bangalore).

Preparation for Acute Toxicity Studies

Rats were deprived of food overnight (but not water 16-18 h) before administration of the, *MAHA MANJISHTADHI KASHAYAM*.

Administration of Doses:

MAHA MANJISHTADHI KASHAYAM was suspended in coconut water and administered to the two 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 before 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, and aggression, 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 monitored for a further 14 days and observations made daily. The toxicological effect was assessed based on 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 the 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, diarrhea, lethargy, sleep and coma. The principles and criteria summarized in the Humane Endpoints Guidance Document are taken into consideration. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress were humanly killed. When animals are killed for human reasons or found dead, the time of death was recorded.

Acute oral toxicity study of MAHA MANJISHTADHI KASHAYAM

Table No. 1: Dose finding experiment and its behavioral Signs of acute oral Toxicity

Observation done:

SL	Group CONTROL	Observation	SL	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	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	7	Change in skin	No significant color
8	Piloerection	Normal	8	Piloerection	Normal
9	Defecation	Normal	9	Defecation	Normal
10	Sensitivity response	Normal	10	Sensitivity	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

Behavior:

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:

The 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 were calculated for the control and the treated dose groups.

Mortality:

Animals were observed for mortality throughout the entire period.

Results:

All data were summarized in tabular form, (Table-1-4) 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.

No of animals in each group:3

Table No. 2: (Observational study Results)

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 No. 3.1: Body weight Observation

DOSE	**DAYS**		
	1	7	14
CONTROL	200.1±65.70	201.3 ± 41.11	201.6 ±02.12
HIGH DOSE	202.3± 6.64	202.7 ±7.42	203.2 ± 2.70
P value (p)*	NS	NS	NS

Table No. 3.2: (Water intake (ml/day) of Wistar albino rats group exposed to *MMK*

DOSE	**DAYS**		
	1	6	14
CONTROL	54 ± 3.20	54±6.10	54.3±5.44
HIGH DOSE	53.5±1.30	53.8±6.70	54.2±5.64
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 No. 4: Food intake (gm/day) of Wistar albino rats group exposed to *MMK*

DOSE	**DAYS**		
	1	7	14
CONTROL	56.03±2.82	56.2±2.96	57.7±8.86
High DOSE	58.6±5.44	58.4±5.20	59.8±6.67

REPEATED DOSE 28-DAY ORAL TOXICITY (407) STUDY OF MMK

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 was treated as a control and other three group were treated with test drug (low, mid, high) for 28 days. Animals were allowed an 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 guidelines three dose levels were selected for the study. They are low dose (X), mid dose (5X), high dose (10X). X is calculated from the acute toxicity dose 2000 mg and the X dose is (20mg/kg), 5X dose is (100mg/kg), 10X dose is (200mg/kg).

Table No. 5:

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

Preparation and Administration of Dose:

MAHA MANJISHTADHI KASHAYAM suspended in with water, It was administered to animals at the dose levels of X, 5X, 10X. 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 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 were calculated for the 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 the 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 overnight. Blood samples were collected from orbital sinus using sodium heparin (200IU/ml) for Bio chemistry and potassium EDTA (1.5 mg/ml) for Hematology as an anticoagulant. Blood samples were centrifuged at 3000 r.p.m. for 10 minutes.

Hematological Investigations:

Haematological parameters were determined using Haematology analyzer.

Biochemical Investigations:

Biochemical parameters were determined using an auto-analyzer.

RESULTS

Repeated Dose 28- day oral toxic study of MAHA MANJISHTADHI KASHAYAM

Table No. 6: Body weight of Wistar albino rats group exposed to *MMK*

DOSE	DAYS				
	1	7	14	21	28
CONTROL	242.4±10.40	243.2 ± 15.04	243.4 ± 15.40	244.6± 16.50	244.2 ± 16.10
LOW DOSE	240.5 ± 55.25	241.7 ± 16.29	241.8± 15.24	242 ±16.30	242.8± 46.06
MID DOSE	248.3± 14.72	248.3 ± 22.20	248.4 ± 17.42	249.2 ± 35.08	249.4 ± 34.10
HIGH DOSE	251.3± 23.51	251.7±33.07	252.4 ± 32.34	253 ± 4.08	253 ± 7.70
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 No. 7: Water intake (ml/day) of Wistar albino rats group exposed to *MMK*

DOSE	DAYS				
	1	6	14	21	28
CONTROL	51.3 ± 3.54	51.4±1.27	51.7±1.31	52.1±1.12	52.4±1.72
LOW DOSE	65.1±1.21	65.6±4.22	66.6±1.02	65.2±2.06	66.4±1.20
MID DOSE	62.1±1.02	62.3±1.21	62.1±2.62	63.4±4.32	63.4±1.64
HIGH DOSE	53.6±6.80	53.2±1.52	53.4±1.74	54.6±1.88	54.8±2.82
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 No. 8: Food intake (gm/day) of Wistar albino rats group exposed to *MMK*

DOSE	DAYS				
	2	7	23	22	28
CONTROL	42±5.21	42.2±4.22	42.8±3.13	43.2±6.72	44±6.80
LOW DOSE	43.6±6.22	43.8±2.42	44.4±1.50	44.5±1.30	44.8±1.12
MID DOSE	44.1±6.70	44.2±2.40	44.6±5.64	45.3±2.40	45.7±1.34
HIGH DOSE	46.4±1.45	46.6±1.34	46.8±2.36	47.2±1.70	47.6±1.62
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 No. 9: Haematological parameters of Wistar albino rats group exposed to *MMK*

Category	Control	Low dose	Mid dose	High dose	P-value (p)*
Haemoglobin(g/dl)	14.5±0.43	14.60±0.32	14.8±0.23	14.84±0.33	N.S
Total WBC (×10^3 l)	12.71±0.40	12.82±0.21	12.94±0.60	13.06±1.40	N.S
Neutrophils (%)	08.12±0.40	08.22±0.32	08.31±1.50	08.04±2.20	N.S
lymphocyte (%)	90.12±1.60	90.14±1.40	90.16±1.44	91.20±1.64	N.S
Monocyte (%)	0.1±0.02	0.1±0.01	0.1±0.04	0.1±0.03	N.S
Eosinophil (%)	0.02±0.02	0.02±0.04	0.02±0.06	0.02±0.06	N.S
Platelets cells10^3/µl	700.26±2.28	702.32±2.42	702.21±2.60	702.42±3.64	N.S
Total RBC 10^6/µl	7.64±0.32	7.65±0.32	7.65±0.04	7.66±0.06	N.S
PCV%	40.30±0.4	40.32±5.30	40.5±2.70	41.2±1.22	N.S
MCHC g/dL	34.7±1.61	34.8±1.32	34.8±1.35	34.13±1.36	N.S
MCV fL(µm^3)	52.7±3.04	52.7±2.40	52.9±2.20	52.9±1.20	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 No. 10: Biochemical Parameters of of Wistar albino rats group exposed to *MMK*

BIOCHEMICAL PARAMETERS	CONTROL	LOW DOSE	MID DOSE	HIGH DOSE	P Value (p)*
GLUCOSE (R) (mg/dl)	98.10±2.40	98.12±1.62	99.9±.08	99.9±5.25	N.S
T.CHOLESTEROL(mg/dl)	109.14±3.10	109.25±2.40	109.30±1.58	110.21±1.60	N.S
TRIGLY(mg/dl)	73.05±1.08	73.11±1.02	73.25±1.42	75.26±1.54	N.S
LDL	68.5±4.13	68.4±1.05	68.3±1.03	69.40±2.44	NS
VLDL	15.2±1.30	15.20±1.71	15.22±1.62	15.24±1.55	NS
HDL	25.22±2.30	25.22±2.60	25.46±1.72	26.56±1.43	NS
Ratio 1(T.CHO/HDL)	4.36±1.10	4.37±1.20	4.64±2.32	4.74±2.63	NS
Ratio 2(LDL/HDL)	2.76±2.33	2.72±1.40	2.79±2.10	2.84±04.02	NS
Albumin (g/dL)	3.9.42±0.50	3.9.62±0.54	3.9.48±4.20	4.02±3.24	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 No. 11: Renal function test of of Wistar albino rats group exposed to *MMK*

PARAMETERS	CONTROL	LOW DOSE	MID DOSE	HIGH DOSE	P Value (p)*
UREA (mg/dl)	24.31±0.10	24.30±0.19	24.26±1.28	25.42±1.02	N.S
CREATININE(mg/dl)	0.7±0.04	0.71±0.06	0.73±0.04	0.74±0.08	N.S
BUN(mg/dL)	15.8±0.04	15.8±0.24	15.8±0.42	15.9±1.02	NS
URIC ACID(mg/dl)	5.04±0.02	5.08±0.20	5.4±0.32	5.6±0.20	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)

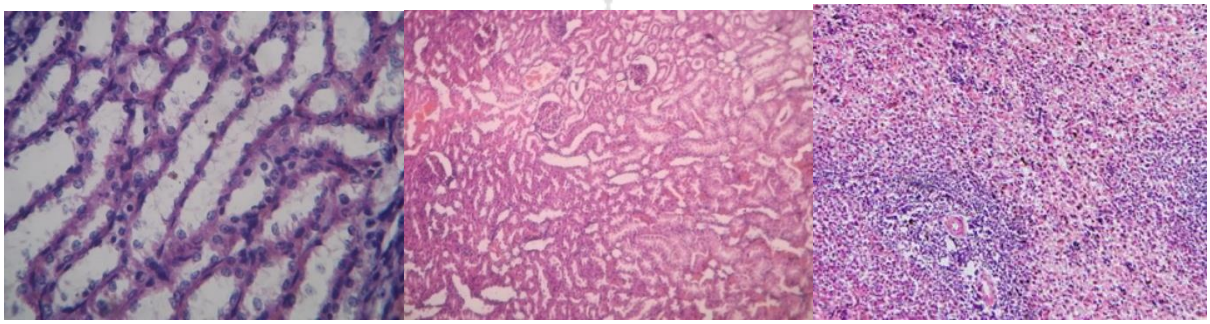
Table No. 12: Liver Function Test of Wistar albino rats group exposed to *MMK*

PARAMETERS	CONTROL	LOW DOSE	MID DOSE	HIGH DOSE	P Value (p)*
T BILIRUBIN(mg/dl).	0.04±0.01	0.04±0.03	0.04±0.03	0.04±0.01	N.S
SGOT/AST(U/L)	51.11±1.43	51.12±0.62	52.24±1.34	53.54±1.63	N.S
SGPT/ALT(U/L)	87.11±1.43	87.24±1.14	88.44±1.36	88.33±0.21	N.S
ALP(U/L)	166.30±2.11	166.1±2.10	166±1.14	167.3±2.01	N.S
T.PROTEIN(g/dL)	6.9±0.14	6.9±0.41	7.00±0.60	7.2±0.41	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.

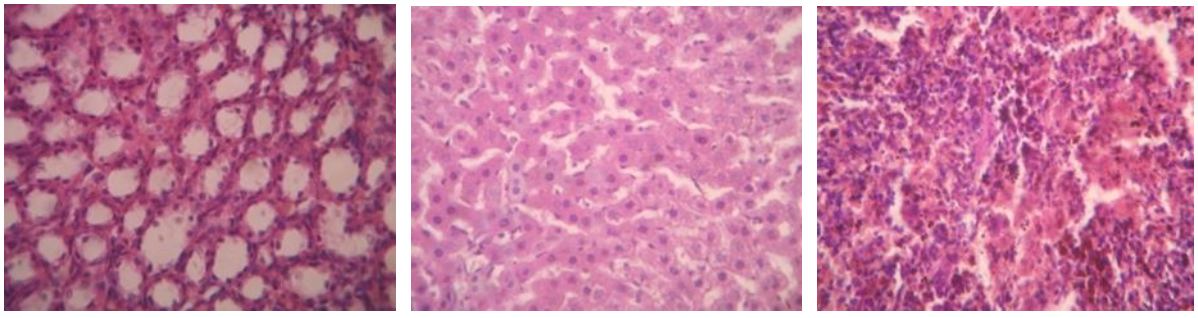
HISTO PATHOLOGYCONTROL GROUP

Kidney Liver Spleen



High dose

Kidney Liver Spleen



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 was sliced 5 or 6µm sections and were dehydrated in an auto technician 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 a microtome and the slides were stained with Haematoxylin-eosin red.

CONCLUSION:

The findings of acute study revealed that this MMK formulation is non-toxic with a single oral dose of 2000 mg/kg/day. The 28 days sub-acute toxicity study, revealed no significant changes with 50 mg/kg/day. Slight changes in biochemical parameters and structural levels were at 100 mg/kg/day and severe cellular changes at 200 mg/kg/day. So, it is concluded that the formulation is safe to use at dose of 50 mg/kg/ day for 28 days whereas the 100 mg/kg/day should be cautiously employed and 200 mg/kg/day should not be recommended. In the future it is recommended to analyze the effects of the individual ingredients on the organs and tissues at different doses.

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