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Pre-clinical toxicological profiling of Siddha formulation *Sambirani Poo Kuligai* by Acute and Sub-acute toxicity studies in accordance with OECD Guidelines

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Abstract

In recent times use of traditional medicines has been conventionally increased globally. Short-term and long-term toxicity studies with rodents are generally conducted for 14 or 28 days. Results of these studies can help to predict appropriate doses of the test substance for future sub – chronic or chronic toxicity studies .It can be used to determine NOELs (No observable effect level) for some toxicology endpoints. According to the recent regulatory guidelines preclinical toxicity evaluation of the siddha formulations is mandatory to ascertain the possibility of adverse event in humans upon short and long term usage of the drugs. The main objective of the toxicity study is to establish the safety margin of the drugs at preclinical level as the siddha preparations being prescribed widely to the larger category of people since several years it's become regulatory essential for the researcher to justify the safety in humans and animals as well. Siddha system of medicine is a traditional practice that concerns the cultural interpretation of health, disease, and illness. In siddha the practice of ethnomedicine is a complex multidisciplinary system constituting the usage of herbs that has been the source of healing for people since several centuries. *Sambirani poo kuligai* (SPK) is poly herbal preparation which comprises of *Styrax benzoin, Felbovinum purifactum, Syzygium aromaticum* and *Piper betel* .The main aim of the present investigation is to establish the safety profile of the test drug SPK by acute and sub-acute oral toxicities in accordance with OECD 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 (100 and 200 mg/kg/day) of the test drug SPK were administered for 28 days followed by this biochemical and hematological 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 SPK in experimental animals. The mean body weight and most of the biochemical and hematological parameters showed normal levels in rats treated with SPK at both the dose levels. The results of the present investigation has provided an evidence based data's that clearly

justified that the siddha drug *Sambirani poo kuligai* has wide margin of safety and it didn't alters any of the physiology, behavioral and other functional parameters of the treated animals.

Keywords: Preclinical toxicity, OECD guidelines,Siddha system, *Sambirani poo kuligai*, Biochemical, Hematological parameters.

1. Introduction

Siddha system is one of the most conservative medical systems in the world. In the field of medicine Siddhars had enlightened the world to save the human lives from various refractive diseases. In Siddha system, the medicines are not only made up of an herbs which includes minerals, metals and other products of various organisms also. Toxicity studies on herbal drugs are commonly used to evaluate the possible health risk of the intrinsic chemical compounds in the plant which could result in adverse effects from the plant [1]. Specifically, acute toxicity and LD_{50} determination have been described as initial steps in the toxicological evaluations of herbal preprations [2], and data from such evaluations provide comprehensive information on the toxicological classification and labelling of such compounds [3]. According to research [4], substances with LD_{50} values of ≥5000 mg/kg b.w. are said to be safe and practically nontoxic.

The basic rationale of using rodents as ideal model for prediction of toxicity is all because of their genomic resemblance, drug metabolism and response towards toxic chemicals and drugs are almost similar to that of the humans. According to several scientific evidences it was proven fact that the rodent liver physiology simulates humans in several pathways. Hence the mechanism of drug metabolism offers several classical information upon administration in rodents. The basic parameters have to be studied for prediction of toxicity in rodents includes body weight, food intake, water intake, social behavior, sensory and motor coordination, muscle strength, exploratory behavior, liver function, kidney function, gastro intestinal motility, respiratory and circulatory function etc.

General toxicity tests measures changes in toxicity depending on dose and time after administration of test substances [5]. To examine the toxicity of a test substance, the toxicity of the test substance to all organ tissues needs to be assessed by acute and subacute toxicity tests using laboratory animals. Acute toxicity tests, also known as single dose toxicity tests, can qualitatively and quantitatively evaluate toxicity that occurs within a short period when a test substance is administered to a test animal in a single dose. Subacute toxicity tests, also known as 4-week dose range finding toxicity tests, measure the toxicity changes after repeated administration of the test substance to the test animal depending on dose and time [6].

For centuries herbal medicines and their formulations have been considered to be safe and effective due to their negligible side effects. This assumption may have influenced the indiscriminate use of these formulations to a large extent amongst the rural populace. These formulations are usually administered over a long period of time without proper dosage monitoring by the experts and lack of awareness of the toxic effects that might result from such prolonged usage [7]. Therefore, scientific knowledge towards oral toxicity is much needed, which will not only help identify doses that could be used subsequently, but also to reveal the possible clinical signs elicited by agents under investigation. The main aim of the present investigation is to establish the safety profile of the test drug SPK by acute and sub-acute oral toxicities in accordance with OECD guidelines.

2. Materials and Methods

2.1. Source of raw drugs:

The Required raw materials were procured from a well reputed indigenous drug shop from Parrys corner, Kanda Samy Temple, Chennai, Tamil Nadu, India. All raw drugs were authenticated by the Botanist and faculties of Gunapadam

department, Government Siddha Medical College, Arumbakkam, Chennai, Tamil Nadu, India. The test drug *Sambirani poo kuligai* was prepared as per Agasthiar Paripuranam 400.

2.2. Ingredients

The siddha formulation *Sambirani poo kuligai* Comprises of the following ingredients

Sambirani [Styrax benzoin]	-	250 g
Korosanai [Felbovinum purifactum]	-	6 g
Kirambu [Syzygium aromaticum]	-	20 g
Vetrilai [Piper betel]	-	50 ml

2.3.Purification of Raw Drug

***Styrax Benzoin* [8]**: The gums were purified by removing the sand, dust and odd particles.

Fel bovinum purifactum: The unwanted debris substances were removed.

***Syzygium aromaticum* [9]**: The flower buds were removed and fried slightly.

Piper betle : The stalk and the middle vein were removed.

2.4.Method of preparing Sambirani poo kuligai [10]

The purified *Styrax benzoin* was powdered well and was placed in a small pot. Then a paper was pasted on the inner surface of the big pot. The big pot was placed over the small pot and their mouths oppose each other. The gap between their mouths were covered by a seven layered muddy wet cloth and they allowed to dry. Then it was subjected to sublimation process for 12 hours (*4 samam*).After finishing sublimation process let the pot undisturbed to give away heat. Followed by this the seal were opened and the sublimed product was scrapped and collected.

2.5.Kuligai Process

Syzygium aromaticum and *Felbovinum* are powdered well and sieved through a white cloth. Finely powdered *Syzygium aromaticum* powder and *Felbovinum* powder are added along with the sublimate. Then all these substances are grounded well with *Piper betle* leaf juice for 48 minutes [2 *Nazhigai*]. The paste was made into pills in the size of seeds of *Abrus precatorius [Kundri size]* which was equivalent to 130 mg, dried in the shade and bottled up.

2.6.Toxicological Profiling of *Sambirani Poo Kuligai*

2.6.1.Animal

Healthy adult Wistar albino rat weighing between 180-200 g 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. [Approval no: IAEC/XLIV/27/CLBMCP/2014]

2.6.2.Acute toxicity Study

Acute toxicity in rats were carried out in accordance with OECD guideline 423.The animals were fasted overnight (12- 16 hrs) with free access to water. The study was conducted with single oral administration of study drug*Sambirani Poo Kuligai* (SPK) 2000mg/kg (p.o). 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 h 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 [11].Body weight was recorded periodically. At the end of the experiment all animals were subjected for gross necropsy and observed for pathological changes.

2.6.3.Sub-Acute toxicity Study

Sub-acute toxicity in rats were carried out in accordance with OECD guideline 407.The animals were randomly divided into control group and drug treated groups First group served as a control and other two group were treated with test drug SPK at the dose of 100 and 200 mg/kg,p.o 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 actate) 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 [12].

2.6.4. 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 analyser.

2.6.5. Biochemical analysis [13]

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.

2.6.6. Histopathological evaluation [14]

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.

2.6.7. Statistical analysis[15]

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 SPK on clinical signs of rats in Acute Oral Toxicity Study

The dose of SPK 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 SPK on clinical signs in Acute oral Toxicity Study

Parameters	Observation
Body weight	Normal
Assessments of posture	Normal
Signs of Convulsion Limb paralysis	Absence (-)
Body tone	Normal
Lacrimation	Absence
Salivation	Absence
Change in skin color	No significant color change
Piloerection	Normal
Defecation	Normal
Sensitivity response	Normal
Locomotion	Normal
Muscle gripness	Normal
Rearing	Mild
Urination	Normal

3.2. Dose finding experiment and its behavioral Signs of Toxicity for SPK in acute toxicity study

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

Table 2: Behavioral Signs of Toxicity for SPK in acute toxicity study

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.Analgesia15.Lacrimation 16. Exophthalmos 17. Diarrhea 18. Writhing 19 Respiration 20. Mortality

3.3.Effect of SPK 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 SPK at the dose of 100 and 200 mg/ kg b.w. The results were tabulated in Table 3.

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

Dose (mg/kg/day)	*Days*				
	1	**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 mg	124.75±0.39	125.38±0.47	127.78±0.44	128.86±0.36	132.33±0.33
200 mg	123.12±0.10	124.0±0.44	125.93±0.21	127.30±0.19	129.46±0.15

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; n = 3 per sex

3.4. Effect of SPK on average organ weight of rats in sub-acute toxicity study

No significant changes on the weight of the vital organs was observed in rats during the 28 consecutive days of treatment via oral route with SPK at the dose 100 and 200 mg/ kg b.w. The results were tabulated in Table 4.

Table. 4. Effect of *Sambirani Poo Kuligai* on Organ weight in Wistar albino rats

Organ	Control	100 mg	200 mg
Liver (gm)	17.50±1.86	15.83±0.73	15.76±0.70
Heart (gm)	1.83±1.73	2.39±0.28	1.87±0.29
Lung (gm)	2.24±1.67	1.81±0.33	2.13±0.17
Spleen (gm)	1.10±1.68	1.35±0.27	1.32±0.19
Brain (gm)	2.04±2.11	1.97±0.19	2.00±0.18
Kidney (gm)	1.90±1.76	1.75±0.25	1.84±0.25

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; n = 3 per sex

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

No statistically significant differences were recorded in hematological parameters of rats treated with SPK at the dose of 100 and 200 mg/ kg b.w.. The results were tabulated in Table 5.

Table 5: Hematological parameters of rats exposed to SPK in Sub-acute Toxicity study

Parameter	Control	100 mg	200mg
RBC (x 10^6/mm^3)	8.29±0.43	6.3±0.29	7.6±0.321
PCV (%)	49.66±0.77	49.76±0.568	52.93±0.44
Hb (g/dl)	15.13±0.39	14.22±0.29	15.31±0.214
WBC(mm^3)	11.75±0.85	9.40±0.25	8.00±0.07
Neutrophils (%)	23.29±0.73	22.96±0.11	16.66±0.61
Lymphocyte %	85.5±0.46	76.40±0.41	78.69±0.26
Eosinophils(%)	4.10±0.23	2.73±0.27	3.13±0.07
Platelets(x 10^3/mm^3)	425.73±1.35	461.61±7.47	549.99±0.92

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; n = 3 per sex

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

No statistically significant differences were recorded in biochemical parameters of rats treated with SPK at the dose of 100 and 200 mg/ kg b.w. The results were tabulated in Table 6.

Table 6: Biochemical parameters of rats exposed to SPK in Sub-acute toxicity study

Parameters	Control	100 mg	200 mg
Protein (g/dl)	8.58±0.68	6.67 ±0.59	7.57 ±0.49
Albumin (g/dl)	5.34±0.40	3.77± 0.19	2.65 ±0.27
BUN (mg/dl)	22.06±1.55	29.32± 0.48	35.14±1.41
Blood sugar (mg/dl)	108.63±0.81	104.93± 3.14	118.48±1.42
Creatinine (mg/dl)	0.85±0.07	0.48 ±0.09	0.65±0.04
Total Cholesterol (mg/dl)	93.21±1.16	122.42± 5.29	100.42±0.97
Triglycerides (mg/dl)	52.58±1.56	53.74 ±0.98	48.75±0.31
SGOT (U/L)	74.35±1.23	139.31± 0.79	124.79± 3.44
SGPT(U/L)	27.07±0.84	121.46± 3.49	72.63±6.15
Alkaline phosphatase (U/L)	104.63±1.14	126.35± 2.52	139.35±1.19

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; n = 3 per sex

3.7. Effect of SPK on urine biochemistry of rats in Sub-acute oral toxicity study

No significant changes were recorded in biochemical parameters of rats treated with SPK at the dose of 100 and 200 mg/ kg b.w. The results were tabulated in Table 7.

Table 7: Effect of SPK on Urine parameters in rats

Parameters	Control	100 mg	200 mg
Colour	Yellow	Yellow	Yellow
Transparency	Clear	Clear	Clear
Specific gravity	1.01	1.01	1.01
Ph	6.4	6.2	7.1
Protein	Nil	Nil	Nil
Glucose	Nil	Nil	Nil
Bilirubin	-ve	- ve	- ve
Ketones	-ve	- ve	- ve
Blood	Absent	Absent	Absent
RBCs	Nil	Nil	Nil
Epithelial cells	Nil	Nil	Nil
Casts	Nil	Nil	Nil

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; n = 3 per sex

4. Discussion

The majority of the people in developing countries use various traditional herbal medicines to treat a number of diseases and ailments [16]. Although, many studies have been undertaken in the past to investigate the pharmacological potential of such remedies, however, rather little work has been done to assess the potential toxicities of such products. There is now growing evidence that many herbal medicines do cause serious toxicity to their users [17,18]. Therefore, much more scientific attention is now being given to assess the potential toxicity of herbal medicines than before.

Siddha formulations offers tremendous advantage in clinical practice against metabolic and lifestyle disorders including neuro degenerative diseases. Often investigation on siddha preparations attempted on reverse pharmacology basis. Hence nearly 80% of the formulation already have proven track record clinically and now several investigation are being made on its preclinical aspect.The use of herbal medicines as complements or alternatives to orthodox medicines has been on the increase. The reasons, which have given rise to this trend, include the cheapness, availability and accessibility of these natural medicines. Besides, there has been the erroneous belief that these medicines are free from adverse effects [19] .On the other hand they have been rejected because many of the acclaimed medicinal values have not been scientifically evaluated and their safety profiles uncertain [20].

Safety pharmacology is a subdivision of pharmacology which focuses on identification and characterization of pharmacological activities that affect the clinical safety of a drug. The guideline recommends assessing effects on functions of cardiovascular, central nervous and respiratory systems, which are referred as the core test battery of safety pharmacology [21,22]. Toxicological evaluation of siddha formulation SPK has provided an evidence based data with respect to C.N.S, A.N.S and C.V.S system on the tested animals. SPK at a dose of 2000 mg/kg had no adverse effect on the treated rats in up to 14 days of observation. There were no significant changes in the weight and the organs of the rats.

The haemopoietic system is one of the most sensitive targets of toxic compounds and an important index of physiological and pathological status in both humans and animals [23]. The hemo toxic nature of the drug exerted by the fluctuation in blood cell count in particular to RBC and WBC cells. Low hemoglobin content reflects the low level of RBC which in turn affects the oxygen carrying capacity of the blood. At the end of the most of the toxicity studies the blood collected from the animal before sacrifice will be subjected to whole blood analysis and also to serological analysis [24]. It was observed from the hematological profiling of the present investigation that there is no significant change in basic hemato cellular components of the rats treated with SPK at both the dose level of 100 and 200 mg/kg.

The changes of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) contents is a sensitive index to reflect the degree of liver cell damage [25]. When the chronic liver injury happened, ALT and AST would be released from the injury of the liver cells, resulting in the increase in the content of serum [26,27]. In addition, Serum urea nitrogen (BUN) reflect glomerular filtration function, when renal parenchymal was damaged, BUN could increase [28,29]. In various organs, liver and kidney are strong for drug affinity and conducive to the elimination of the drug, but also have a certain role in the accumulation [30,31]. There is no significant changes were observed in the serological profile of rats treated with SPK at the dose of 100 and 200mg/kg. From this it may be concluded that the formulation SPK doesn’t change the normal physiology of the vital organs such as liver and kidney.Organ weight is the most important index for the predication of internal organ toxicity caused by the drug. In the present toxicity study investigation it was observed that there is no significant difference in the vital organ weight of the treated rats when compared to that of the control group animals.

5. Conclusion

Toxicity profiling of siddha preparations are become highly essential in order to prove the safety of the formulation upon short and long term administration in humans. Results of toxicity study render some useful information to the investigators with respect to the effect of the drug on CNS, CVS, ANS and other metabolic organs. In conclusion, this study presented the results on the acute and sub-acute toxicity of siddha formulation *Sambirani Poo Kuligai*that can be very useful for future *in-vivo* and clinical studies. Results of acute toxicity study revealed that test drug SPK was well tolerated at the dose of 2000mg/kg in the tested rats. There were no biologically significant, treatment related adverse effects on body weights, hematology and clinical biochemistry parameters of animals treated with SPK at the dose level of 100 and 200 mg/kg for the period of 28 consecutive days. By considering the data's obtained from the investigation it may be concluded that siddha formulation *Sambirani Poo Kuligai*is relatively non-toxic and has wide safety margin for short and long term administration.

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