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Toxicity studies of a herbomineral formulation Vasanthakusumakara Mathirai in rats

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Abstract

Siddha system was the ancient system of medicine which was endowment by Siddhars to the world. This system has herbals, metals and minerals preparations. The metals and minerals are toxic in nature. But in Siddha preparation the metals and minerals were purified (Suthi) before preparation as medicine. To evaluate the safety, the toxicological studies were done. Vasanthakusumakara Mathirai was a Siddha herbomineral formulation. This study was done for establish acute and repeated 28 days toxicity. In acute single oral dose there was no abnormal result was observed. In repeated 28 days toxicity study the haematological, biochemical parameter and urine analysis shows that the biological values were within normal laboratory limits. Here the Vasanthakusumakara Mathirai was a safe herbomineral formulation which can be used for many chronic diseases like asthma, tuberculosis. This result conserves the way for the future validation of the drug action.

Keywords: Vasanthakusumakara mathirai, Acute toxicity, Repeated 28 days toxicity, OECD guidelines.

Introduction

Siddha was a traditional system of medicine practising in south India mainly in Tamilnadu. This antique system was flourished by the great Siddhars, who has a legitimized knowledge. Siddha system contains herbals, minerals, metals formulations. For metals and minerals the toxicity study should be done to prove its safety. Toxicological studies were used to justify the drug's safety, dose fixation etc. Now a day, a drug wants to undergo scientific parameters like standardization, toxicity and pharmacological activity to validate worldwide.

Vasanthakusumakara Mathirai a herbomineral preparation used to recover bronchial asthma, tuberculosis, fever etc. Being a herbomineral formulation and long term use for the chronic disease, the drug want to undergoes acute and repeated 28 days toxicological studies.

The acute toxic method was a stepwise procedure with the use of 3 animals of a single sex per step. Depending on the mortality and the moribund status of the animals, on average 2-4 steps may be necessary to allow judgment on the acute toxicity of the test substance. Morbid animals or animals obviously in pain or showing signs of severe and enduring distress shall be humanely killed, and are considered in the interpretation of the test results in the same way as animals that died on 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%.

Materials and Methods

Acute oral toxicity on rats – (OECD-423 guidelines)

Acute toxicity study was carried out as per OECD guideline (Organization for Economic Co - operation and Development, Guideline-423.

The experimental protocol was approved by the institutional ethical committee (IAEC) under CPCSEA (approval no: IAEC/XLIV/22/CLBMCP/2014) by the institution C. L. Biad Metha college of pharmacy, Thuraipakkam, Chennai.

Animal: Healthy wistar albino female rat weighing 200–220 gm

Selection of animal species:

The preferred rodent species was rat, although other rodent species may be used. Healthy young adult animals of commonly used laboratory strain Swiss albino rat were obtained from Animal house of king's institute, Guindy, Chennai. Females should be nulliparous and non-pregnant.

Each animal at the commencement of its dosing should be between 8 and 12 weeks old and its weight should fall in an interval within±20 % of the mean weight of the animals. The studies were conducted in the animal house of C. L. Baid Metha College of pharmacy, Thuraipakkam, Chennai.

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.

Administration of doses

VKM was prepared as per the classical Siddha literature was suspended in 2% CMC with uniform mixing and was administered to the groups of Wistar albino rats. It was given in a single oral dose by gavages using a feeding needle. Animals were fasted prior to dosing. Following the period of fasting, the animals were weighed and then the test substance was administered. After the substance has been administered, food was withheld for a further 3-4 hours. The principle of laboratory animal care was followed. Observations were made and recorded systematically and continuously observed as per the guideline after substance administration.

The visual observations included skin changes, mobility, aggressively, sensitivity to sound and pain, as well as respiratory movements. They were deprived of food, but not water 16–18 hours prior to the administration of the test suspension. Finally, the number of survivors was noted after 24 hours and these animals were then maintained for a further 14 days and observations made daily. The toxicological effect was assessed on the basis of mortality.

Number of animals and dose levels

Since this *VKM* has been under practice for long time and likely to be non-toxic, a limit test at one dose level of 2000 mg/kg body weight will be carried out with 6 animals (3 animals per step).

Duration of Study : 48 hours
Evaluation : 14 Days

Limit test

The limit test was primarily used in situations where the experimenter has information indicating that the test material was likely to be nontoxic, i.e., having toxicity only above regulatory limit doses. A limit test at one dose level of 2000 mg/kg body weight was carried out with three animals per step. The test substance-related mortality was not produced in animals, so further testing at the next lower level need not be carried out.

Observations

- The animals were observed individually after dosing at least once during the first 30mins and periodically during the first 24 hours.
- Special attention: First 1-4 hours after administration of drug, and
- It was observed daily thereafter for a total of 14 days, except when they needed to be removed from the study and killed humanely for animal welfare reasons or are found dead.

a. Mortality

Animals will be observed intensively at 0.5, 2.0, 4.0, 6.0, 12.0, 24.0 and 48.0 hours following drug administration on day 1 of the experiment and daily twice thereafter for 14 days.

b. Body weight

Body weights will be recorded at Day 1, 2, 7 and 14 of the study

c. Cage-side observation

These include changes in skin and fur, eyes and mucous membranes and also respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour patterns. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

d. Gross necropsy

All animals (including those which die during the test period are removed from the study) will be subjected to gross necropsy. Gross necropsy includes examination of the external surface of the body, all orifices, cranial, thoracic and abdominal cavities and their contents, brain, eye, thymus, lungs, heart, spleen, liver, kidneys, adrenals, testes and uterus of all animals

Data and reporting

All the data were summarised in tabular form showing the animals used, number of animals displaying signs of toxicity, the number animals found dead during the test or killed for humane reasons, a description and the time course of toxic effects and reversibility, and necroscopic findings.

Test substance and Vehicle

In order to ensure the uniformity in drug distribution in the medium the suspension was made by mixing *VKM* with 2% CMC solution and it was found suitable for dose accuracy.

Justification for choice of vehicle

The vehicle selected as per the standard guideline was pharmacologically inert and easy to employ for new drug development and evaluation technique [(1)].

Repeated dose 28 days oral toxicity study on rats – (OECD-407 guidelines)

Justification for Dose Selection

The results of acute toxicity studies in Wistar albino rats indicated that *VKM* was non-toxic and no behavioral changes was observed up to the dose level of 2000 mg/kg body weight. On the basis of body surface area ratio between rat and human, the doses selected for the study were100mg/kg, 200 mg/kg and 400 mg/kg body weight. The oral route was selected for use because oral route was considered to be a proposed therapeutic route [(2)].

Preparation and administration of dose

VKM at three doses respectively was suspended in 2 ml of 2% CMC in distilled water. It was administered to animals at the dose levels of 100, 200 and 400 mg/kg. The test substance suspensions were freshly prepared every day for 28 days. The control animals were administered vehicle only. Administration was by oral (gavage), once daily for 28 consecutive days.

Randomization, Numbering and Grouping of Animals

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

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

Body Weight*:*

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

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.

Functional Observations:

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

Laboratory Investigations:

Following laboratory investigations were carried out on day 29 in animal's fasted over-night. Blood samples were collected from orbital sinus using sodium heparin (200IU/ml) for Blood chemistry and potassium EDTA (1.5 mg/ml) for Haematology as anticoagulant. Blood samples were centrifuged at 3000 r.p.m. for 10 minutes. On 28th day of the experiment, 24 hours urine samples were collected by placing the animals in the metabolic cage with free access to tap water but no feed was given.

The urine was free from fecal contamination. Toluene was used as a preservative while collecting the sample. The sediments present in the urine were removed by centrifugation and the collected urine was used for biochemical estimations. On 29th day, the animals were fasted for approximately 18 hours, then slightly anesthetized with ether and blood samples were collected from the retro-orbital plexus into two tubes: one with EDTA for immediate analysis of haematological parameters, the other without any anticoagulant and was centrifuged at 4000 rpm at 4 °C for 10 minutes to obtain the serum. Serum was stored at 20 °C until analyzed for biochemical parameters.

Haematological Investigations:

Blood samples of control and experimental rats was analyzed for hemoglobin content, total red blood corpuscles (RBC), white blood corpuscles (WBC) count and packed cell volume (PCV).

Biochemical Investigations:

Serum was used for the estimation of biochemical parameters. Samples of control and experimental rats were analyzed for protein, bilirubin, urea, BUN, creatinine, triglyceride, cholesterol and glucose levels was carried using standard methods. Activities of glutamate oxaloacetate transaminase/ Aspartate aminotransferase (GOT/AST), glutamate pyruvate transaminase/

Alanine amino transferase (GPT/ALT) and alkaline phosphatase were estimated as per the colorimetric procedure.

Urine analysis:

Urine samples were collected on end of treatment for estimation of normal parameters. The estimations were performed using appropriate methodology.

Necropsy:

All the animals were sacrificed on day 29. Necropsy of all animals was carried out and the weights of the organs including liver, kidneys, spleen, brain, heart, and lungs were recorded. The relative organ weight of each animal was then calculated as follows;

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of animal on sacrifice day (g)}} \times 100$$

Statistical analysis:

Findings such as clinical signs of intoxication, body weight changes, food consumption, hematology and blood chemistry were subjected to One-way ANOVA followed by Dunnet's multi comparision test using a computer software programme GRAPH PAD INSTAT-3 version.

Results

Acute oral toxicity in rats

Dose finding experiment and its behavioural Signs of Toxicity for *Vasanthakusumakara Mathirai*

Table: 12 Observation of acute toxicity study

Group	Day
Body weight	Normal
Assessments of posture	Normal
Signs of Convulsion, Limb paralysis	Absence (-)
Body tone	Normal
Lacrimation	Absence
Salivation	Absence
Change in skin colour	No significant colour change
Pile erection	Normal
Defecation	Normal
Sensitivity response	Normal
Locomotion	Normal
Muscle grippness	Normal
Rearing	Mild
Urination	Normal

Table: 13 Dose finding experiment and its behavioural Signs of Toxicity for *Vasanthakusumakara Mathirai*

Dose mg/kg	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2000	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-

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.Musclerelaxant*
13. Hypnosis *14.Analgesia* *15. Lacrimation*
16. *Exophthalmos* *17. Diarrhoea* *18. Writhing*
19. Respiration *20. Mortality*

In the acute toxicity study, the rats were treated with different concentration of *Vasanthakusumakara Mathirai* from the range of 5mg/kg to 2000mg/kg which did not produce signs of toxicity, behavioural changes, and mortality in the test groups as compared to the controls when observed during 14 days of the acute toxicity experimental period.

These results showed that a single oral dose of the extract showed no mortality of these rats even under higher dosage levels indicating the high margin of safety of this extract.

In acute toxicity test the *Vasanthakusumakara Mathirai* was found to be non toxic at the dose level of 2000mg/ kg body weight.

Sub-acute oral toxicity 28 days repeated dose study in rats

Table: 14 Body weight (g) changes of rats exposed to *Vasanthakusumakara Mathirai*

Dose (mg/kg/day)	*Days*				
	0	**7**	**14**	**21**	**28**
Control	120.59±0.92	122.79±0.87	123.52±1.18	127.24±1.12	131.25±1.05
100	125.92±0.63	128.14±1.01	131.19±1.30	133.82±1.18	138.62±1.27
200	127.53±3.83	135.17±1.34	139.30±1.35	141.34±1.58	146.55±1.50

*Values are expressed as mean ± SEM (Dunnet's test) *p<0.05 – Significant, **p<0.01-Highly significant, ***p<0.001-Extremely significant n=3*

Table: 15 Effect of *Vasanthakusumakara Mathirai* on Organ weight in rats

Organ	Control	100 mg/kg	200 mg/kg
Liver (g)	3.07±0.20	3.43±0.58	2.97±0.41
Heart (g)	0.32±0.04	0.34±0.06	0.28±0.05
Lung (g)	0.28±0.05	0.32±0.07	0.31±0.09
Spleen (g)	0.25±0.06	0.26±0.08	0.23±0.05
Brain (g)	0.37±0.05	0.46±0.08	0.43±0.05
Kidney (g)	0.76±0.05	0.76±0.09	0.72±0.05

*Values are expressed as mean ± SEM (Dunnet's test) *p<0.05 – Significant, **p<0.01-Highly significant, ***p<0.001-Extremely significant n=3*

Table: 16 Effect of *Vasanthakusumakara Mathirai* on Haematological parameters in rats

Parameter	Control	100 mg/kg	200 mg/kg
RBC ($\times 10^6/mm^3$)	8.29±0.43	8.25±0.55	8.9±0.73
PCV (%)	49.66±0.77	50.21±0.99	51.3±1.16
Hb (%)	15.13±0.39	14.83±0.41	14.3±0.31
WBC ($\times 10^3/mm^3$)	11.75±0.85	11.98±0.98	12.21±1.11
Neutrophils (%)	23.29±0.73	22.39±0.64	22.12±0.57
Eosinophills (%)	4.1±0.23	3.73±0.33	2.2±0.55
Lymphocyte (%)	85.5±0.46	86.03±0.58	86.4±0.7
Platelets ($\times 10^3/mm^3$)	425.73±1.35	445.47±5.60	452.83±3.70

*Values are expressed as mean ± SEM (Dunnet's test) *p<0.05 – Significant, **p<0.01-Highly significant, ***p<0.001-Extremely significant n=3*

Table: 17 Effect of *Vasanthakusumakara Mathirai* on Biochemical parameters in rats

Parameters	Control	100 mg/kg	200 mg/kg
Glucose (mg/dl)	108.63±0.81	111.72±0.83	114.28±1.16
BUN (mg/dl)	22.06±1.55	25.48±1.41	26.88±1.36
Creatinine (mg/dl)	0.85±0.07	0.96±0.07	1.01±0.10
SGOT (U/L)	74.35±1.23	72.50±1.24	70.68±0.95
SGPT(U/L)	27.07±0.84	24.22±1.28	22.24±1.15
ALP (U/L)	104.63±1.14	100.98±1.40	100.21±1.15
Protein (g/dl)	8.58±0.68	6.64±0.94	5.62±0.78
Albumin (g/dl)	5.34±0.40	4.45±0.66	3.47±0.35
Total Cholesterol (mg/dl)	93.21±1.16	94.50±0.92	96.14±1.20
Triglycerides (mg/dl)	52.58±1.56	51.60±1.38	50.45±1.69

*Values are expressed as mean ± SEM (Dunnet's test) *p<0.05 – Significant, **p<0.01-Highly significant, ***p<0.001-Extremely significant n=3*

Table: 18 Effect of *Vasanthakusumakara Mathirai* on Urine parameters in rats

Parameters	Control	100 mg/kg	200 mg/kg
Colour	Yellow	Yellow	Yellow
Transparency	Clear	Clear	Turbid
Specific gravity	1.01	1.02	1.04
Ph	7.2	7.4	6.9
Protein	Nil	Nil	Nil
Glucose	Nil	Nil	Nil
Bilirubin	-ve	-ve	-ve
Ketone	-ve	-ve	-ve
Blood	Absent	Absent	Absent
RBCs	Nil	Nil	Nil
Epithelialcells	Nil	Nil	Nil
Casts	Nil	Nil	Nil

Discussion

The dose selected for the sub acute toxicity study was 100mg, 200mg/kg of Vasanthakusumakara *Mathirai*.

All the animals were free of intoxicating signs throughout the dosing period of 28 days. No physical changes were observed throughout the dosing period. No mortality was observed during the whole experiment.

No abnormal deviations were observed. No significant changes were observed in the values of different parameters studied when compared with controls and values obtained were within normal biological and laboratory limits.

The weights of organs recorded did not show any significant differences in the treatment and the control group indicating that *Vasanthakusumakara Mathirai* was not toxic to kidney, liver and spleen.

There was no significant changes were observed in haemoglobin (Hb), red blood cell (RBC), white blood cell (WBC), packed cell volume (PCV), Erythrocyte sedimentation rate (ESR) in all the treated groups as compared to respective control groups.

From the acute and sub-acute toxicity studies the drug produced some significant changes, but the values were found within normal limits. So the drug *Vasanthakusumakara Mathirai* was nontoxic and safe.

Conclusion

Toxicological studies have been done according to OECD guidelines to know the acute and sub acute toxicity of the drug. There was no mortality rate in acute single oral dose study. In 28 days repeated oral study, the haematological and biochemical parameters showed slight various between 100 and 200mg but within the limits when compared to control. The body weight of the animals has slight change (increased in body weight). Thus, it will establish the safety and potency of the drug when administrated for long time.

Vasanthakusumakara Mathirai could be conformed as no-observed-adverse effect level (NOAEL) drug as it acts harmless under normal usage and to be of no toxicological concern. So, it can be concluded that the VKM prescribed for therapeutic use in human.

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