Non-Phenolic Diarylheptanoid from *Curcuma comosa* Protects Against Thioacetamide-Induced Acute Hepatotoxicity in Mice

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ABSTRACT

*Curcuma comosa* Roxb. (*C. comosa*, Zingiberaceae) is a medicinal herb containing diarylheptanoids with anti-oxidative and anti-inflammatory activities. The crude extract and its phenolic diarylheptanoid were shown to have hepatoprotection *in vitro*. The present study investigated the active principles and their underlying mechanisms that provided protection against thioacetamide (TA)-induced hepatotoxicity *in vivo*. Hepatic injury was induced in adult male mice by a single injection of TA (50 mg/kg BW, i.p.). *C. comosa* ethanol extract (5-500 mg/kg BW, p.o.), isolated diarylheptanoids: (3R)-1,7-diphenyl-(4E,6E)-4,6-heptadien-3-ol, *(D-049)* or (3S)-1-(3,4-dihydroxy-phenyl)-7-phenyl-(6E)-6-hepten-3-ol, *(D-092)*, (1-25 mg/kg BW, i.p.), was given prior to receiving TA. Changes in plasma activity of alanine transaminase (ALT), hepatic glutathione (GSH) content, the activities of superoxide dismutase (SOD) and catalase (CAT), and the expression levels of tumor necrosis factor (TNF-α) and cytochrome P450 2E1 (CYP 2E1) were determined at 24 h after TA-treatment. *C. comosa* extract suppressed the elevation of plasma ALT level in the TA-induced acute hepatotoxicity with increases in hepatic SOD and CAT activities. The protective effect was observed at 1 and 6 h prior to receiving TA and the effective dose was at 25 mg/kg BW. For pretreatment with diarylheptanoids, only non-phenolic diarylheptanoid, D-049 (5-25 mg/kg BW), provided the protection, but not phenolic diarylheptanoid, D-092. Moreover, D-049 suppressed the expression of pro-inflammatory cytokine TNF-α and CYP 2E1. These findings suggest that D-049 is an active principle in *C. comosa* that contributes hepatoprotection against TA-induced oxidative damage. It may mediate through its increased intracellular antioxidant enzymatic detoxification, which subsequently decrease the formation of bioactive metabolites of TA.

1. INTRODUCTION

*Curcuma comosa*, Roxb. is a plant in Zingiberaceae family which possess various pharmacological actions, including antioxidant, anti-inflammatory, immunodulatory and estrogenic-
like effects. C. comosa rhizome extract has been demonstrated to protect against cisplatin-induced nephrotoxicity and carbon tetrachloride-induced hepatotoxicity in mice by restoring glutathione content and increases in antioxidative enzymes. The C. comosa extract contains a number of diarylheptanoids in both non-phenolic and phenolic forms. A phenolic diarylheptanoid named (3S)-2,4-dihydroxyphenyl-1-phenyl-1-hepten-3-ol (D-092) exhibits high antioxidant activity comparable to those of Vitamin C and trolox by using DPPH assay which may provide antioxidant activity against the cisplatin-induced nephrotoxicity. Recently, our in vitro study has focused on the protective activity of diarylheptanoids from C. comosa using isolated rat hepatocytes. Hydroxyl diarylheptanoids (D-091 and D-092) show the protection against tert-butyl hydroperoxide (t-BHP)-induced injury in a primary rat hepatocytes model whereas the non-phenolic diarylheptanoid does not have the effect. However, the active constituents of C. comosa extract in providing the hepatoprotection has not been investigated in animals.

Thioacetamide (TA) is a potent hepatotoxic agent of which its toxicity requires an activation by CYP 2E1 in the hepatocytes. Its S-oxide form is a highly reactive toxic intermediate that is further converted into reactive S,S-dioxide form. In mice, acute single dose exposure to 50 mg/kg BW TA causes massive centrilobular necrosis after intraperitoneal (i.p.) administration, and this is caused through the generation of reactive oxygen species (ROS). ROS that interacts with the cellular components such as protein, lipid and nucleic acids, leads to subsequent protein dysfunction, lipid peroxidation and DNA damage. This damage then triggers the signaling cascade that activates the release of pro-inflammatory cytokines such as TNF-α and several others. It culminates in the progression of liver injury. To counteract the liver injury, intracellular antioxidant enzymes such as superoxide dismutase (SOD) is activated to prevent the ROS from causing cellular damage. As an experimental hepatic toxicant, various drugs have been tested against TA in vivo, providing insight into the mechanisms of action and biological effects. The present study aimed to investigate the effects and mechanisms of C. comosa extract and its diarylheptanoids on the protection against TA-induced hepatotoxicity in mice. The results indicated that the compound(s) that demonstrated high activity in an in vitro assay should be evaluated in vivo in order to further establish relevance in clinical application.

2. MATERIALS AND METHODS

2.1. Plant materials and chemicals

Preparations of C. comosa standard extract and isolation of DPHD were conducted as previously described. Rhizomes of C. comosa were purchased from Kampangsan District, Nakhonpathom, Thailand. The plant taxonomy was identified by Dr. Puangpaka Sootornchainaksaeng and Dr. Thaya Jenjittikul (2010) and a voucher herbarium specimen was deposited at the Department of Plant Science, Faculty of Science, Mahidol University, Bangkok (Voucher herbarium number SCMU300). The rhizomes were sliced, dried, and ground to powder. The powder was first extracted with hexane and then with ethanol. Nonphenolic diarylheptanoid (D-049), (3R)-1,7-diphenyl-(4E,6E)-4,6-heptadien-3-ol, and phenolic diarylheptanoid (D-092), (3S)-1-(3,4-dihydroxyphenyl)-7-phenyl-(6E)-6-heptadien-3-ol (Figure 1), were isolated from the ethanol extract. C. comosa ethanol extract contains D-049 and D-092 at approximately 155.5 and 79.6 mg/g extract, respectively. Thioacetamide was purchased from Fluka AG (Buchs, Switzerland). All other chemicals were of analytical grade.

2.2. Animals and experiments

Adult male ICR mice weighing between 30-35 g (approximately 6-8 weeks of age) were obtained from the National Laboratory Animal Center (Salaya, Nakhon Pathom, Thailand). They were housed in a translucent plastic cage with sawdust bedding, and maintained in a controlled room with a 12-hour light-dark cycle, temperature 21-22°C. The relative humidity was approximately 50-60%. They were allowed free access to food (mouse pellets, C.P. Mice Feed, Pokphand Animal Fed Co., Ltd., Bangkok, Thailand) and water ad libitum. The animals were allowed to acclimatize for 1 week before the experiment. They were fasted overnight (12 h) prior to the treatment, and they were sacrificed by sodium pentobarbital (50 mg/kg BW, i.p.). All experimental protocols were approved by the Faculty of Science, Mahidol University Animal Care and Use Committee (SCMU-ACUC Review).
Animals were randomly assigned into various experimental groups: control, TA-induced acute hepatotoxicity (50 mg/kg BW, i.p.), pretreatment with *C. comosa* ethanol extract (5-500 mg/kg BW, oral gavage) from 24 h to 1 h prior to TA injection, or pretreatment with diarylheptanoid compounds (D-049 or D-092) (1-25 mg/kg BW, i.p.) prior to TA injection. Final volume of all administrations was 0.3 ml. TA was dissolved in distilled water, and serve as its vehicle control. *C. comosa* extract was diluted in 10% DMSO in olive oil to serve as the vehicle control for the extract group. Diarylheptanoid was dissolved in 10% DMSO in water to serve as the vehicle control.

Twenty-four hours after TA injection, the animals were anesthetized with thiopenthal (50 mg/kg BW), and blood samples were collected from posterior vena cava. Plasma was then obtained and stored at -80˚C until the analysis of plasma alanine transaminase (ALT) activity. For the liver, it was rapidly removed, a portion of each liver was rinsed with cold 0.9% NaCl, and was frozen in liquid nitrogen and stored at -80˚C until further biochemical analyses.

### 2.3. Biochemical analyses

Plasma ALT activity was determined according to the method described previously by Reitman and Frankel (1957) with modification using α-ketoglutaric acid and dl-alanine as substrates. The activities of superoxide dismutase (SOD) and catalase (CAT) in the cytosol were assessed as previously described by Winterbourne (1975) and Lück (1965), respectively. The hepatic glutathione (GSH) content was also determined following the method by Akerboom (1981). The total amount of proteins was measured by bicinchoninic acid protein assay.

### 2.4. Total RNA extraction and RT-PCR of TNF-α and CYP 2E1

Total RNA was extracted from the liver tissue using TRIzol® Reagent. In RT-PCR reaction, RNA was first reverse transcribed into cDNA using QIAGEN OneStep RT-PCR kit (QIAGEN, Germany). A volume of 1 µg of RNA from each sample was added into the PCR reaction, containing 5× QIAGEN OneStep RT-PCR buffer, dNTP mixture containing 10 mM of each dNTP, enzyme mix, 10 µM primer, and appropriate volume of RNase-free water to perform a reaction in a total volume of 20 µl. The thermal amplification cycle was as follow: denaturation at 95˚C for 30 s, annealing at 55˚C for 30 s and extension at 72˚C for 45 s. PCR reaction was amplified at 35, 28, and 22 cycles for TNF-α, CYP 2E1, and β-actin, respectively, using GeneAmp® PCR System 2400 (Norwalk, CT, USA). β-actin was amplified to verify equal loading and normalize the relative expression. The following oligonucleotide primers were used:

- **β-actin**: Fw: 5'-GAG GGA AAT CGT GCG TGA CAT-3' and Rv: 5'-ACA TCT GCT GGA AGG TGG ACA-3',
- **TNF-α**: Fw: 5'-AAC TTC GGG GTG ATC GGT
CC-3’and Rv: 5’-TGG GGG CTG GGT AGA GAA TG-3’. CYP 2E1: Fw: 5’-CCT CCT CCT CGT ATC CAT CTG GAA A-3’ and Rv: 5’-GTC ACA GCT GGC TCA GCA TTT TCC T-3’.

The amplification products underwent electrophoresis on a 2% agarose gel. The gels were visualized by ethidium bromide staining, and the images were acquired using an image analysis system (Syngene Chemi Genius, MD, USA). The intensity of specific PCR band was quantified in relation to β-actin bands using Gene Tools Analysis software (Syngene Chemi Genius, MD, USA).

2.5. Statistical Analysis

Data were expressed as mean ± the standard error of means (S.E.M.). The statistical differences among the groups were analyzed by using one-way analysis of variance (ANOVA) followed by Newman-Keuls Comparison Test. Value of $P < 0.05$ was considered to be statistically significant.

3. RESULTS

3.1. Time-course effect of C. comosa extract against TA-induced acute hepatotoxicity

Hepatotoxicity of TA was initially evaluated at 24 h after a single intraperitoneal injection of various doses of TA, and TA at a dose of 50 mg/kg BW was chosen as it clearly increased plasma activity of ALT, indicating its acute hepatotoxicity. In Figure 2a, twenty-four hours after TA injection, plasma ALT activity was significantly increased from 14.2±1.2 IU/L in normal control to 146.4±8.3 IU/L ($P < 0.01$). To determine the optimum time-course of C. comosa treatment, the C. comosa ethanol extract (500 mg/kg BW) was orally given at various time points prior to TA administration. The protective effect of the extract was found at 1 and 6 h prior to TA administration in which plasma ALT was comparable to that of normal control. The protective effect disappeared when the extract was given at 12 and 24 h before TA administration by which plasma ALT was as high as the untreated TA-control. Treatment with the extract twice at 24 and 1 h prior to TA gave a similar protective effect as that of a single dose at 1 or 6 h before TA.

To assess the underlying mechanism by which the C. comosa extract protected against the TA-induced hepatotoxicity, intracellular anti-oxidant system in the liver was determined at 24 h after TA. In Figure 2b, SOD activity in control group was approximately 305±29 units/mg protein, and it was significantly decreased by TA to 163±13 units/mg protein ($P < 0.01$). Pretreatment with the C. comosa extract prior to TA significantly restored the suppression of SOD activity by TA, particularly when it was given a single dose at 1 or 6 h, or twice at 24 and 1 h. CAT activity which was slightly decreased by TA was also increased by C. comosa extract. It is noted that the extract markedly increased CAT activity to the level higher than the control (Figure 2c). The level of CAT remained high after TA administration, particularly by twice administrations at 24 and 1 h prior to TA. However, hepatic GSH concentration at 24 h after TA was not significantly altered (Figure 2d). This observation indicated the potential of C. comosa to modulate cellular anti-oxidant system to detoxify TA as well as to protect against liver injuries. The effectiveness of protective effect was at 1 or 6 h or twice at 24 and 1 h prior to TA which inversely correlated to the levels of ALT. As in our earlier study, the significant hepatoprotection of C. comosa extract against CCl₄ was observed from 2 h to peak during 12-24 h. Based on the effectiveness for time course of pretreatment in the earlier study and in the current study, two administrations of C. comosa extract at 24, and 1 h before TA were selected. Thus, further studies were conducted by pretreatment twice at 24 and 1 h.
Dose response effect of *C. comosa* extract and Diarylheptanoids (D-049 and D-092) against TA-induced acute hepatotoxicity

To determine the minimum effective dose of the *C. comosa* extract on the protection against the TA-induced acute hepatotoxicity, various doses of the extract were orally given to the animals. The extract from a dose of 25 mg/kg BW onward to 500 mg/kg BW significantly protected against TA-induced hepatotoxicity (*Figure 3a*) (*P* < 0.01). The protective potential of two major isolated diarylheptanoids (D-049 and D-092) from *C. comosa* was evaluated. Interestingly, pretreatment with non-phenolic diarylheptanoid, D-049 at 1, 5, 10, or 25 mg/kg BW, i.p., significantly suppressed the elevation of plasma ALT level as compared to TA treated group (*P* < 0.05) (*Figure 3b*). The maximal protection against the TA-induced liver injury of D-049 was observed at a dose of 5 mg/kg BW onward. In contrast to D-049, the phenolic diarylheptanoid, D-092 did not protect against the TA-induced hepatotoxicity even at a high dose of 25 mg/kg BW, suggesting that non-phenolic diarylheptanoid, D-049 is one of the active constituents in the *C. comosa* extract that provides hepatoprotection to TA-induced hepatotoxicity.
Figure 3. Dose-response effect of pretreatment with *C. comosa* ethanol extract (a), D-049 and D-092 (b) on plasma ALT activity in thioacetamide (TA)-induced hepatotoxicity. Mice orally received various doses of *C. comosa*, and intraperitoneal injection of D-049 or D-092 at 24 and 1 h prior to a single dose of TA (50 mg/kg BW, i.p.). Each value represents mean ± SEM (N=6). **P < 0.01 significantly different compared to control. ”**P < 0.01 significantly different compared to TA treated group.
3.3. Effect of D-049 on intracellular activities of SOD, CAT and GSH content in the TA-induced hepatotoxicity

To assess the underlying mechanism by which D-049 protected against the TA-induced hepatotoxicity, the intracellular antioxidant system of the liver was analyzed. In Figure 4, SOD activity in control group is approximately 304±29 units/mg protein, and it is significantly decreased by TA administration to 163±13 units/mg protein (P < 0.01). Pretreatment with D-049 significantly increased the SOD activity as compared to TA-treated group (P < 0.01) (Figure 4). However, the CAT activity and hepatic GSH content were not significantly affected at 24 h after TA (data not shown). More importantly, D-049 at a dose of 25 mg/kg BW alone significantly increased hepatic SOD activity (P < 0.05). This observation indicated the potential of C. comosa to directly modulate cellular antioxidant system.

![Figure 4](image_url)

**Figure 4.** Dose-response effect of D-049 pretreatment on superoxide dismutase activity (SOD) in thioacetamide (TA)-induced hepatotoxicity. Mice intraperitoneally received various doses of D-049 at 24 and 1 h prior to a single dose of TA (50 mg/kg BW, i.p.). Each value represents mean ± SEM (N=6).

*P < 0.05, **P < 0.01 significantly different compared to control.

3.4. Effect of D-049 on the mRNA expressions of TNF-α and CYP 2E1 in the TA-induced hepatotoxicity

To further investigate the molecular mechanism of action of D-049, the mRNA expression of TNF-α was determined. TA alone markedly up-regulated the mRNA expression of TNF-α at 24 h after treatment which suggested hepatic inflammation (Figure 5a). Treatment with D-049 alone (25 mg/kg BW) did not alter the expression of TNF-α in D-049-treated control group. Pretreatment with D-049 at doses of 1 and 25 mg/kg BW prior to TA significantly attenuated the up-regulation of TNF-α as compared to TA-treated group (P < 0.05). The expression of CYP 2E1 mRNA, an enzyme that catalyzes the bioactivation of TA was then investigated. The level of CYP 2E1 significantly decreased in the TA-treated group as compared to control (Figure 5b). Pretreatment with D-049 at a dose of 25 mg/kg BW restored CYP 2E1 level, whereas a dose of 1 mg/kg BW was not able to prevent this reduction. It should also be noted that D-049 at a dose of 25 mg/kg BW alone significantly increased the CYP 2E1 mRNA level.
Figure 5. Effect of D-049 pretreatment on hepatic expressions of TNF-α mRNA (a) and CYP 2E1 mRNA (b) in thioacetamide (TA)-induced hepatotoxicity. Mice intraperitoneally received D-049 twice at 24 and 1 h prior to a single dose of TA (50 mg/kg BW, i.p.). Changes in TNF-α mRNA and CYP 2E1 mRNA expression were determined at 24 h after TA administration. Each value represents mean ± SEM (N=3).

## P < 0.01 significantly different compared to control.

* P < 0.05, ** P < 0.01 significantly different compared to TA treated group.

4. DISCUSSION

The present study demonstrated that both the C. comosa ethanol extract and non-phenolic compound, D-049, effectively protected against TA-induced hepatotoxicity, indicating that D-049 is an active principle providing the hepatoprotection in the extract. The protective effect of D-049 was accompanied with an increase in SOD activity which is the anti-oxidant enzyme catalyzing the dismutation of the superoxide radical into hydrogen peroxide and oxygen\(^{23}\). With regard to the rapid protective action occurring within 1-6 prior to TA, it is likely that the compound has the ability to promptly activate intracellular antioxidant system in the liver to counteract the oxidative free radical of TA metabolite. In corresponding to the protective effect of D-049, the up-regulation of proinflammatory cytokine TNF-α mRNA expression induced by TA was suppressed (Figure 5a). Surprisingly, the phenolic diarylheptanoid, D-092, that has previously been reported to provide protective effects to many oxidative stresses in \textit{in vitro} systems does not have any protection against TA treatment in mice of the present study\(^{2,24,25}\). It may be inactivated by metabolic biotransformation in the body. The protective effect may mediate through its rapid increasing intracellular enzymatic detoxification, which subsequently decrease the formation of bioactive metabolites of TA.

TA has been used as a common compound to induce hepatotoxicity in experimental animals. Following TA administration, mice develop severe liver damage. The toxicity of TA requires two steps of hepatic metabolism by which TA is firstly bioactivated to a highly reactive toxic metabolite thioacetamide-S-oxide (TASO) and further to a very reactive compound of thioacetamide-S-S-dioxide (TASO\(_2\)). Their binding to macromolecules in the cell is a critical step to initiate hepatocellular damage. The bioactivation of TA is carried out by hepatic cytochrome P450 monooxygenases (CYP 2E1) enzyme\(^{12,26,27}\). After an intraperitoneal injection, plasma TA levels has been reported to peaked by 5 min\(^{28}\). The formation of TASO was also rapid that it was detected in plasma by 5 min.

In the present study, the duration of pretreatment with the extract as well as the compound has a great impact to its protective action. The effective protection was observed at approximately 1–6 h prior to thioacetamide treatment. It is not clear why such time period is required. As oxidation of TA occur shortly after administration, this duration may reflect the full induction period required for the expression of
the protective mechanism particularly the hepatic anti-oxidant system. At the time of sacrifice, the levels of CAT and GSH were not different from the normal control. It would be of high interest for further study. Thus, our finding reiterates two possible mechanisms of protection by C. comosa extract and D-049 compound that include their intrinsic free radical scavenging effect and an increase in intracellular antioxidant detoxification system. The protective effect of D-049 at 5 mg/kg was comparable to the effect of the extract at 25 mg/kg. This result may due to the similar amount of D-049 in the extract. In considering the amount of D-049 content in the extract, the 25 mg of the extract should contain approximately 6.22 mg D-049 as one gram of the C. comosa ethanol extract contains compound D-049 at approximately 155.5 mg. This may account for the comparable effect of extract to that of D-049 at 5 mg/kg.

In response to cell injury, ROS from TA trigger pro-inflammatory cytokine expression such as TNF-α, interleukin (IL-1) and cellular adhesion molecules\(^{29}\). Oxidative stress then affects many cellular functions by altering gene expression through the activation of transcription factor NF-κB. An earlier study suggested that an activation of NF-κB pathway has been implicated in the pathogenesis of liver injury by TA\(^{14}\). It was apparently increased at 2 h after TA administration\(^{14}\). Pro-inflammatory cytokines such as iNOS and TNF-α are the downstream molecules in the NF-κB pathway. In this study, the up-regulation of TNF-α in animals receiving TA was apparent (Figure 5a), and is consistent with earlier report\(^{29}\). Pretreatment with D-049 effectively suppressed the up-regulation of TNF-α, and this finding is in line with the administration of other medicinal plants\(^{31}\).

In the present study, TA induced hepatic damage with decreased CYP 2E1 mRNA expression (Figure 5b). TA is a good ligand of CYP2E1. The inhibition of CYP 2E1 activity by TA was likely due to the initial binding of CYP 2E1 with formation of highly bioactive metabolite of TA, in turn, activated and destroyed the CYP 450 system. The bioactive metabolite might covalently bind to the enzyme’s active site, causing subsequent suicidal inactivation of the CYP pathway as reported in other hepatotoxic studies\(^{5,26,27}\). It is intriguing that D-049 alone significantly increased the expression of CYP 2E1 mRNA, an implication that it may enhance bioactivation of TA and hepatotoxicity. It was not clear how D-049 protected against TA-hepatotoxicity, whereas its induction of CYP 2E1 led to an increase activation of the hepatotoxicant. However, it is worth noting that D-049 treatment is characterized by a marked persistent increase in SOD activity (Figure 4). The enrichment of SOD in Zingiberaceae plants has been well documented including C. comosa\(^{22}\). A recent proteomic analysis of C. comosa found SOD and ascorbate peroxidase as a part of the protein profile\(^{33}\). Thus, their presence and their anti-oxidative role are undisputed. Although the role of C. comosa extract and the isolated compound in inducing the expression of antioxidant enzymes remains elusive, it is possible that the active constituent(s) in C. comosa directly stimulate gene expression of SOD.

In this study, it was surprised that the phenolic diarylheptanoid (D-092), which is structurally similar to D-049, did not exert hepatoprotection. This finding challenged our understanding of the report by Suksen et al. (2016), who showed that phenolic diarylheptanoids protected against t-BHP-induced toxicity in vitro\(^{6}\). Other studies also showed active biological properties of compound D-092 in vitro, and the properties include the suppression of microglial cell activation, and anti-apoptotic property in astroglial cell\(^{34}\). The difference in activities between in vitro and in vivo of D-092 might be a result of the metabolism of this compound in the body. The polar phenolic diarylheptanoid is hydrophilic, which may also be readily applicable in in vitro model. However, the compound may be biotransformed in the body, resulting in pharmacological activity alteration in vivo. The observed differential response is similar to the previous report by Winuthayanon et al. (2009), whereby similar compounds from C. comosa exhibited estrogenic-like activity in vitro, but were ineffective in vivo\(^{35}\). The differential effect might be influenced by the different experimental model, metabolism of the compound(s), and the mechanism of action of each hepatotoxican. Therefore, further study is required to truly establish our understanding of the herbal remedies that have been used without substantial scientific investigation. The compound(s) that demonstrated biological activity in vitro should also be evaluated in vivo, which will be more relevant in clinical use.
5. CONCLUSIONS

In conclusion, non-phenolic diarylheptanoid, D-049, is an active principle of *C. comosa* that provided the hepatoprotective effect against TA in mice. The mechanism of action may be related to the direct scavenging effect as well as an increase in intracellular enzymatic antioxidant system in the liver. This is the first report of *C. comosa*’s therapeutic potential against acute TA-induced hepatotoxicity, the natural antioxidant against ROS and highlights the essence of biological model relevance in clinical application for assay the pharmacological activities of natural products.

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Conflict of interest
Authors declare no conflict of interest in this study.

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None to declare

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