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Evaluation of the acute and sub-acute toxicity of the ethanolic extract of *Pericampylus glaucus* (Lam.) Merr. in BALB/c mice

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ABSTRACT

Objective: To evaluate the safety dose range of ethanolic extract from the leaves of *Pericampylus glaucus* (Lam.) Merr. by acute and sub-acute oral toxicity study on animal model.

Methods: The acute and sub-acute toxicity study was carried out as per Organization for Economic Co-operation and Development guidelines 423 and 407. In acute toxicity study, the oral dose (300, 2000 and 4000 mg/kg) of tested plant extract was administered to three groups in single dose and general behavior, adverse effects and mortality were determined up to 72 h and compared to normal group. In sub-acute study, the tested crude plant extract was administered orally at doses of 600 and 1000 mg/kg for 28 days to the two animals groups and their body weight, hematological, serum hepatic biochemical parameters were evaluated and compared to normal group by sacrificing all group animals.

Results: In acute toxicity, all treated groups' revealed neither mortality nor any significant alteration in behavior only drowsiness, sedation and lethargy were observed in two group, *i.e.* 2000 and 4000 mg/kg of the tested plant extract. In sub-acute toxicity study no change in hematological, biochemical parameter and organ body weight were observed during study compared to the normal group. The kidney function parameters [serum glutamic-oxaloacetic transaminase (aspartate transaminase), serum glutamic pyruvic transaminase (alanine transaminase)] were significantly increased following administration of tested crude plant extract (600, 1000 mg/kg).

Conclusions: The result indicates that the oral administration of *Pericampylus glaucus* (Lam.) Merr. extract did not produce any significant toxic effect in BALB/c mice. Hence, the extract can be utilized safely for therapeutic use in pharmaceutical formulations.

1. Introduction

The use of traditional medicine is an important part in Malaysian culture and was practiced by ancients long before the introduction of modern medicine. A complete report on the Malay traditional medicinal plants were compiled in a book entitled "A Dictionary of the Economic Products of the Malay Peninsula". This book had become the starting point for the phytochemists and ethno-botanist to do some studies and research relating to the medicinal plants. All of these works add a comprehensive knowledge to the account of Malaysian medicinal plants[1,2]. In Malaysia, the plants of the Menispermaceae family are known to be rich source of bioactive compounds that have significant role in curing of various diseases. The genus *Pericampylus glaucus* (Lam.) Merr. (*P. glaucus*) belongs to the family of Menispermaceae and is commonly found in ground and forest area of the whole Malaysia. It also occurs in Thailand, India, China, Indonesia, Myanmar, Taiwan, Philippine, and Vietnam[3]. In Malaysia the traditional name of the plant is commonly known as "akar chuping"[4]. The plant is climber shrubs and the young stems are often long slender and yellowish in

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colors, while the old stems are glabrescent. The leaves are spiral, simple with no stipules. The petioles are 2–4 cm long. The blade is papery, velvety underneath, yellowish or glaucous and 2.9 cm × 3.1 cm–5.5 cm × 6.0 cm. The apex is round and the base is flat and the margin is crenate. The blade has 3 to 5 pairs of secondary nerves and can be seen. The fruits are glaucous berries, horseshoe-shaped and their diameter is 6–7 mm. The seeds are spiny with 3 mm × 5 mm lengthy[5]. In traditional system of medicine various parts of the plant are used in variety of diseases. In Malaysia, the roots are traditionally claimed to be effective against diabetes[6]. In Taiwan, the stems and roots are effectively used to stop bleeding, inflammation, arthritis, sore throat, abdominal pain, productive cough, colds, headache, abdominal distention, diaphoretic[7]. In Bangladesh, the plant is also connected for various ailment and are claimed to be effective for the relief of fever, loss of movement tongue, muscles pain, joint pain, muscle disorder, hepatic disorder, diabetes, malaria and edema[8]. In Indonesia, the plant is used to counteract hair loss and to resolve swelling of the spleen[3]. The fruits are claimed for constipation[9]. The leaves and stem of the plant are used by Vietnam people against snake biting[10]. In China, the boiling roots of the plant are used for treatment of cough, laryngitis, pulmonary disease and the leaves are used for fractures[11]. Despite the traditional uses of the plant, there is little *in vitro* investigations have been published. The alkaloids isolated from plant have been proved a significant effect against chronic diseases like hepatitis B and HIV virus[11]. Triterpenes isolated from plant have been reported *in vitro* anticancer activity[12]. Nowadays in all over the world people give preference to plant origin drugs as a source for medication, because of undesirable effects of synthetic drugs, which are believed to be suitable for chronic treatment. Traditional plants might provide new compounds, which can counter the high cost and toxic effects of the current medicines for many rural populations in developing countries. As no work has been done on ingestion of *P. glaucus* at high doses, the systemic approach in evaluating their efficacy and safety profile is needed. Therefore, the present study was aimed to evaluate the safety of *P. glaucus* leaves extract with acute and sub-acute toxicity tests in BALB/c mice.

2. Materials and methods

2.1. Collection and extraction of the plant

The whole plant of *P. glaucus* was collected in the month of September 2014 from village Kampung Jeram Kedah, Negeri Sembilan, Malaysia and authenticated by Ms. Tan Ai Lee at Forest Research Institute Malaysia, Malaysia where voucher specimen herbarium with number (SBID: 014/14) was deposited at the Faculty of Pharmacy. After washing the plant with running water, the leaves were separated and dried in shade for 20 days at room temperature. After shade drying, the leaves were grinded through blender and converted into coarse of powder. The powder was extracted by continuous hot extraction using the Soxhlet apparatus at a temperature of 78 °C for 48 h using 95% ethanol. The extract was then concentrated under reduce pressure through rotary evaporator (N-10000, Eyela, Japan). The extracts were collected and preserved in a desiccator until used for further studies.

2.2. Test animal

Adult healthy BALB/c mice weighting 20–30 g were used and kept in the animal house of the Department of Pharmacology, Lincoln University College, Malaysia. The animals were kept in plastic cages (34 × 47 × 18 cm^3) at animal house, in an air conditioned environment with five mice in each cage and maintained at room temperature of (25 ± 2) °C with relative humidity (60% ± 10%) under 12 h night and light cycle. The animals used for the experiment were approved by animal ethics committee of the Lincoln University College, Malaysia.

2.3. Acute toxicity study

The oral acute toxicity study of ethanolic extract of *P. glaucus* was evaluated according to Organization for Economic Co-operation and Development (OECD) guideline 423 on BALB/c mice (20–30 g)[13], where the limit test dose of 4 000 mg/kg was used. All the animals were kept at overnight fasting before to every experiment with free excess to water. The animals were divided into four groups, each comprising 5 animals. The 1st group served as a negative control, while 2nd, 3rd and 4th was considered as tested groups received orally *P. glaucus* (dissolved in normal saline) extract at dose of 300 mg/kg, 2 000 mg/kg and 4 000 mg/kg. Before dose administration, the body weight of each animal was determined and the dose was calculated according to the body weight. The animals were observed for any toxic effect for first 4 h after the treatment period. Further animals were investigated for a period of 3 days for any toxic effect. Behavioral changes and other parameters such as body weight, urinations, food intake, water intake, respiration, convulsion, tremor, temperature, constipations, changes in eye and skin colors, *etc*.

2.4. Sub-acute toxicity study

The oral sub-acute toxicity study was carried out according to OECD guideline 407[14]. Adult healthy BALB/c mice (20–30 g) of each sex were divided into 3 groups of 5 animals each and were placed under standard conditions. Group I was considered as control and the other two groups which were considered as tested groups received the plant extract at a dose of 600 and 1 000 mg/kg body weight respectively for 28 consecutive days[15].

2.5. Hematological and biochemical examination

On 29th day, all the animals were sacrificed by an anesthesia (chloroform) after an overnight fasting (8 h). The blood sample was collected into test tube with and without ethylene diamine tetra acetic acid as an anticoagulant respectively for biochemical and hematological parameters. The blood without the ethylene diamine tetra acetic acid was evaluated for biochemical analysis, allowed to clot after centrifugation at 2 500 r/min for 15 min to obtain serum and stored at −20 °C until assayed for biochemical estimation. After collecting the blood, all the vital organs such as liver, kidney, heart, pancreas and small intestine were separated, weighed each organ on electronic balance and relative organ body weight of both test treated groups were determined and compared to control group. The relative organ weight (ROW) of each organ was calculated as follows[16]:

ROW = (Absolute organ weight (g)
/Rat body weight on sacrifice day) × 100

2.6. Effect of plant extract on hematological parameters

Red blood cell count, hematocrit, mean cell volume, hemoglobin, white blood cell count, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, monocyte, neutrophil, lymphocyte and platelet count of the control and plant treated groups were determined and compared with control group using an automatic haemotology analyzer (Sysmex K21, Tokyo, Japan).

2.7. Effect of plant extract on serum biochemical parameters

The biochemical analysis were done on serum after centrifugation of collected blood and the following parameters like aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase, high density lipoprotein, total bilirubin (T-BIL), total protein, albumin, urea and creatinine level were determined for both control and extract treated groups. All analyses were determined on using clinical chemistry analyzer (Vital Scientific, Netherlands).

2.8. Statistical analysis

Statistical analysis was performed as mean of variance ± SEM (n = 5) followed by ANOVA test using Graph Pad Prism and for multiple comparison test among the groups, Bonferroni test was performed. A probability level of $P < 0.001$ was accepted statistically.

3. Results

3.1. Acute toxicity study

The acute toxic effect of ethanolic extract was determined as per the OECD guideline 423, where the limit test dose of 4000 mg/kg was used. No treatment related toxic symptom or mortality were observed after oral administration of the tested plant extract at a dose of 300, 2000 and 4000 mg/kg. The general behavioral of the extract treated animals and control group was observed first for short period (4 h) followed by long period (72 h), did not display any drug related changes in behavior, breathing, skin effects, water consumption, impairment in food intake and temperature. Therefore, the extract seems to be safe at a dose level of 4000 mg/kg, and the LD_{50} was considered be >4000 mg/kg. However, there were sign of sedation, lethargy and drowsiness after the administration of plant extract at dose of 0.6 g/kg and 1.0 g/kg, compared to control group. The parameters observed for acute toxicity study after the administration of the test plant extract compared with normal group are presented in (Table 1).

3.2. Sub-acute toxicity study

The sub-acute toxic study of the tested plant extract was determined as per OECD guideline 407. All the tested group animals treated with plant extract at a dose of 600 and 1000 mg/kg daily survived throughout the 28 days. No clinical toxicity signs were observed in the plant treated group compared to the control group.

Table 1
General appearance and behavioral observations of acute toxicity study for control and treated groups.

Observation	Control group	300 mg/kg	2000 mg/kg	4000 mg/kg
Digestion	NO	NO	NO	NO
Body weight	Normal	Not change	Not change	Not change
Temperature	Normal	Normal	Normal	Normal
Food intake	Normal	Normal	Normal	Normal
Urination	Normal	No effect	No effect	No effect
Rate of respiration	Normal	No effect	No Effect	No effect
Change in skin	No effect	No effect	No effect	No effect
Drowsiness	Not present	Not present	Present	Present
Sedation	No effect	No effect	Observed	Observed
Eye color	No effect	No effect	No effect	No effect
Diarrhea	Not present	Not present	Not present	Not present
General physique	Normal	Normal	Lethargy	Lethargy
Coma	Not present	Not present	Not present	Not present
Death	Alive	Alive	Alive	Alive

NO: Not observed.

3.3. Effect of plant extract on relative organ body weight

There was no significant difference in average organs and relative organs weight between control and extract treated group at a dose of 600 and 1000 mg/kg. The effect plant tested extract on principal organ weights relative to body weight are shown in Tables 2 and 3 and Figures 1–3. There were no significant

Table 2
Effect of oral administration of ethanol extract of *P. glaucus* on average organ weight (g) of mice.

Organ	Average organ weight		
	Normal	600 mg/kg	1000 mg/kg
Liver	0.872 ± 0.016	0.866 ± 0.016	0.890 ± 0.053
Kidney	0.338 ± 0.011	0.333 ± 0.030	0.355 ± 0.020
Pancreas	0.134 ± 0.011	0.141 ± 0.013	0.129 ± 0.007
Heart	0.161 ± 0.003	0.152 ± 0.010	0.146 ± 0.021
Intestine	0.663 ± 0.019	0.652 ± 0.020	0.662 ± 0.022
Average body weight on the sacrifice day	25.530 ± 0.140	25.370 ± 0.009	27.500 ± 0.003

Values are expressed as mean ± SEM. $P > 0.05$ when compared to control group.

Table 3
Effect of oral administration of ethanol extract of *P. glaucus* on relative organs weight (g) of mice.

Organ	Relative organs weight		
	Normal group	600 mg/kg extract	1000 mg/kg extract
Liver	3.410 ± 0.016	3.343 ± 0.016	3.230 ± 0.053
Kidney	1.320 ± 0.011	1.310 ± 0.030	1.290 ± 0.020
Pancreas	0.524 ± 0.011	0.554 ± 0.013	0.470 ± 0.007
Heart	0.630 ± 0.003	0.599 ± 0.020	0.530 ± 0.021
Intestine	2.590 ± 0.019	2.560 ± 0.020	2.400 ± 0.022

Values are expressed as mean ± SEM. $P > 0.05$ when compared to control.

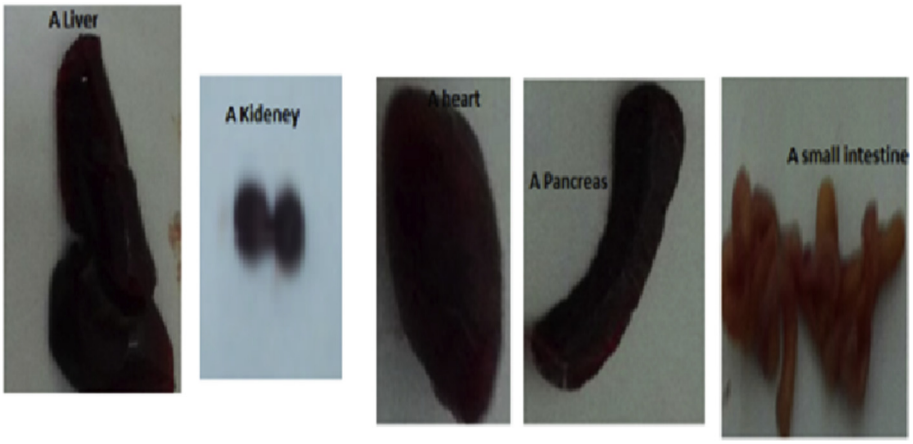

Figure 1. Group-II vital organ of control group.

Figure 2. Group-II treated with 600 mg/kg extract.

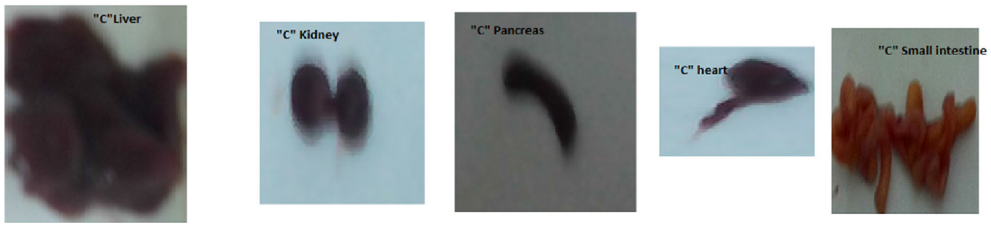

Figure 3. Vital organ of Group-III treated with 1000 mg/kg of *P. glaucus* extract.

differences in the changes of each weight. The results revealed that, the vital organs such as liver, kidney, heart, pancreas and small intestine were not adversely affected throughout the treatment by extract. The average and relative organ weight of tested plant extract and control treated groups shown statistically non-significant differences ($P > 0.05$).

3.4. Effect of plant extract on hematological parameters

The results of the hematological tests are summarized in (Table 4 and Figure 1). All the tested hematological parameters including total blood count, hemoglobin, red blood cell, total white blood cell, neutrophil, monocyte, lymphocyte, packed cell volume, and platelet count were within normal limits compared to control group. No toxicologically significant differences ($P > 0.05$) between treated animals with the plant extract and control were found. There were generally no significant differences noted between control and treated groups for the hematological parameters measured.

3.5. Effect of plant extract on biochemical parameters

The results of the various biochemical tests on the experimentally treated animals with the plant extract and normal group are summarized in (Table 5 and Figure 2). Oral administration of the plant treated extract at a dose of 600 and 1 000 mg/kg did not cause significant changes in serum biochemical parameters such as albumin, total protein, globulin, T-BIL, urea, sodium, creatinine and uric acid levels when compared to control group. However, SGOT (AST) and SGPT (ALT) were statistically difference in plant treated group at dose of 600 and 1 000 mg/kg when compared to control group mice. There was a significant increase in (AST) and SGPT (ALT) level ($P < 0.001$).

Table 4
Effect of oral *P. glaucus* extract on hematological parameters.

Parameters	Normal group	600 mg/kg extract	1 000 mg/kg extract
Total RBC (10^{12}/L)	8.625 ± 0.517	10.170 ± 0.933	9.050 ± 0.710
Hemoglobin (g/L)	11.500 ± 0.610	10.220 ± 1.520	12.270 ± 1.300
PCV (L/L)	41.500 ± 2.020	39.500 ± 2.100	38.460 ± 2.940
MCV (fL)	46.250 ± 1.315	42.250 ± 3.940	49.300 ± 2.500
MCH (pg)	15.000 ± 1.000	14.500 ± 0.645	15.760 ± 0.564
MCHC (g/dL)	33.500 ± 2.900	35.500 ± 0.913	36.000 ± 0.500
Platelet count (10^9/L)	308.000 ± 33.800	326.500 ± 27.600	292.010 ± 34.600
WBC (10^9/L)	11.500 ± 1.041	9.000 ± 1.034	10.210 ± 1.470
Neutrophil (%)	26.250 ± 2.680	27.500 ± 1.930	28.000 ± 2.790
Lymphocyte (%)	53.250 ± 5.150	60.250 ± 4.190	58.250 ± 2.600
Monocyte (%)	4.000 ± 1.080	3.500 ± 1.190	2.500 ± 0.645

Values are expressed as mean ± SEM. $P > 0.05$ when compared to normal control group.

Table 5
Effect of oral *P. glaucus* extract on biochemical parameters.

Parameters	Normal group	600 mg/kg extract	1 000 mg/kg extract
Urea (mg/dL)	32.50 ± 3.20	28.70 ± 0.40	27.60 ± 0.10
Creatinine (mg/dL)	1.05 ± 0.15	0.80 ± 0.40	0.80 ± 0.70
Uric acid (mg/dL)	0.51 ± 0.39	0.15 ± 0.05	0.19 ± 0.01
Total cholesterol (mg/dL)	115.90 ± 14.01	107.00 ± 0.50	104.50 ± 17.30
Total protein (g/dL)	6.30 ± 0.90	6.25 ± 0.95	5.85 ± 0.35
Bilirubin (g/dL)	0.35 ± 0.05	0.75 ± 0.25	0.50 ± 0.20
Albumin (g/dL)	2.80 ± 0.30	2.50 ± 0.10	3.50 ± 0.45
Globulin (g/dL)	34.00 ± 3.00	29.00 ± 1.00	29.00 ± 0.05
SGOT (AST) (IU/L)	114.00 ± 25.00	342.50 ± 27.50***	314.50 ± 16.50***
SGPT (ALT) (IU/L)	36.50 ± 1.50	154.50 ± 42.50***	165.50 ± 31.00***

Data are expresses as mean ± SEM. ***$P < 0.001$ when compared to control group.

4. Discussion

According to the World Health Organization, 80% of the remote area population rely on traditional medicine and the history of medicinal plants used by human as a medicine is about 60 000 year old[17]. The uses of medicinal plants as a source of drugs in primary health care have become popular universally, particularly in developing countries as a safe because of natural source. The bioactive compound isolated from herbal plants are believe to be harmless without causing any side effect on health, and thus is widely used as OTC-medication[18]. Plant origin drugs are known to play a vital role in the management various chronic diseases and have received a great preference by researcher as alternatives source to allopathic pharmaceutical drugs in recent times[19]. The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human and environment. Malaysia has plenty of medicinal and more than 1 300 medicinal plant species are found in Peninsular Malaysia alone and the use of medicinal plants as a source of drugs by Malaysian has always been some thousands of year ago[20]. However, there is a lack of proven scientific studies on the toxicity and adverse effect of these treatments. Therefore, the present research was aimed to evaluate the ethanol leaf extract of *P. glaucus* for acute and sub-acute toxicity study and to identify the range of dose that could be used for further studies. The oral acute toxicity study of the tested plant extract was carried out on BALB/c mice at single dose of 300, 2 000 and 4 000 mg/kg body weight and was continuously monitored for first 4 h, followed for a period of 72 h for any toxic effect after the treatment period. No major changes in behavior and mortality were observed in all groups. However, sedation, lethargy and drowsiness were confirmed in 2 000 and 4 000 mg/kg body weight in treated group. The extract seems to be safe at a dose level of 4 000 mg/kg, and the LD_{50} is considered be >4 000 mg/kg. Any pharmaceutical drug or compound with the oral LD_{50} higher than 1 000 mg/kg could be considered safe and low toxic[21]. This suggests that the ethanolic extract of *P. glaucus* is practically non-toxic in single dose of level 4 000 mg/kg body weight. However in case of multiple dose uses in the treatment of the chronic disorder like cancer, diabetes or hyperlipidemia, whether it will be safe and have no effect on relative organ weight, hematological and biochemical parameters that can be confirmed from its sub-acute toxicity study. A sub-acute toxicity study was therefore carried out with doses of 0.6 and 1 g/kg of extract as per OECD guideline[22]. Decreases or increases in the body weights are associated with toxic effects of chemicals and drugs. However, scientific evidence confirmed that increases or decreases in the body weights are accompanied with accumulation of fats and physiological adaptation responses to the plant extracts rather than to the toxic effects of chemicals or drugs that lead to decrease appetite and, hence, lower caloric intake by the animal[23]. The relative weight of the vital organs like liver, kidney, heart, pancreas and small intestine were found normal indicating no toxic effect in both control and treated group and was statistically non-significant differences ($P > 0.05$). The absence of any significant differences in the liver, kidney, heart and small intestine weight provides support for the safety of *P. glaucus*. After 28 days of

treatment with tested plant extract, the hematological parameters showed no significance $P > 0.05$ when compared to control group. The bone marrow is responsible for the production of the blood cell and some phytochemicals isolated from plant have affect red blood cell levels[24]. Hence, the tested plant extract may not have harmful effects on bone marrow function and justify the fact that at all doses of *P. glaucus* does not induce anemia, making it safe. Similarly, estimation of serum biochemical parameters in treated animals showed non-significance ($P > 0.05$) compared to control group. However, the transaminases enzyme SGOT (AST) and SGPT (ALT) were observed positive and showed a remarkable significant elevation ($P < 0.001$) in plant treated animal for 600 and 1 000 mg/kg extract as compared to respective control group. Many studies have confirmed that elevated serum levels of hepatic enzymes, transaminases (SGPT and SGOT) are not a directly linked for liver injury but increase levels are responsible to cause inflammation, cellular leakage and damage of cell membrane to cells in the liver[25]. The main target organ for drug or bioactive active compound is liver where exposed to the foreign substances being absorbed in intestines and metabolized to other compounds which may or may not be hepatotoxic to the mice[26]. Therefore, the increase in liver hepatic enzyme (SGPT and SGOT) after administration of the ethanolic plant extract might be because of certain phytochemical compound that might have toxic potential on liver with increasing dose and result liver injury. However, these changes may not be toxicologically significant, as they were not corroborated by the biochemical findings (ALT, AST). Further specific assays of toxicity and more histological study could provide more information regarding to the toxic effect of the extract on liver.

This study provides very important data on the acute and sub-acute toxicity profile of the ethanolic extract of *P. glaucus* that should be very useful for any future *in vivo* and clinical study of this plant medicine. *P. glaucus* extract was found to be less toxic when oral sub-acute toxicities in mice were performed. These results showed that the use of extract of *P. glaucus* is safe and explained the extensive use of the plant as a traditional medicine in Malaysia.

Conflict of interest statement

The authors report no conflict of interest.

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