Department of Pharmacognosy[1], School of Pharmacy, Second Military Medical University, Shanghai; Institute of Botany[2], Jiangsu Province and Chinese Academy of Sciences, Nanjing; Center for Disease Control and Prevention[3], Jinan Military Region, Shandong, People's Republic of China

Hepatotoxicity of kaurene glycosides from *Xanthium strumarium* L. fruits in mice

YANG WANG[1,2], TING HAN[1], LI-MING XUE[1], PING HAN[3], QIAO-YAN ZHANG[3], BAO-KANG HUANG[1], HONG ZHANG[1], QIAN-LIANG MING[1], WEI PENG[1], LU-PING QIN[1]

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Lu-Ping Qin, Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, Shanghai 200433, People's Republic of China;
qinsmmu@126.com
Ting Han, Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, Shanghai 200433, People's Republic of China
than927@163.com

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The fruit of *Xanthium strumarium* L. (Cang-Er-Zi) is a traditional Chinese medicine that is used in curing nasal diseases and headache according to the *Chinese Pharmacopoeia*. However, clinical utilization of *Xanthium strumarium* is relatively limited because of its toxicity. The present investigation was carried out to evaluate the toxic effects on acute liver injury in mice of the two kaurene glycosides (atractyloside and carbxyatractyloside), which are main toxic constituents isolated from Fructus Xanthii on acute liver injury in mice. Histopathological examinations revealed that there were not obviously visible injury in lungs, heart, spleen, and the central nervous system in the mice by intraperitoneal injection of atractyloside (ATR, at the doses 50,125 and 200 mg/kg) and carbxyatractyloside (CATR, at the doses 50,100 and 150 mg/kg) for 5 days. However, it revealed extensive liver injuries compared with the normal group. In the determination of enzyme levels in serum, intraperitoneal injection of ATR and CATR resulted in significantly elevated serum alanine aminotransferase (ALT), asparate aminotransferase (AST), alkaline phosphatase (ALP) activities compared to controls. In the hepatic oxidative stress level, antioxidant-related enzyme activity assays showed that ATR and CATR administration significantly increased hepatic malondialdehyde (MDA) concentration, as well as decreased superoxide dismutase (SOD), catalase (CAT) activities and glutathione (GSH) concentration, and this was in good agreement with the results of serum aminotransferase activity and histopathological examinations. Taken together, our results demonstrate that kaurene glycosides induce hepatotoxicity in mice by way of its induction of oxidative stress as lipid peroxidation in liver, which merited further studies. Therefore, these toxic constituents explain, at least in part, the hepatotoxicity of *X. strumarium* L. in traditional medicine.

1. Introduction

The fruit of *Xanthium strumarium* L., commonly known as cocklebur fruit with Chinese name "Cang-Er-Zi", is recorded in the current Chinese Pharmacopoeia, which was popularly used as traditional Chinese medicine for treating nasal sinusitis, headache caused by wind-cold, urticaria and arthritis. However, clinical utilization of *X. strumarium* L. is relatively limited because of its toxicity. It is reportedly hepatotoxic in humans and farm animals (Zhang and Zhang 2003; Goodwin et al. 1992; Witte et al. 1990; Cole et al. 1980). The main toxic components in *X. strumarium* L. are regarded as atractyloside (ATR, 1) (Georgiou et al. 1988) and carbxyatractyloside (CATR, 2) (Cole et al. 1980), which also exist in *Callilepsis laureola* (Mokhobo 1976) and *Atractylis gummifera* (Larrey 1997). The underlying mechanisms to explain the hepatotoxic effects remain unclear, although the hepatotoxic effects of ATR has been documented. Moreover, studies on hepatotoxicity of CATR are absent and thus further investigation is needed.

Fatal liver necrosis in Africa and the Mediterranean regions is common in children following ingestion of traditional herbal remedies that contain atractyloside (ATR), a diterpenoid glycoside. ATR is a well described, potent hepatotoxin (Georgiou et al. 1988); and most of the toxicity data on this compound have been derived primarily from human poisonings arising from ingestion of ATR-containing plants (Wainwright et al. 1977). The toxicity of ATR is reportedly caused by the inhibition of energetic pathway (Allmann et al. 1967) and GSH depletion in liver slices (Obatomi et al. 1998). ATR is an important biochemical tool because it inhibits oxidative phosphorylation in the outer membranes of isolated mitochondria. However, this may not be the only mechanism by which it causes toxicity.

There are reports that oxidative stress plays a crucial role in hepatotoxicity induced by alcohol, carbon tetrachloride, chromium, etc. (Bagchi et al. 2002; Conde de la Rosa et al. 2008). ROS produced during oxidative stress are highly reactive, and may therefore modify and inactivate lipids, proteins, DNA, RNA and induce cellular dysfunctions. To prevent ROS-induced cellular

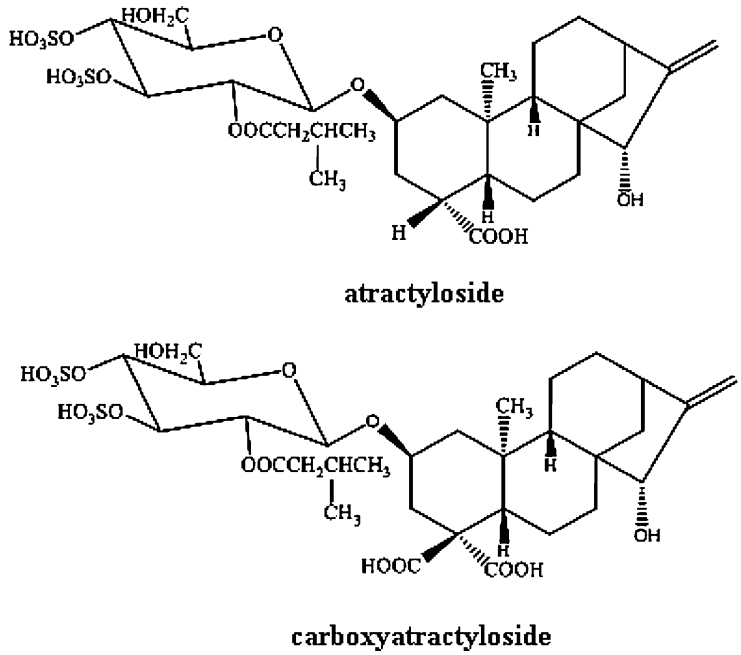

Fig. 1: Chemical structures of atractyloside (ATR) and carboxyatractyloside (CATR).

damage, the body has developed the antioxidant system, which includes antioxidant enzymes such as catalase, superoxide dismutase and low-molecular antioxidants such as glutathione and some plasma protein. Generally, cellular antioxidants maintain a point of balance and prevent the damage of the organism induced by ROS.

As part of our continuing investigation of this plant, we isolated and identified two kaurene glycosides compounds. According to the Chinese Pharmacopoeia, the clinical dose for *X. strumarium* L. is 3–9 g/d, which does not cause side effects. *X. strumarium* L. can cause toxicity in long-term high doses (Zhang and Zhang 2003; Niu 2002). Our preliminary surveys have shown that atractyloside (ATR) and carbxyatractyloside (CATR) are the toxic components, which can produce similar toxic reactions with *X. strumarium* L. in high doses. So the present study focuses on the hepatotoxic evaluation and the possible mechanism of the two kaurene glycosidic compounds in mice. The present study was designed to observe the oxidative injury in liver induced by ATR and CATR and aims to provide a scientific basis for clinical safety evaluation of the plant.

2. Investigations and results

2.1. Histopathological change

In order to estimate toxicity of the two compounds, we prepared three different doses of each tested compound. There was no obviously visible injury in the lung, heart, spleen, kidney and the central nervous system for histopathological examination in the mice after intraperitoneal injection of ATR (at the doses 50,125 and 200 mg/kg) and CATR (at the doses 50,100 and 150 mg/kg) for 5 days. ATR and CATR induced obvious toxic liver injury according to the results of histopathological examination (Fig. 2). The normal liver histological sections showed that hepatocytes were well-preserved and uniform cytoplasm, prominent nucleus, nucleolus and central central veins were visible (Fig. 2-A). No changes were identified in 50 mg/kg ATR groups at a light microscopic level (Fig. 2-B). Compared with the normal group, liver tissue from mice treated with ATR at doses of 200 mg/kg body wt. revealed extensive liver injuries, characterized by severe hepatocellular degeneration and necrosis around the central vein, fatty changes, inflammatory cell infiltration, congestion, cytoplasmic vacuolation, massive fatty degeneration and the loss of cellular boundaries (Fig. 2-D). In addition, the histopathological hepatic lesions induced by administration of CATR were similar to ATR. Hepatocellular changes ranged

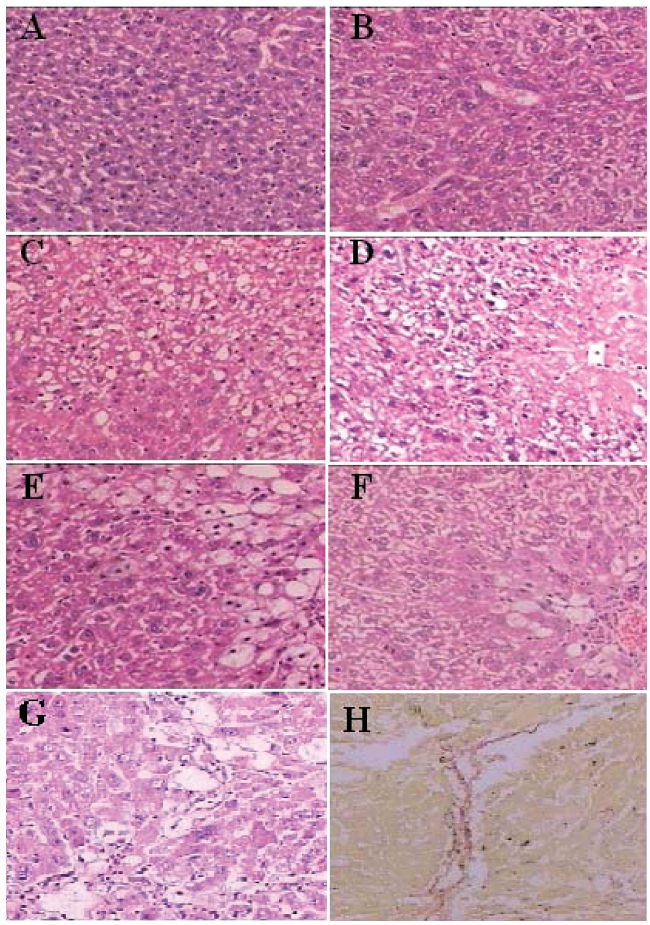

Fig. 2: Histopathlogy of the liver (×100).
A: Normal control. B: ATR(50 mg/kg) C: ATR (125 mg/kg) D: ATR (200 mg/kg)
E: CATR (50 mg/kg) F: CATR (100 mg/kg) G-H: CATR (150 mg/kg).

Table 1: Effects of ATR and CATR on serum transaminase level

Groups	ALT(u/l)	AST(u/l)	ALP(u/l)
Normal control	30.0 ± 4.8	93.8 ± 17.5	183.0 ± 20.8
ATR(50 mg/kg)	36.1 ± 11.4	97.4 ± 6.1	185.8 ± 15.4
ATR (125 mg/kg)	$39.9 \pm 6.4^{*}$	100.9 ± 12.5	198.4 ± 24.9
ATR (200 mg/kg)	$141.5 \pm 31.8^{**}$	$491.2 \pm 86.5^{**}$	$242.6 \pm 33.4^{*}$
CATR (50 mg/kg)	33.6 ± 5.2	101.6 ± 17.0	186.2 ± 19.9
CATR (100 mg/kg)	39.1 ± 10.3	$150.2 \pm 94.3^{*}$	$197.6 \pm 33.7^{*}$
CATR (150 mg/kg)	$107.7 \pm 13.4^{*}$	$561.8 \pm 64.1^{**}$	$268.2 \pm 24.9^{*}$

Values are expressed as mean ± SD of ten mice in each group
* $P < 0.05$,
** $P < 0.01$, compared with normal control group

from middle to marked centrilobular hepatocellular necrosis and vacuolation, fibrosis in portal areas was evident in all animals with CATR at doses of 150 mg/kg body wt (Fig. 2-G). In addition, special stains of collagen fibers showed that most liver cells was edematous, with obvious fibrosis (Fig. 2-H). Some animals were noted to show middle hepatocellular vacuolation and periportal hepatocytes necrosis and multifocal inflammation that was associated with single cell necrosis. These results improved ATR and CATR induced hepatocellular injury at the high doses.

2.2. Serum ALT, AST and ALP level

The results are presented in Table 1, which shows the effects of the tested compounds on the clinical chemistry parameters at different doses. No liver injury was found at doses of 50 mg/kg and 125 mg/kg body wt. of ATR. On the other hand, significant changes in clinical chemistry parameters were observed by intraperitoneal administration of ATR at the dose of 200 mg/kg. The serum ALT, AST and ALP levels in the 200 mg/kg ATR treated group were increased by 371.7% ($P < 0.01$), 423.7% ($P < 0.01$) and 32.6% ($P < 0.05$), respectively, compared to controls. The activities of serum ALT, AST and ALP after intraperitoneal administration of CATR at doses of 100 mg/kg body wt. were much higher than those in the normal group. Intraperitoneal administration of CATR at doses of 150 mg/kg body wt. gave rise to serum transaminase level of ALT 259.0% ($P < 0.05$), AST 498.9% ($P < 0.01$) and ALP 46.6% ($P < 0.05$), respectively, indicating that the toxic effect of CATR displayed in a dose-dependent manner. The presented data provide pharmacological support for the hepatotoxic effects of ATR and CATR.

2.3. Biochemical parameters of liver tissue

MDA is one of the main end products of LPO, and our results show that the MDA concentration was significantly increased in ATR (200 mg/kg)-treated livers (see Table 2), which indicates ATR induced LPO injury in liver. SOD, GST and CAT are all important antioxidative enzymes in mammalian cells. The SOD, GST and CAT activities were decreased in ATR (200 mg/kg)-treated livers. As shown in Table 2, the MDA concentration was significantly increased in CATR (150 mg/kg)-treated livers. The hepatic SOD activity in the CATR (150 mg/kg) group was significantly reduced by 32.9% when compared with the control group. The hepatic GSH content and CAT activity was also found significantly ($P < 0.01$) decreased in the CATR (150 mg/kg) group when compared with the vehicle control group. Our results indicate that oxidative stress like lipid peroxidation may be involved in ATR and CATR-induced injury in liver.

3. Discussion

In this study, we observed the toxicity induced by ATR and CATR via serum transaminase assay, tissue pathological observation and biochemical parameters of liver tissue. The results of our work showed that ATR and CATR induced hepatocellular injury. When liver was injured, the membrane permeability of liver parenchyma cell intensified, the activities of ALT and AST in serum increased sharply as a consequence. Serum aminotransferase activities have long been considered as sensitive indicators of hepatic injury. In the present study, ATR and CATR administration caused a dramatic elevation in serum ALT, AST, ALP activities and liver tissue histopathological change, indicating hepatotoxicity induced by ATR and CATR.

In the hepatic oxidative stress level, antioxidant-related enzyme activity assays showed that ATR and CATR administration significantly increased hepatic MDA concentration, as well as decreased SOD, CAT and GSH, and this was in good agreement with the results of serum aminotransferase activity and histopathological examinations. As one of the most important products of lipid peroxidation, MDA is used as an indicator of tissue damage involving a series of chain reactions. Lipid peroxidation has been implicated in the pathogenesis of increased membrane rigidity, osmotic fragility, reduced erythrocyte survival and perturbations in lipid fluidity (Ohkawaetal et al. 1979). The observation of elevated levels of hepatic MDA in ATR and CATR-treated mice (high doses toxin group) in the present study is consistent with this hypothesis. A major defense mechanism involves the antioxidant enzymes, including SOD, CAT, and GSH which convert active oxygen molecules into non-toxic compounds. The enzymatic antioxidant defense systems are the natural protector against lipid peroxidation (Zimmermann et al. 1973). Administration of ATR and CATR leads to generation of peroxy radical O2, which is associated with inactivation of CAT, GSH, SOD enzymes. This probably explains the significantly reduced activities of CAT, GSH, SOD observed in mice.

Table 2: Effects of ATR and CATR on biochemical parameters of liver tissue

Groups	MDA (nmol/mgprot)	SOD (U/mgprot)	GSH (mgGSH/gprot)	CAT (U/mgprot)
Normal control	2.0 ± 0.5	109.5 ± 7.8	5.6 ± 1.7	110.4 ± 21.4
ATR(50 mg/kg)	1.9 ± 1.6	101.2 ± 15.4	3.9 ± 2.3	$106.3 \pm 13.5^{*}$
ATR (125 mg/kg)	3.1 ± 0.3	$89.8 \pm 6.26^{**}$	$4.4 \pm 1.9^{**}$	$95.4 \pm 9.7^{**}$
ATR (200 mg/kg)	$3.8 \pm 1.5^{**}$	$73.5 \pm 7.38^{**}$	3.8 ± 1.4	74.7 ± 20.2
CATR (50 mg/kg)	2.1 ± 0.4	98.2 ± 10.2	4.7 ± 0.6	96.8 ± 11.2
CATR (100 mg/kg)	$2.3 \pm 1.7^{*}$	95.4 ± 11.5	4.8 ± 2.2	83.4 ± 12.5
CATR (150 mg/kg)	$3.0 \pm 0.9^{**}$	$76.9 \pm 14.3^{**}$	$3.7 \pm 1.8^{**}$	$67.1 \pm 8.7^{**}$

Values are expressed as mean ± SD of ten mice in each group
* $P < 0.05$,
** $P < 0.01$, compared with normal control group

In conclusion, our results demonstrate that kaurene glycosides induce hepatotoxicity in mice by induction of oxidative stress like lipid peroxidation in the liver, which merited further studies. Therefore, these toxic constituents explain the hepatotoxicity of *X. strumarium* L. in traditional medicine and provide a scientific basis for the clinical safety evaluation of the plant. The focus of future research should be directed on whether other plant components can materially infuence kaurene glycosides toxicity. In addition, there is also the need to provide appropriate therapeutic intervention strategies to limit or eliminate its toxicity in human poisoning.

4. Experimental

4.1. Drugs and chemicals

Ethanol (CP), petroleum ether (AR), chloroform (AR), ethyl acetate (AR), *n*-butanol (AR), dehydrated alcohol (AR), methanol (AR), NaCl (AR) was purchased from Sinopharm Chemical Reagent Co., LtdS. China. Superoxide dismutase (SOD), reduced glutathione (GSH) and protein quantization measurement kits were purchased from Nanjing Jiancheng Bioengineering Institute.

4.2. Plant material

The ripe fruits of *X. strumarium* L. were collected from a local research farm in Sunqiao town, Shanghai, in China, and authenticated by Prof. Lu-ping Qin, Second Military Medical University, Shanghai, China. The voucher specimen of the plant was deposited at the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, Shanghai, PR China (No.CE20031107).

4.3. Experimental animals

Experimental groups consisted of 10 ICR mice (approximately 25 g each, in half respectively male and female) per group. They were housed at 21 ± 1 °C under a 12 h light/12 h dark cycle and had free access to standard pellet diet (Purina chow) and tap water. The procedures in this study were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of the institution. All efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

4.4. Extraction, isolation and preparation of pure compounds

Dried fruits of *Xanthium strumarium* L. (30 kg) were ground and extracted with 75% aqueous ethanol by infiltration. The solvent was evaporated under vacuum to afford 1650 g crude extract. Then the extract was suspended in water and partitioned with petroleum ether, chloroform, ethyl acetate, *n*-butanol and aqueous fraction successively. The aqueous fraction (335 g) was subjected to repeated column chromatography over silica gel column chromatography and eluted with ethyl acetate–methanol (20:1∼1:5). Combination of similar fractions on the basis of TLC analysis afforded 5 fractions (1 to 5). Fraction 2 was purified by Sephadex LH-20 and eluted with (70% aqueous methanol) to get compound **1** (atractyloside). Chromatography of fraction 3 on Sephadex LH-20 (60% aqueous methanol) gave two fractions (3-I, 3-II). Fraction 3-I was further chromatographed on Sephadex LH-20 (50% aqueous methanol) to give compound **2** (carboxyatractyloside).
For pharmacological studies, these two compounds were dissolved in water. The doses employed are expressed as mg per kg body weight.

4.5. Chemical analysis

From the aqueous fraction, two kaurene glycosides (Fig. 1) were obtained and identified as atractyloside (**1**) and carboxyatractyloside (**2**), both of which have been previously reported and isolated. The structures were elucidated unambiguously by spectroscopic methods including 1D and 2D NMR analysis and also by comparing experimental data with literature data (MacLeod et al. 1990 Wang et al. 1983).
Compound 1: white powder (159 mg); 1H and 13C NMR (Wang et al., 1983); EIMS *m/z* 727 $[M\text{-}H]^-$; HREIMS: *m/z* 726.2231 (calcd. for $C_{30}H_{46}O_{16}S_2$, 726.2222).
Compound 2: white powder (130 mg); 1H and 13C NMR (MacLeod et al., 1990); EIMS *m/z* 771 $[M\text{-}H]^-$; HREIMS: *m/z* 770.2116 (calcd. for $C_{31}H_{46}O_{18}S_2$, 770.2120).

4.6. Treatment protocol

Mice were fasted for 12 h before treatment with drugs, but had free access to tap water. They received different doses of drugs or sterile saline vehicle by intraperitoneal administration. Food was returned to the mice after treatment, and 5 days later all mice were sacrificed, and then the livers, hearts, lungs, spleens, kidneys and brains of the mice were collected. The volume was adjusted with sterile saline to the appropriate final concentration.
The dose selection for the test samples was based on the results of preliminary experiments. Our previous study has found that the half lethal dose (LD_{50}) of ATR on mice was 398.3 mg/kg and the 95% confidence interval of LD_{50} (Feiller correction) was 360.9–439.4 mg/kg by intraperitoneal administration. The half lethal dose (LD_{50}) of CATR on mice was 203.5 mg/kg and the 95% confidence interval of LD_{50} (Feiller correction) was 176.4–228.7 mg/kg (data not shown). We found that serum ALT, AST level achieved the highest after treated with 200 mg/kg ATR for 5 days, so we selected the high dose 200 mg/kg of ATR and 150 mg/kg of CATR as the administration doses and 5 days as the incubation time.

4.7. Histopathological examinations

Tissue sections was dissected and fixed in 10% formalin, then processed routinely, embedded in paraffin, sectioned to 4 μm thickness, deparaffinized, rehydrated using standard techniques, stained with hematoxylin and eosin (H&E). The extent of liver necrosis and steatosis was evaluated by assessing morphological changes in liver sections.

4.8. Determination of enzyme levels in serum

Serum alanine aminotransferase (ALT), asparate aminotransferase (AST), and alkaline phosphatase (ALP) were measured on an automatic analyzer (HITACHI 7600–020, Japan) using diagnostic reagent kit.

4.9. Determination of biological parameters of liver tissue

Each right hepatic lobe sample was washed thoroughly in ice-cold saline to remove the blood after thawing, blotted the saline gently using filter paper. 0.15 g of each sample was placed in 1.35 ml saline and ten percent of homogenate in the vitric homogenizer at 20 °C was prepared. The homogenate was centrifuged at 3000 rpm for 10 min, the supernatant was used for the estimation of malondialdehyde (MDA), superoxide dismutase (SOD) activities, and reduced glutathione (GSH), catalase (CAT) and protein content.
Lipid peroxidation (LPO) in the tissues was determined by a previously reported method (Hogberg et al. 1974). Malondialdehyde (MDA) formed as an end product of the lipid peroxidation served as an index of the intensity of lipid peroxidation. MDA reacts with TBA to generate a pink-colored product, which has absorbance at 532 nm. Catalase activity was measured by the decay of hydrogen peroxide according to the previous reported method (Aebi 1984). The SOD, GSH, and proteins were estimated using kits according to the manufacturer's instructions. The tissue activity was calculated based on tissue protein concentration.

4.10. Statistical analysis

Data was expressed as mean ± SD. Significant differences were determined by Student *t*-test. $P \leqq 0.05$ were considered as statistically significant difference.

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