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Acute and Sub-acute Toxicity Evaluation of Siddha Formulation Perungaya Chooranam in Wistar Rats

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Abstract

Siddha system of medicine has gained people's satisfaction with its therapeutic outcomes and there are perceptions that herbal medicines are inherently safe. Furthermore, the dissatisfaction of patients towards allopathic medicine in terms of efficacy and safety has also prompted the use of traditional Siddha preparations. 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. It provides a base for fixation of dose to the pharmacological study. Toxicity study further reveals the information about LD50 and also the dose which causes lethal effect and also safe therapeutic dose. Perungaya Chooranam (PC) is widely used Siddha remedy for clinical management of several disease including gastrointestinal issues in humans. The main aim of the present investigation is to evaluate the formulation PC 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, 200 and 400 mg/kg/day) of the test drug PC were administered for 28 days and biochemical, hematological and histopathological parameters were evaluated. Results of the present investigation clearly showed that there was no sign of toxicity and no mortality after single and repeated administration of *the test drug PC at varying doses in tested rats.* There was no significant difference in mean general behavioral pattern including body weight, biochemical, hematological and serological observation 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 PC.Single and repeated oral administration of the siddha drug PC may be safe and considered as relatively non-toxic at the varying doses of 20, 200 and 400 mg/kg/day dose level. From the results obtained from the present preclinical investigation it was concluded the acute or sub-acute oral administration of the test drug PC is considerably very safe and may render clinical benefits in patients upon short and long term usage.

Keywords: Siddha system, Perungaya Chooranam, OECD, Acute, Sub-acute Biochemical, Hematological and Histological examination.

1. Introduction

Traditional medicine, also called complementary or alternative medicine, natural medicine, nonconventional medicine, or holistic medicine, has always maintained its popularity worldwide. Over the last decade, we have seen its increasing use in many developed and developing countries. Currently, as a main part of traditional medicine, herbal medicine can be prescribed as drugs by doctors in some countries, such as China, India, and Germany, but only be used as dietary supplements in other countries, such as the United States.

Siddha medicine is one of the most ancient medical systems of India. Siddha is the mother medicine of ancient Tamils/Dravidians of peninsular South India. The word Siddha means established truth. The persons who were associated with establishing such a Siddha school of thought were known as Siddhars. They recorded their mystic findings in medicine, yoga, and astrology in Tamil. Fundamental Principles of Siddha include theories of Five Elements (Panchabootham), and Three Forces/Faults (Mukkuttram). The Eight Methods of Examination (Envagai Thervukal) is used to determine diagnosis, etiology, treatment and prognosis.

The safety and efficacy of herbal medicine, as well as quality control have become important concerns for both health authorities and the public. Health-care professionals, providers, and consumers are calling for regulations of products of herbal medicine [2]. Some countries, such as Japan and China, have applied GMP management to herbal manufactories. This will not only ensure the safety of herbal products, but also promote recognition of traditional medicine and their products, and further define their role in modern health-care systems.

With recent increasing interest in alternative or herbal medicine for the prevention and treatment of various illnesses, there is increasing concern about the safety of medicinal plants. There are general and herb-specific concerns regarding herbs and their potential to produce adverse effects. Accidental herbal adverse effects may occur as a result of collecting wrong raw materials and inappropriate preparation due to a lack of knowledge on active and toxic components in the materials and pharmaceutical quality control, or overdosed or over-lasting administration due to the mistaken belief that herbal remedies are harmless. Unfortunately, many countries have no official regulations for quality control on the manufacturing or labeling claims of herbal remedies and dietary supplements [3].The main objective of the present research work is to evaluate the short and long term safety of the Siddha formulation Perungaya Chooranam by acute and sub-acute toxicity studies in rodent.

2. Materials and Methods

2.1. 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 $\pm$ 2^0Cand 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: XLVIII/07/CLBMCP/2016

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 *Perungaya Chooranam* (PC) 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 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 [4].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 consists of 12 animals (6 Males and 6 Females). First group served as a control and other three group were treated with test drug PC (20, 200 and 400 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 sign 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 [5].

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 [6]

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 [7]

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[8]

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 PC on clinical signs of rats in Acute Oral Toxicity Study

The dose of PC 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 Perungaya 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 PC on Body weight of rats in acute toxicity study

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

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

Dose	Days		
	1	7	14
Control	320.2±42.30	322.4 ± 60.10	323.6 ±52.10
PC 2000 mg/kg	302.4± 1.21	302 ± 2.04	304.2 ± 2.10
P value (p)*	NS	NS	NS

3.3.Effect of PC 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 PC at low, mid and high dose of 20, 200 and 400 mg/ kg b.w. The results were tabulated in Table 3.

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

Dose	Days				
	1	7	14	21	28
Control	235.2±18.46	236.5 ± 35.10	236.6 ± 45.60	238.7± 56.16	238.4 ± 66.15
Low dose	248.2 ± 65.24	250.7 ± 66.28	254.6± 55.34	256 ±56.34	256.8± 35.36
Mid dose	252.4± 18.34	253.3 ± 16.24	253.4 ± 14.12	255.2 ± 15.20	256.4 ± 54.10
High dose	261.6± 62.24	261.4±42.22	262.4 ± 52.24	263 ± 54.28	264 ± 74.60
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 PC 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 PC at low, mid and high dose of 20, 200 and 400 mg/ kg b.w.The results were tabulated in Table 4 and 5.

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

Dose	Days				
	1	7	14	21	28
Control	34±4.14	34.2±6.12	34.3±2.18	34.2±1.14	34±5.62
Low dose	36.3±1.64	36.3±1.51	36.2±1.51	36.5±1.62	36.5±1.22
Mid dose	34.1±2.12	34.2±3.50	34.2±2.14	34.2±2.16	35.2±1.64
High dose	32.4±1.62	32.1±1.64	32.6±2.36	32.6±1.20	36.4±2.32
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 Perungaya Chooranam on water intake of rats in Sub-acute toxicity study

Dose	Days				
	1	7	14	21	28
Control	60.1 ± 8.72	60±1.52	60.2±1.40	61±1.32	61.4±1.62
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	64.1±1.81	64.2±1.32	64.4±1.14	64.6±1.62	65.8±2.02
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 PC on Hematological parameters of rats in Sub-acute oral toxicity study

No statistically significant differences were recorded in hematological parameters of rats treated with PC at low, mid and high dose of20, 200 and 400 mg/ kg b.w. The results were tabulated in Table 6.

Table 6: Haematological parameters of rats exposed to Perungaya Chooranam

Category	Control	Low dose	Mid dose	High dose	P value (p)*
Haemoglobin(g/dl)	13.4±0.71	13.30±0.14	13.4±0.13	13.72±0.13	N.S
Total WBC (×10^3 l)	09.41±0.22	09.32±0.22	09.34±0.22	09.30±1.10	N.S
Neutrophils (%)	21.13±0.60	21.02±0.52	22.11±1.42	22.02±2.71	N.S
Lymphocyte (%)	82.10±1.26	82.12±1.42	83.10±2.44	83.20±2.54	N.S
Monocyte (%)	1.1±0.03	1.1±0.01	1.2±0.04	1.1±0.03	N.S
Eosinophil (%)	0.8±0.03	0.8±0.04	0.9±0.05	0.9±0.08	N.S
Platelets cells10^3/µl	900.17±3.18	902.11±4.62	902.11±2.20	902.22±2.64	N.S
Total RBC 10^6/µl	9.32±0.11	9.47±0.33	9.50±0.64	9.60±0.46	N.S
PCV%	48.10±0.2	48.62±5.30	48.8±4.70	48.4±.71	N.S
MCHC g/dL	36.5±1.61	36.2±1.51	36.8±1.30	36.13±1.60	N.S
MCV fL(µm^3)	58.2±2.02	58.2±1.80	58.7±1.10	59.7±1.30	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 PC on Biochemical parameters of rats in Sub-acute oral toxicity study

No statistically significant differences were recorded in biochemical parameters of rats treated with PC at low, mid and high dose of 20, 200 and 400 mg/ kg b.w. The results were tabulated in Table 7 -9.

Table 7: Biochemical parameters of rats exposed to Perungaya Chooranam

Biochemical Parameters	Control	Low dose	Mid dose	High dose	P Value (p)*
Glucose (R) (mg/dl)	138.10±2.02	138.12±2.10	138.9±12.06	138.12±5.25	N.S
T.Cholesterol (mg/dl)	140.14±5.10	140.15±5.20	142.40±1.68	143.21±1.10	N.S
Trigly(mg/dl)	74.15±1.82	74.11±1.32	74.15±1.22	76.16±1.21	N.S
LDL	78.6±2.13	78.7±2.05	78.10±1.03	78.40±01.32	NS
VLDL	14.2±1.52	14.20±2.41	14.02±1.32	14.04±12.15	NS
HDL	28.12±4.32	28.32±2.50	28.46±1.20	28.51±1.23	NS
Ratio 1(T.CHO/HDL)	3.73±1.16	3.72±1.80	3.73±1.32	3.74±2.33	NS
Ratio 2(LDL/HDL)	1.92±1.22	1.92±1.20	1.93±2.20	1.94±06.02	NS
Albumin (g/dL)	6.21±0.22	6.22±0.52	6.4±7.20	6.55±6.48	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 Perungaya Chooranam

Parameters	Control	Low dose	Mid dose	High dose	P Value (p)*
Urea (mg/dl)	14.50±0.29	14.50±0.29	14.46±1.18	14.42±1.22	N.S
Creatinine (mg/dl)	0.42±0.02	0.41±0.04	0.43±0.03	0.44±0.09	N.S
BUN(mg/dL)	19.1±0.02	19.10±0.34	19.6±0.42	19.26±1.02	NS
Uric acid(mg/dl)	4.02±0.04	4.06±0.21	4.4±0.12	4.20±0.10	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 9: Liver Function Test of rats exposed to Perungaya Chooranam

Parameters	Control	Low dose	Mid dose	High dose	P Value (p)*
T Bilirubin (mg/dl)	0.08±0.01	0.08±0.03	0.08±0.03	0.08±0.01	N.S
SGOT/AST(U/L)	64.11±1.53	64.12±0.22	64.24±1.54	65.74±1.53	N.S
SGPT/ALT(U/L)	79.21±1.02	79.34±1.04	79.44±1.16	79.38±0.21	N.S
ALP(U/L)	137.11±2.21	137±2.20	139±1.24	140.03±6.02	N.S
T.Protein(g/dL)	7.2.40±0.14	7.2±0.41	7.2±0.60	7.3±0.61	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 PC on Histopathological changes of rats in Sub-acute oral toxicity study

No abnormality were detected in the histopathological analysis of organs (Brain, Heart, Kidney, Liver and Lung) retrieved from the rats treated with PC at high dose of 400 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

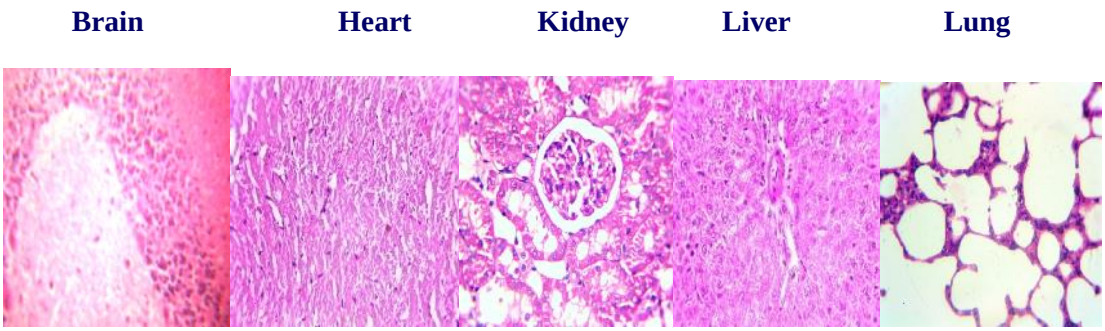
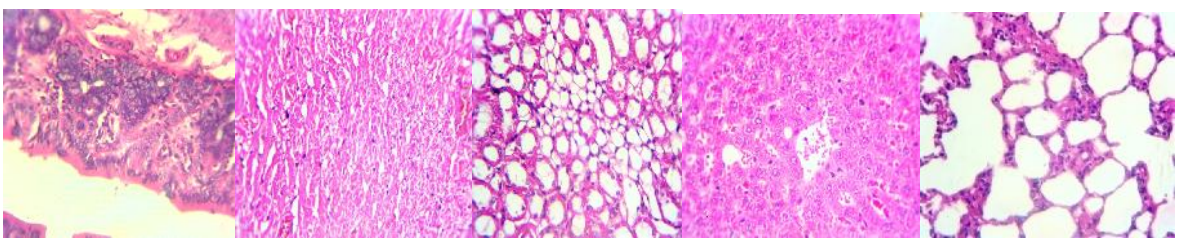


Figure 2: Histopathological representation organs of PC treated rats in Sub-acute oral toxicity study



4. Discussion

Traditional medicines are believed to be safer than chemical products. Therefore, toxicity studies of such formulations do not usually receive as much attention as studies of chemical products. However, some herbal based formulations are potentially toxic and may be harmful to human health. Therefore, scientific knowledge towards oral toxicity of Siddha formulation 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. In the acute toxicity study Siddha formulation Perungaya Chooranam administered at the dose of 2000 mg/kg by oral route and was observed for 14 days. Result of the study shows that treatment with PC did not cause any death in rats. Treated animals showed no evidence of toxicity. Further there is no evidence of behavioral changes, neuro toxicity and cardiovascular toxicity was observed in this study. There were no abnormalities observed on the behavior performance, posture, gait and pupil change; no bizarre behaviors such as salivation and muscle trembling were observed.

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 test drug PC administered at the dose of 20, 200 and 400 mg/kg for 28 days. The body weight changes serve as a sensitive indication of general health status of animals [9,10]. After 28 days of treatment of the PC, all the animals exhibited a normal increment in body weight. It can be stated that leaves of PC did not interfere with the normal metabolism of animals. The significant increment in food and water intake is considered as being responsible for augmentation in body weight gain.Results of the hematological parameters showed no significant changes in the level of RBC, WBC, hemoglobin,platelets, granulocytes and agranulocytes in the treated groups as compared with the control group.

The changes of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) contents is a sensitive index to reflect the degree of liver cell damage [11,12]. 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 [13,14]. In sub-acute study, there were no changes on the common markers of liver function such as ALT and AST and bilirubin. Thus, it may be presumed that test drug PC did not cause significant damage on liver.

In addition, Serum urea nitrogen (BUN) reflect glomerular filtration function, when renal parenchymal was damaged, BUN could increase [15, 16]

Treatment with PC showed no significant changes in blood urea nitrogen (BUN), Uric acid and creatinine when compare to the control group rats. The levels of BUN and creatinine are good indicators of renal function. It was proven from this result that treatment with PC did not attributed to liver and renal functions and this is further confirmed by the histopathological findings of this organ.

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 [17, 18]. Histopathological observation of the vital organs harvested from the control and drug treated group's revealed Regular marginal alignment on the neurons with no signs of oedema or degeneration were observed in the brain. No evidence of pyknotic nucleus was observed in heart. Lungs show normal airway and bronchial secretion. Bronchial blood vessels and connective tissue appears normal with no signs of pulmonary oedema. In liver periportal zone appears normal. No evidence of phagocytosis in intracytoplasmic region. Appearance of proximal and distal convoluted tubules was normal with no evidence of atrophy. Lumen of distal convoluted tubule and collecting duct was normal in the kidney.

5. Conclusion

In conclusion, this study presented the results on the acute and sub-acute toxicity of Siddha formulation Perungaya chooranam that can be very useful for future *in-vivo* and clinical studies. Results of acute toxicity study revealed that test drug PC 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, food consumption, hematology and clinical biochemistry parameters of animals at all groups when compared to control groups. Similarly, there were no histopathological changes in treated animals. By considering the data's obtained from the investigation it may be concluded that Siddha formulation Perungaya chooranam is relatively non-toxic and has high safety margin for short and long term administration.

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