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Safety Evaluation of Siddha Formulation Sangu Chunnam by Acute and Sub-Acute Toxicity Studies in Rats

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Abstract

Siddha system of medicine is believed as a brilliant achievement and symbol of Tamil culture which originated in Southern parts of India. Siddha medicine invented from Dravidian culture and is grown in the time of Indus valley civilization. It is believed that in ancient time, the system was developed by eighteen Siddhar (a class of Tamil sages). In many aspects Siddha system has own philosophy and concept, holistic approach, and lifestyle oriented measures. The majority of the people in developing countries like India use traditional Siddha medicines to treat a number of diseases and ailments. Although, many studies have been undertaken in the past to investigate the potential of Siddha remedies, however, rather little work has been done to assess the safety of such products. Indian drug regulatory authorities strongly recommends the need of the safety profiling of several Siddha formulation as it may be commercialized to global standard.

Sangu Chunnam (SC) is traditional Siddha preparation as indicated for management of versatile clinical condition in humans as per Siddha vedic literature.The main aim of the present investigation is to evaluate the formulation SC 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, 100 and 200 mg/kg/day) of the test drug SC 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 SC 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 SC.Single and repeated oral administration of the Siddha drug SCmay be safe and considered as relatively non-toxic at the varying doses of 20, 100 and 200 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 SC is considerably very safe and doesn't induce any toxicity in the treated animals.

Keywords: Siddha system , Sangu Chunnam, OECD, Preclinical, Acute, Sub-acute.

1. Introduction

India has the unique distinction of having six recognized systems of medicine in this category. They are-Ayurveda, Siddha, Unani and Yoga, Naturopathy and Homoeopathy. Though Homoeopathy came to India in 18th Century, it completely assimilated in to the Indian culture and got enriched like any other traditional system hence it is considered as part of Indian Systems of Medicine [1]. The mineral and metal-based drugs in Siddha System are categorized under the following categories: 1. *Uppu (Lavanam)*- drugs that are dissolved in water and get decrepitated when put into the fire giving rise to vapor. 2. *Pashanam* : drugs that are water insoluble but give off vapors when put in to fire 3. *Uparasam*: Similar to pashanam chemically but have different actions. 4. *Ratnas and uparatnas*, which include drugs based on precious and semi-precious stones 5. *Loham* - metals and metal alloys that do not dissolve in water but melt when put in to fire and solidify on cooling. 6. *Rasam*: drugs that are soft, sublime when put in to fire changing into small crystals or amorphous powders. *7. Gandhakam:* sulphur is insoluble in water and burns off when put into fire. From the above basic drugs compound preparations are derived. From the animal kingdom thirty-five products have been included in the materia medica. It is much similar to preparations used in Ayurveda. Numbers of plant-based preparations are also used in Siddha system of medicine they are quite similar in profile to those mentioned in Ayurveda [2].

Toxicity testing is approaching a pivotal point where it is poised to take advantage of the revolution in biology and biotechnology. The current system is the product of an approach that has addressed advances in science by incrementally expanding test protocols or by adding new tests without evaluating the testing system in light of overall risk-assessment and risk-management needs. That approach has led to a system that is somewhat cumbersome with respect to the cost of testing, the use of laboratory animals, and the time needed to generate and review data. In combination with varied statutory requirements for testing, it has also resulted in a system in which there are substantial differences in chemical testing, with many chemicals not being tested at all despite potential human exposure to them [3].

A principal aim of toxicological research, therefore, is to identify potential risk factors before exposure of humans to toxic substances, in particular where the damage or disease occurs after a long latency period.

A paradigm change is therefore needed here to establish the concept of preventive toxicology.The main objective of the present research work is to evaluate the short and long term safety of the Siddha formulation Sangu Chunnam by acute and sub-acute toxicity studies in rodent.

2. Materials and Methods

2.1.Animal

Healthy adult Wistar albino rats 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/06/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 *Sangu Chunnam* (SC) 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 consist of 12 animals (6 Males and 6 Females). First group served as a control and other three group were treated with test drug SC (20, 100 and 200 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 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 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 analyser.

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 SC on clinical signs of rats in Acute Oral Toxicity Study

The dose of SC 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 Sangu Chunnam 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 SC on Body weight of rats in acute toxicity study

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

Table 2:Effect of Sangu Chunnam on Body weight of rats in acute toxicity study

Dose	Days		
	1	7	14
Control	280.2±42.30	281.4 ± 64.12	282.6 ±26.18
SC 2000 mg/kg	270.4± 21.24	271 ± 3.64	271.4 ± 2
P value (p)*	NS	NS	NS

3.3. Effect of SC 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 SC at low, mid and high dose of 20, 100 and 200 mg/ kg b.w. The results were tabulated in Table 3.

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

Dose	Days				
	1	7	14	21	28
Control	290.2±24.22	291.4 ± 14.24	291.5 ± 25.40	292.5± 35.46	292.4 ± 45.15
Low dose	265.2 ± 46.14	265.4 ± 27.20	267.6± 66.74	268 ± 62.18	268.8± 54.34
Mid dose	270.4± 04.24	270.3 ± 46.54	271.2 ± 68.16	271.4 ± 54.26	272.4 ± 64.70
High dose	250.6± 64.94	250.6 ± 50.53	251.4 ± 52.44	251 ± 24.68	252 ± 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 SC 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 SC at low, mid and high dose of 20, 100 and 200 mg/ kg b.w.The results were tabulated in Table 4 and 5.

Table 4:Effect of Sangu Chunnam on food intake of rats in Sub-acute toxicity study

Dose	Days				
	1	7	14	21	28
Control	36±4.12	36.2±3.12	37.3±2.84	37.2±1.41	38±2.43
Low dose	38.2±1.41	38.3±1.13	38.1±1.21	39.5±1.23	39.5±1.26
Mid dose	35.1±3.32	35.2±3.04	35.2±2.42	36.2±2.61	37.2±1.42
High dose	37.1±1.32	37.1±1.41	37.6±2.62	38.2±1.10	39.6±3.42
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 Sangu Chunnam on water intake of rats in Sub-acute toxicity study

Dose	Days				
	1	7	14	21	28
Control	60.2 ± 1.21	60.6±6.12	62.2±4.10	62±4.12	64.6±1.32
Low dose	62.1±1.10	62.6±2.42	62.9±1.72	63.2±6.86	64.4±1.54
Mid dose	58.1±1.26	58.3±3.21	59.1±6.41	59.4±1.72	59.4±1.82
High dose	54.1±1.41	54.2±1.42	54.4±1.44	54.6±1.52	55.8±2.82
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 SC on Hematological parameters of rats in Sub-acute oral toxicity study

No statistically significant differences were recorded in hematological parameters of rats treated with SC at low, mid and high dose of 20, 100 and 200 mg/ kg b.w.. The results were tabulated in Table 6.

Table 6: Haematological parameters of rats exposed to Sangu Chunnam

Category	Control	Low dose	Mid dose	High dose	P value (p)*
Haemoglobin (g/dl)	15.8±0.68	15.60±0.84	15.8±0.26	15.92±0.65	N.S
Total WBC ($\times10^3$ l)	8.71±0.32	8.75±0.26	8.68±0.27	8.60±1.22	N.S
Neutrophils (%)	29.22±0.01	30.02±0.10	31.11±1.12	32.02±1.02	N.S
lymphocyte (%)	58.12±1.32	58.12±1.12	58.10±2.33	58.20±2.62	N.S
Monocyte (%)	.06±0.02	.06±0.04	.06±0.01	.06±0.06	N.S
Eosinophil (%)	0.2±0.04	0.2±0.02	0.2±0.01	0.2±0.06	N.S
Platelets cells10^3/µl	543.14±3.43	543.41±4.12	544.13±4.0	545.12±2.54	N.S
Total RBC 10^6/µl	7.68±0.12	7.76±0.43	7.69±0.48	7.75±0.26	N.S
PCV%	49.42±0.2	49.42±1.12	49±1.22	49.60±2.21	N.S
MCHC g/dL	31.8±1.32	31.24±1.20	32.18±1.10	32.33±1.12	N.S
MCV fL(µm^3)	57.3±3.20	57.2±1.20	57.9±1.24	57.8±1.22	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 SC on Biochemical parameters of rats in Sub-acute oral toxicity study

No statistically significant differences were recorded in biochemical parameters of rats treated with SC at low, mid and high dose of 20, 100 and 200 mg/ kg b.w. The results were tabulated in Table 7 -9.

Table 7: Biochemical parameters of rats exposed to Sangu Chunnam

Biochemical parameters	Control	Low dose	Mid dose	High dose	P Value (p)*
Glucose (R) (mg/dl)	105.14±8.2	105.16±4.10	106.02±11.10	106.12±6.2	N.S
T.Cholesterol (mg/dl)	108.16±1.42	108.25±1.20	109.62±1.18	109.24±1.63	N.S
Trigly(mg/dl)	64.16±1.42	64.12±1.22	66.16±1.22	66.16±1.22*	N.S
LDL	69.6±2.13	69.12±2.34	69±1.32	.24±12.12	NS
VLDL	13.4±1.32	13.42±4.24	13.24±2.84	13.54±14.16	NS
HDL	22.16±6.12	22.42±2.20	23.18±2.26	24.18±22.12	NS
Ratio 1(T.CHO/HDL)	4.61±1.12	4.62±1.24	4.64±1.14	4.64±2.30	NS
Ratio 2(LDL/HDL)	2.40±1.14	2.41±1.12	2.41±2.20	2.46±10.02	NS
Albumin (g/dL)	4.43±0.16	4.53±0.32	4.44±10.32	4.42±10.48	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 Sangu Chunnam

Parameters	Control	Low dose	Mid dose	High dose	P Value (p)*
Urea (mg/dl)	21.30±0.99	21.20±0.36	21.16±1.18	21.48±1.21	N.S
Creatinine(mg/dl)	0.42±0.02	0.41±0.04	0.42±0.06	0.44±0.08	N.S
Bun(mg/dL)	14.1±0.11	14.10±0.60	14±0.32	14.46±1.12	NS
Uric Acid(mg/dl)	5.00±0.34	5.06±0.21	5.7±0.14*	5.62±0.26	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 Sangu Chunnam

Parameters	Control	Low dose	Mid dose	High dose	P Value (p)*
T Bilirubin(mg/dl).	0.03±0.03	0.03±0.02	0.04±0.02	0.04±0.04	N.S
SGOT/AST(U/L)	139.15±1.33	139.34±0.32	140.01±1.62	140.75±1.02	N.S
SGPT/ALT(U/L)	72.12±1.18	72.22±1.34	72.14±1.28	72.46±0.61	N.S
ALP(U/L)	129.22±3.16	129±12.14	130±14.04*	130.23±11.15*	N.S
T.Protein(g/dL)	8.12±0.34	8.18±0.12	8.16±0.14	8.54±0.49	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 SC on Histopathological changes of rats in Sub-acute oral toxicity study

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

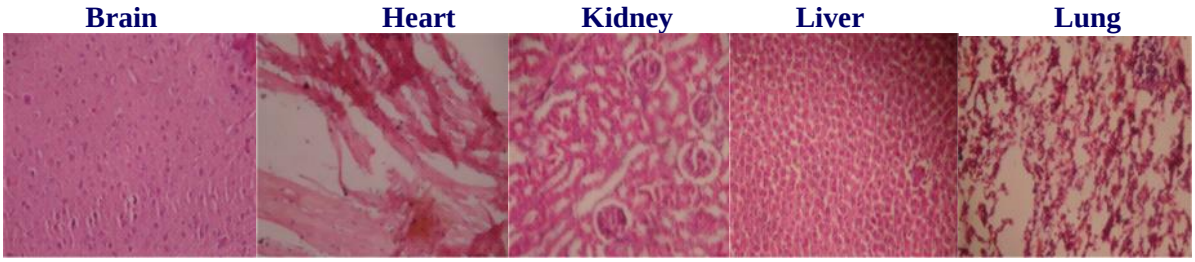

Figure 2: Histopathological representation organs of SC treated rats in Sub-acute oral toxicity study

4. Discussion

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. Acute toxicity is defined as the unwanted effect (s) that occurs either immediately or at a short time interval after a single or multiple administration of such substance within 24 hours. The unwanted (or adverse) effect is any effect that produces functional impairments in organs and/or biochemical lesions, which could alter the functioning of the organism in general or individual organs [9]. Studies of acute toxicity however tends to establish the dose-dependent unwanted (or adverse) effect (s),

which may take place and this includes all information that is important in the assessment of acute toxicity including mortality.

The acute toxicity study may provide initial information on the mode of toxic action of an agent, acts as the basis for classification and helps in deciding the dose of drugs in animal studies. Moreover, if a high dose is found to be survivable, no further acute testing will be conducted [10]. In this study, Siddha formulation Sangu Chunnam administered at the dose of 2000 mg/kg had no adverse effect on the treated rats in up to 14 days of observation. No significant change in the body weight, behavioral and sensory parameters were observed in acute toxicity study.

Apart from mortality, other biological effects and the time of onset, duration and degree of recovery on survived animals, are also important in acute toxicity evaluation. Acute toxicity study solely gives information about LD_{50}, therapeutic index and the degree of safety of a drug under investigation [11]. Since there is no treatment-related toxicity was observed during the acute toxicity evaluation, further testing of sub-acute toxicity study was conducted to evaluate the 28-day repeated daily dose of the srug SC on rats. The selected doses (20, 100 and 200 mg/kg) in this study were informed by the averages of daily consumed regimen of the test drug SC on humans. Results of the study reveals that 28-day daily dose treatment with the SC elicited no clinical signs of toxicity, morbidity, or mortality across all the treatment groups hence it may concluded that the formulation SC is safe at the tested doses over the observation period.

There is no significant difference observed in the RBC indices which suggested that the SC does not affect erythropoiesis, morphology, or osmotic fragility of red lood cells. WBC's are the first line of cellular defines that respond to infectious agents, tissue injury, or any inflammation. Furthermore, no significant changes were observed in neutrophils, lymphocytes, and monocytes in rats treated with SC at all three dose levels suggesting that the drug SC might not have exerted challenge on the immune system of the animals.

Additionally, most of the biochemical parameters were not altered. No relevant changes were found in levels of ALT, AST, ALP, or creatinine, which are good indicators of liver and kidney functions [12]. No gross lesions were found in histopathology examinations. Therefore, this study indicates that treatment with SC do not cause dose dependent toxicity in the tested animals. Kidney damage may be ascertained by measurements of urea, uric acid, creatinine levels and their deviations from normal in their serum concentrations are a tentative pointer to nephrotic injury [13]. In this sub-acute toxicity study, there is no significant difference observed in the kidney function indices in the SC drug treated animals is suggestive of normal renal function and further supports the nontoxic tendency of the medicine.

Histopathology of kidney, liver, brain, lung and heart of rats treated with SC at the dose of 20, 100 and 200 mg/kg ,b.w. shows normal histomorphological structure of all vital organs were when compare to that of the control. Arrangement of the neurons appears intact with no signs of degeneration or apoptotic changes were observed. Myocardial fiber mass appears denser with no signs of degeneration or fibrosis were observed. Microscopic examination of lung revealed normal alveoli and alveolar sac with no signs of infiltration. Appearance of portal triad was normal with no signs of inflammatory cell infiltration. Liver parenchyma appears normal with no evidence of necrosis were observed. Appearance of proximal and distal convolutes tubules was normal. No evidence of atrophy was observed. Further there is no significant degeneration or inflammatory changes observed in any of the vital organs of control and drug treated groups.

5. Conclusion

The results strongly suggest that the Siddha drug Sangu Chunnam is safe and well tolerated at the tested oral doses since no deleterious changes were observed in animal macro-parameters, behavior and habits, hematology, serum biochemistry and histopathological parameters. The overall result of toxicological profiling of SC provides valuable evidence based data that shows the drug is relatively non-toxic, causes no apparent organ damage or mortality in both the short term and long term administration on study animals. Hence from the results, it was concluded that the drug SC were safe and no significant toxicity related events will be encountered while using this for long term treatment for the chronic disease condition.

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