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PRECLINICAL SAFETY EVALUATION OF SIDDHA FORMULATION KEELVAYU NIVARANA CHOORANAM BY ACUTE AND SUB-ACUTE TOXICITY STUDIES IN WISTAR RATS

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ABSTRACT

Toxicity assessment is a major problem in drug and environmental chemical development. This has been well documented in the drug industry where poor preclinical and clinical safety assessment correlations. Toxicity bioassays or animal tests are important components of human toxicity assessment, but rarely address specific chemical pathways of toxicity. Thus, there is a critical need for bioanalytical platforms to establish the chemistry of metabolic toxicity pathways to augment traditional bioassays. Siddha systems of medicine have been used for thousands of years in worldwide traditional medicines for their potential health benefits. Although they are generally presumed safe unless a significant risk has been identified in humans, increasing number of case reports notify acute or chronic intoxications resulting from their use. Keelvayu Nivarana Chooranam (KVNC) is a poly herbal preparation which comprises of certain biologically significant herbs like Hemidesmus indicus, Smilax chinensis, Withania somnifera and Alpinia officinarum. The main aim of the present investigation is to establish the safety profile of the test drug KVNC in rodent model acute and sub-acute oral toxicities in accordance with OECD regulatory guidelines. Acute toxicity testing is carried out to determine the effect of a single dose on a particular animal species. In the present study acute toxicity sere been carried out by single oral administration of 2000mg/kg. In the sub-acute study, repeated doses (20,100 and 200mg/kg) of the test drug KVNC were administered for 28 days followed by this biochemical and hematological parameters were evaluated. Results of the study have revealed that there was no sign of toxicity and no mortality after single and repeated administration of the test drug KVNC in experimental animals. A normal haematological and serological profile of KVNC treated groups in sub-acute toxicity study further justified the non-toxic nature of the formulation. There is no significant changes were observe in the body weight, ,food intake, water intake, relative organ weight and also in histological analysis of the samples retrieved from the drug treated rats. Since there was no reduction in body and relative organ weights of the treated animals at any of the tested dose it was concluded that the siddha drug KVNC is nontoxic and has wide safety margin.

KEYWORDS: Toxicity assessment, OECD guidelines, Siddha system, Keelvayu Nivarana Chooranam, Hematological profile, serology.

1.INTRODUCTION

In general perception about herbal remedies or traditional a drug are very safe and devoid of adverse effects is not only untrue, but also misleading. Herbs have been shown to be capable of producing a wide range of undesirable or adverse reactions some of which are capable of causing serious injuries, life-threatening conditions, and even death. During the evaluation of the toxic characteristics of traditional medicines and herbal preparations, the determination of LD_{50} is usually an initial step to be conducted. Data from the acute toxicity study may (a) serve as the basis for classification (b) provide initial information on the mode of toxic action of a substance; (c) help arrive at a dose of a new compound;

(d) help in dose determination in animal studies; and (e) help determine LD_{50} values that provide many indices of potential types of drug activity.[1] Moreover, if a high dose (e.g., 2000- 5000 mg/kg) is found to be survivable, no further acute testing will be conducted.[2]

Although, the assessment of the safety of herbal medicines has become an important issue for consumers, regulatory authorities, and healthcare professionals, analysis of adverse events related to the use of these products is much more complex than in the case of conventional pharmaceuticals.[3-4] It is also recognized that evaluation of safety is complicated by factors such as the geographical origin of plant material, different processing techniques, route of administration, and compatibility with other medicines.[5]

Over the years, the use of herbs in the treatment of illnesses has been very successful and its historic usage has been useful in drug discovery development. Herbal prescriptions and natural remedies are commonly employed in developing countries for the treatment of various diseases, this practice being an alternative way to compensate for some perceived deficiencies in orthodox pharmacotherapy.[6-7] The popularity and availability of the traditional remedies have generated concerns regarding the safety, efficacy and responsibility of practitioners using traditional remedies.[8] Herbal remedies are considered safer and less damaging to the human body than synthetic drugs.[9] However, the lack of standardization has been a major concern regarding use of herbal medicines.[10-11] Although herbal supplements may be considered to be safe, some are known to be toxic at high doses and others may have potentially adverse effect after prolonged use. The main aim of the present investigation is to carry out the acute and sub-acute toxicity studies of siddha formulation KVNC on rats.

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.

2.2. Ingredients

The siddha formulation *Keelvayu Nivarana Chooranam* Comprises of the following ingredients

1. *Nannariverpattai (Hemidesmus indicus*) - 116 g
2. *Parangipattai* (*Smilax chinensis*) - 116 g
3. *Seemai Amukara (Withania somnifera)* - 116 g
4. *Chittaraththai (Alpinia officinarum*) - 58 g

2.3. Method of Purification

Nannariverpattai were washed in the running tap water to remove the soil and impurities. *Parangipattai* was dried and powdered and then it was purified by *Pittaviyal* method (steam cooking in milk). A mud pot was taken and it was half filled by milk and half filled by pure water. The mouth of the pot was sealed by a cloth. This *chooranam* then placed over the cloth and the pot was heated. The same drug was later dried and powdered then sieved again.*Amukara* was dried and powdered and then it was purified by *Pittaviyal* method (steam cooking in milk). A mud pot was taken and it was half filled by milk and half filled by pure water. The mouth of the pot was sealed by a cloth. This *chooranam* then placed over the cloth and the pot was heated. The same drug was later dried and powdered then sieved again. *Chittaraththai* was washed in the running tap water to remove the soil and impurities.

2.4. Formulation of *Keelvayu Nivarana Chooranam*

All the above purified ingredients were powdered in an iron mortar separately and it was sieved by a cotton cloth. Then these powders were mixed together and bottled up. It was labeled as *Keelvayu Nivarana Chooranam* (KVNC). The prepared test drug was stored in a clean, air tight glass container. The contents were inspected frequently to avoid moisture and insects. Adult dosage of the drug was 1 gm twice a day.

2.5.Toxicological Profiling of *Keelvayu Nivarana Chooranam*

2.5.1.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 ± 2^0 C and 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 No: IAEC/XLVIII/05/CLBMCP/ 2016].

2.5.2. 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 *Keelvayu Nivarana Chooranam* (KVNC). 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.[12] Body weight was recorded periodically. At the end of the experiment all animals were subjected for gross necropsy and observed for pathological changes.

2.5.3. 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 KVNC at the dose of 20,100 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 28th day, the animals were fasted for overnight with free access to water. On 29th 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.[13]

2.5.4. 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.

2.5.5. Biochemical analysis[14]

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.

2.5.6. Histopathological evaluation[15]

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.

2.5.7. Statistical analysis[16]

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 KVNC on clinical signs of rats in Acute Oral Toxicity Study

In acute toxicological testing, the trial drug KVNC was administered at the dose higher than the therapeutic dose, and the effect is observed for 14 days. The dose of KVNC 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 was observed for (24-48 h) and a long period of (14 days). The results were tabulated in Table 1.

Table 1: Effect of KVNC 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 KVNC in acute toxicity study

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

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

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

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

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

Table 3: Effect on KVNC on change in mean body weight of rats in acute toxicity study.

DOSE	DAYS		
	1	7	14
Control	176.21± 3.22	177.2± 4.27	179.2 ± 4.82
KVNC 2000 mg/kg	172.5± 3.18	174.2± 3.26	175.4 ± 3.27

N.S- Not Significant, ** (p > 0.01), *(p >0.05), n = 6 values are mean ± S.D (One way ANOVA followed by Dunnett's test.

3.4.Effect of KVNC 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 KVNC at the dose of 20, 100 and 200 mg/ kg b.w. The results were tabulated in Table 4.

Table 4:Effect of KVNC on Body weight of rats in Sub-acute toxicity study.

Dose (mg/kg/day)	*Days*				
	1	7	14	21	28
Control	165.6± 2.76	166.4 ± 3.42	167.7 ± 3.26	169.2 ± 3.73	170.7 ± 1.31
20 mg/kg	165.2 ± 4.12	166.7 ± 2.64	166.9± 1.51	172.9 ± 1.66	174.42± 2.76
100 mg/kg	168.6± 1.24	168.9 ± 4.74	170.4 ± 8.92	171.1 ± 6.36	174.7 ± 9.12
200 mg/kg	171.4± 3.74	173.6 ± 6.32	174.6 ± 2.86	175.1± 8.82	175.92 ± 6.42

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; n = 12 of 6 per sex

3.5. Effect of KVNC on average food and water intake of rats in sub-acute toxicity study

No significant changes on the average food and water intake of rats during the 28 consecutive days of treatment via oral route with KVNC at the dose 20,100 and 200 mg/ kg b.w. The results were tabulated in Table 5 and Table 6.

Table 5: Effect of KVNC on average food intake (gms/day) of rats in sub-acute toxicity study.

Dose (mg/kg/day)	*Days*				
	1	7	14	21	28
Control	37.12 ±5.37	38.5±3.22	39.5±3.37	38.5±3.37	37.12±3.12
20 mg/kg	33.7±2.12	35.3±1.42	35.9±1.68	36.4±2.62	35.9±8.42
100 mg/kg	34.2±3.64	35.9±3.64	36.2±6.15	37.4±2.18	35.2±2.64
200 mg/kg	36.2±2.14	36.2±2.18	37.6±2.14	38.2±4.28	39.2±2.18

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; n = 12 of 6 per sex.

Table. 6. Effect of KVNC on average water intake (ml/day) of rats in sub-acute toxicity study

Dose (mg/kg/day)	*Days*				
	1	7	14	21	28
Control	31.5 ± 8.95	32.0 ±6.23	28.5±6.23	29.12±8.19	31.5±3.96
20 mg/kg	29.5±3.31	29.9±6.62	31.7±4.02	32.2±4.29	34.9±3.13
100 mg/kg	31.7±3.93	32.3±3.11	34.1±2.83	32.4±4.11	34.4±2.14
200 mg/kg	32.1±1.12	33.2±2.43	34.7±2.53	35.2±1.89	36.4±2.45

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; n = 12 of 6 per sex.

3.6. Effect of KVNC on Hematological parameters of rats in Sub-acute oral toxicity study

No statistically significant differences were recorded in hematological parameters of rats treated with KVNC at the dose of 20, 100 and 200 mg/ kg b.w. The results were tabulated in Table 7.

Table 7: Hematological parameters of rats exposed to KVNC in Sub-acute Toxicity study

Parameter	Control	KVNC 20mg/kg	KVNC 100mg/kg	KVNC 200mg/kg
Hb (g/dl)	14.8±1.88	12.98±1.28	13.01±1.26	14.18±3.96
Total WBC ×10^3	10.91±2.59	12.25±3.53	12.18±3.61	12.96±3.47
Neutrophils (%)	32.65±1.06	34.23±2.54	34.91±1.36	33.40±2.80
Lymphocyte (%)	69.34±2.48	70.22±3.42	71.48±2.66	71.20±3.96
Monocyte (%)	0.78±0.17	0.81±0.12	0.84±0.11	0.95±0.16
Eosinophil (%)	0.64±0.09	0.19±0.12	0.78±0.06	0.42±0.04
Platelets cells10^3/µl	687.17±8.76	698.71±8.16	705.18±4.0	712.16±4.6
Total RBC 10^6/µl	7.99±0.12	6.82±1.87	6.92±0.59	6.18±0.72
PCV%	37.79±0.6	36.35±1.53	38.2±1.18	36.82±2.14
MCHC g/dl	33.6±2.23	34.19±1.19	35.18±1.92	34.13±1.94
MCV (µm^3)	49.17±3.64	48.20±1.24	49.28±1.24	49.99±1.84

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; n = 12 of 6 per sex.

3.7. Effect of KVNC on biochemical parameters 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 20,100 and 200 mg/ kg b.w. The results were tabulated in Table 8.

Parameter	Control	KVNC 20mg/kg	KVNC 100mg/kg	KVNC 200mg/kg
Glucose (r) (mg/dl)	76.45±13.4	76.16±8.54	79.64±9.20	77.42±11.6
T.Cholesterol mg/dl)	115.26±1.83	112.45±1.13	112.42±1.98	115.22±1.83
Tri Glycerides mg/dl)	46.35±1.48	45.32±1.48	45.58±1.26	46.66±1.45
LDL	72.81±2.13	70.14±2.34	71.8±2.94	72.64±6.12
VLDL	15.2±2.44	14.42±4.63	14.44±6.64	14.94±5.14
HDL	26.66±6.88	27.96±2.34	27.88±5. 66	29.78±6.22
Ratio 1(T.CHO/HDL)	4.42±2.44	4.36±1.44	4.84±2.44	4.86±1.92
Ratio 2(LDL/HDL)	2.83±4.22	3.02±1.52	2.96±4.80	2.86±3.82
Albumin(g/dL)	3.63±0.17	3.13±1.12	3.10±1.92	2.94±3.86

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; n = 12 of 6 per sex.

3.8. Effect of KVNC on Renal function test parameters of rats in Sub-acute oral toxicity study

No significant changes were recorded in RFT parameters of rats treated with SPK at the dose of 20,100 and 200 mg/ kg b.w. The results were tabulated in Table 9.

Parameter	Control	KVNC 20mg/kg	KVNC 100mg/kg	KVNC 200mg/kg
Urea (mg/dl)	13.35±0.99	12.91±1.86	13.16±1.98	13.18±3.92
Creatinine(mg/dl)	0.28±0.08	0.16±1.16	0.12±0.14	0.18±1.22
Bun(mg/dl)	15.02±0.10	14.80±1.20	14.66±0.44	15.10±2.32
Uric Acid(mg/dl)	5.17±0.35	5.25±1.43	5.02±1.35	5.18±1.08

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; n = 12 of 6 per sex.

3.9. Effect of KVNC on Liver function test parameters of rats in Sub-acute oral toxicity study

No significant changes were recorded in LFT parameters of rats treated with SPK at the dose of 20,100 and 200 mg/ kg b.w. The results were tabulated in Table 10.

Parameter	Control	KVNC 20mg/kg	KVNC 100mg/kg	KVNC 200mg/kg
T.BILIRUBIN(mg/dl)	0.48±0.07	0.43±1.26	0.64±1.28	0.68±1.25
AST(U/L)	79.95±1.39	77.15±1.31	78.71±1.83	80.35±3.03
ALT(U/L)	31.23±1.28	31.81±3.52	30.14±3.18	31.9±1.88
ALP(U/L)	143.25±8.70	141.9±8.17	142.16±4.10	144.33±4.25
T.PROTEIN(g/dL)	5.32±0.38	5.28±0.34	5.21±1.33	5.13±1.06

Values are expressed as mean ± S.E.M (Dunnett's test). *P<0.05, **P<0.01, ***P<0.001 vs control; n = 12 of 6 per sex.

3.10. Effect of KVNC on Histopathological changes of rats in Sub-acute oral toxicity study

From the results of the histological analysis it was evident that there is no significant abnormality were detected in the histopathological analysis of organs (Kidney, Liver and Spleen) retrieved from the rats treated with KVNC at high dose of 200 mg/ kg b.w. The results were illustrated in figure 1 and 2.

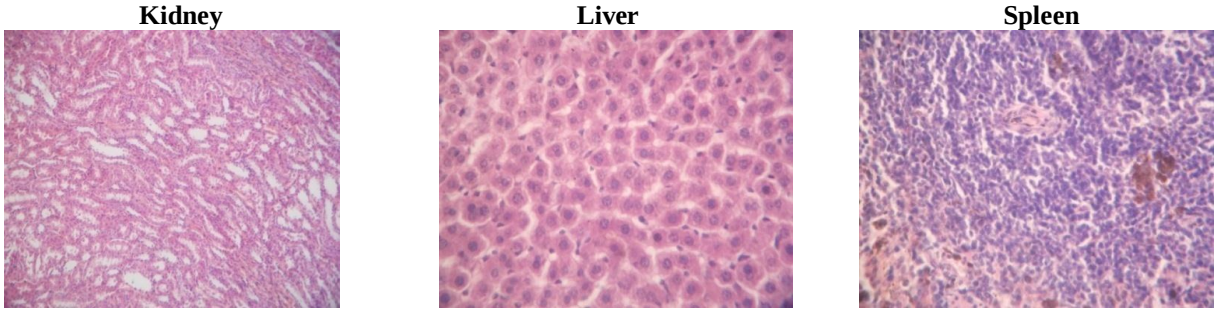

Figure 1: Histopathological representation organs of control group rats in Sub-acute oral toxicity study.

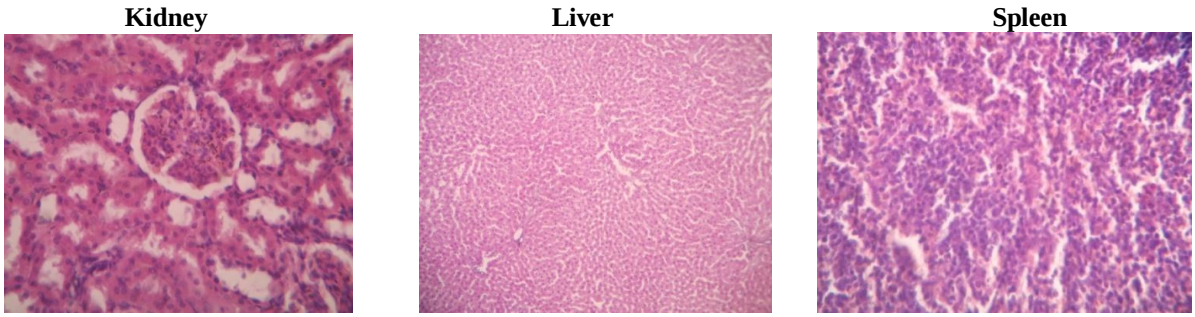

Figure 2: Histopathological representation organs of KVNC treated rats in Sub-acute oral toxicity study.

4. DISCUSSION

Natural products are believed to be safer than chemical products. Therefore, toxicity studies of natural substances do not usually receive as much attention as studies of chemical products. However, some natural substances are potentially toxic and may be harmful to human health.[17] In addition, issues regarding the actual safety of natural substances are constantly discussed.[18-19] Therefore, systematic safety studies are also essential for compounds that are natural-based medicines or functional health foods

The significance of the acute toxicity study in rodents reveals some evidence based data's on highest possible dose which was considerably much higher than the normal therapeutic dose. In the present investigation treatment with KVNC at the dose of 2000mg/kg didn't revealed any adverse event in the animals such as mortality, clinical signs, Body weight and sensory responses pertains to C.N.S and A.N.S

Certain test substance reveals potential toxicity upon long term administration in order to predict the possible safety of the KVNC formulation repeated dose toxicity testing was carried out for a minimum of 28 days. The KVNC is administered daily for a certain period through the oral route. Baseline parameters such as the behavioral and biochemical parameters of the animals was recorded. These will be helpful in calculating percentage changes. The interpretation of human safety details is essential in repeated dose toxicity studies.[20]

Result analysis of the sub-acute toxicity study has clearly shown that the formulation KVNC was absolutely safe on treated rats. There is no significant changes were

observed in the basic behavioral tendency of the animal including body weight, food and water intake.

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.[9] 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. In the current study hematological and serological data's projects no significant variations with respect to blood cell count, KFT, RFT, Lipid profile and other metabolic markers. Internal organ toxicity potential of the drug was ensured after justification of histological report. In the present investigation treated with KVNC even at highest dose of 200mg/kg shown no abnormalities including organ morphology, gross observation, weight and microscopic histological architecture. These evidences of sub-acute study substantiates the wide safety margin of the formulation KVNC on long term usage at clinical level.

5.CONCLUSION

Medicines from herbal origin have been used as a source of remedy for the prevention, cure and treatment of different ailments in Traditional Systems of Medicine. Therapeutically important herbs in alternate complimentary medicines have been extensively explored recently, for their benefits and various applications in herbal health supplements. Acute and sub-acute toxicity studies of *Keelvayu Nivarana Chooranam* on rats reveal absolute safety of the siddha formulation. Further there is no adverse events were observed in any of the observed parameters. Hence it was concluded that the siddha formulation *Keelvayu Nivarana Chooranam* have wide margin of safety on both short and long-term usage.

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