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Research Article

Molluscicidal Activity and Biochemical Interactions of Copper Sulfate against *Theba pisana* (Müller)

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Authors' Contributions
EIG, GMA and AFG presented the concept and planned the methodology. GMA and AFG conducted investigation, performed analysis and wrote the manuscript. GMA, ESHS and AFG arranged resources. ESHS supervised the reseach.

Keywords
Theba pisana, Copper sulfate, Molluscicide, Lethal effect, biochemical indicators

Abstract | The white garden snail, *Theba pisana* is one of the widely distributed gastropods and is a dangerous agricultural pest for many plants. Copper sulfate ($CuSO_4$) is extensively used for controlling a number of molluscs in many areas. In this study, $CuSO_4$ toxicity indices against *T. pisana* after 24, 48 and 72 h using the topical application technique were estimated. Additionally, *in vivo* evaluation of acetylcholinesterase (AChE), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in *T. pisana* intoxicated with two sublethal doses of $CuSO_4$ (0.25 and 0.5 of LD_{50}) after 24, 48 and 72 h were examined. The results indicated that the LD_{50} values of $CuSO_4$ were 166.5, 92.59 and 70.63 µg/g b.w for 24, 48 and 72 h, respectively. The biochemical effects of $CuSO_4$ led to a significant increment of AChE activities in treated snails after all tested times. While the tested compound inhibited ALP activities in all treated animal groups. AST and ALT activities in treated snails were significantly altered by tested doses of $CuSO_4$. This study suggests that the activity of these enzymes modulation may be one of the biochemical mechanisms of $CuSO_4$ toxicity.

Novelty Statement | This is the first study to investigate the sub-lethal effects of $CuSO_4$ on biochemical interactions of land snail, *Theba pisana*. Our findings will contribute to understand the biochemical defects of $CuSO_4$.

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Introduction

Most land snail species are members of the subclass Pulmonata, Gastropoda (phylum, Mollusca) that have acclimated to subsist far from aquatic environment. *Theba pisana*, commonly known as the white garden snail or the Mediterranean snail, has become a serious agricultural pest in many parts of the world (Odendaal *et al.*, 2008), and causing severe damage to several plant including vegetables and ornamentals (El-Okda *et al.*, 1983; Godan, 1983). Their damages are not only due to snail population density, but also to the feeding activity that varies from one species to another (Abdelgalil *et al.*, 2018), and to contamination with their bodies, faces or mucus, deteriorating the quality of the product besides the economic loss (Iglesias *et al.*, 2003).

A range of cultural, chemical and biological measures

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are used for the management of land molluscan pests, however, copper-based fungicides including copper sulfate ($CuSO_4$) have been historically widely used for fungal pests control. These chemicals are not registered to control snails, but they have some lethality to land snails and slugs (El-Wakil and Mesbah, 1995; Radwan, 1998; El-Shahat *et al.*, 2009), also protect plants from snail's attack by its repellent effect (Capinera and Dickens 2016).

It is well known that the toxicity of $CuSO_4$ depends on the copper ion content. The copper ion binds to proteins, leading an ultimate disorder in the structure of DNA and proteins, eventually causing their non-functioning. Land snails physiologically need to consume copper ions to produce the protein hemocyanin. This copper-containing protein is the main protein of molluscan hemolymph and its function is that it can transport oxygen through the hemolymph in a various snail species (Machałowski and Jesionowski, 2021). Land snails must meet their Cu requirements by taking up metal from the soil or the plants they feed on (Gomot and Pihan, 1997). But, snails are extremely sensitive to uptake of additional quantities of copper. Too much amounts of copper can damage the normal action of the enzymes and skin cells as well as prevent oxygen from circulating in their bodies and thus exert toxic effects on snails.

As a result of exposing land snails to $CuSO_4$ stress, the ecotoxicological effect of copper pollution (as model) in terrestrial ecosystems is achieving, and at the same time its toxicological implications against harmful land snail pests in agriculture and horticulture (El-Wakil and Mesbah, 1995; Radwan, 1998; Nica *et al.*, 2013). Generally, the biochemical changes induced by copper exposure provide qualitative data regarding how terrestrial snail is physiologically capable for regulating copper in its body (Dallinger *et al.*, 2005). Numerous biomarkers of pollution could be examined in snails. AChE activity considered as a popular biomarker of pollutant exposure and, in general, as a sensor of neurotoxicity (Deidda *et al.*, 2021). ALP is a marker enzyme for lysosomal membranes, and any disturbance in the membrane properties in response to toxins in hepatic tissues could change the ALP activity (Atencio *et al.*, 2008). Aminotransferases (AST and ALT) are probable biomarkers of organisms exposed to xenobiotics and any changes of enzymatic activity in organisms exposed to xenobiotics reflect the homeostatic regulations in metabolic pathways as portion of the common adaptive syndrome (Giesy *et al.*, 1988).

Previous studies have already investigated the lethal effects of $CuSO_4$ on pulmonate snails not only to freshwater snails (Waheed *et al.*, 2017), but also to land gastropods (Radwan, 1998; El-Gendy *et al.*, 2009). However, the sublethal acute effects of this compound on biochemical interactions of land snails is lacking. Therefore, this study sheds light on the biochemical interactions of AChE, ALP, AST and ALT in the land snail, *T. pisana* with sublethal doses (0.25 and 0.5 of LD_{50}) of $CuSO_4$ after 24, 48 and 72 h exposure through a contact toxicity testing. Our findings will aid in understanding the biochemical defects in snails caused by the exposure to $CuSO_4$.

Materials and Methods

Chemicals used

Copper sulfate, $CuSO4.5H_2O$ (purity 99%) was purchased from Merck, Darmstadt. Acetylthiocholine iodide (AChI), Dithiodinitrobenzoic (DTNB) and bovine serum albumin, were of the best purity grade available, supplied by Sigma Company.

Animals used

Adult snail, *Theba pisana*, with 0.91 ± 0.008 g body weight and 15.7 ± 0.074 mm shell diameter, were gathered from un-treated parterre in province of Alexandria, Egypt. The collected snails were kept under laboratory conditions (about 27 °C and 63–65% RH) in ventilated cages (45 ×35 × 35 cm) for at least 2 weeks adaptation prior to the trials and fed with leaves of lettuce *ad libitum*.

Toxicity test

In this test, stock solution of 0.01 g technical grades of $CuSO_4$ in 1 mL deionized water was used. The $CuSO_4$ stock solution was serially diluted to procure the desired doses. The examined doses were selected based on preliminary trials to identify the range of doses. The tested doses were 64, 80, 96, 112, 128, 144 and 160 microgram per gram body weight (µg/g b.w). The topical application technique was applied according to Hussein *et al.* (1994); Radwan *et al.* (2008). Three replicates for each treatment (10 snails for each replicate) were done. The control group was treated with deionized water. The snails in each replicate were kept in plastic box (10 cm diameter x 7 cm height) covered with a rubber band-fixed cloth net to prevent the escaping of snails. Post 24, 48 and 72 h of treatment, the mortality counts were recorded and mortality percentages were calculated. The snail has lost its response to a thin stainless steel needle was considered dead (WHO, 1965).

Enzymes assays

To study the effect of $CuSO_4$ on some enzymes as biochemical markers of *T. pisana* soft tissue, two sub-lethal doses of 0.25 and 0.5 LD_{50} (41.62 and 83.25 µg/g b.w) after 24 h, (23.14 and 46.29 µg/g b.w) after 48 h and (17.65 and 35.31 µg/g b.w) after 72 h exposure were topically applied. Three replicates for each treatment of the sublethal doses (10 snails in each) were used.

Sample preparation

Post 24, 48 and 72 h of exposure, the surviving snail shells from each group were removed and the soft tissues were weighted and homogenized in 5 vol ice-cold normal saline (w/v ratio) using a Polytron Kinemetica

homogenizer for 60s. The homogenates were centrifuged under cooling at 5000 xg for 30 min utilizing a IEC-CRU 5000 cooling centrifuge. The obtained supernatants were utilized to measure the activities of AChE, ALP, AST and ALT.

AChE activity

Utilizing acetylthiocholine iodide (ASChI) substrate, AChE activity was determined based on the procedure of Ellman *et al*. (1961).

ALP activity

ALP activity was measured upon to the DGKC (1972) method utilizing Diamond Diagnostic Kits.

AST activity

AST activity was estimated utilizing Diamond Diagnostic Kits based on Reitman and Frankel (1957) method.

ALT activity

ALT activity was tested based on the procedure explained by Reitman and Frankel (1957) utilizing Diamond Diagnostic Kits.

Protein content

The protein content was assayed upon to the procedure of Lowry *et al*. (1951).

Statistical analysis

The mortality percentage data of all replicates were utilized for calculating the lethal dose (LD) values after correction the mortality percentage data according to Abbott's formula:

$$M\%=([X-Y]/X)\times 100$$

Where M% is mortality percentage, X is the live snails number in the control and Y is the live snails number in the treatment (Abbott, 1925). The LD_{50} values expressed as µg/g b.w with confidence limits (CL) and slope for each exposure time were calculated utilizing probit analysis program established by Finney (1971). The biochemical results were expressed as a mean ± standard error (SE). Results were performed by one-way analysis of variance (ANOVA). The statistical analysis of biochemical data was carried out utilizing CoStat program, Version 2.6 (2002). The comparison of means was done at $p \leq 0.05$.

Results and Discussion

Toxicity assay

The mortality percentage of snails, the acute toxicity indices as slopes of the LD-P regression lines, chi square and the confidence limits of the LD_{50} values are shown in Table 1. The obtained data demonstrated that the highest $CuSO_4$ dose recorded mortality percentages of 57.14, 90.47 and 100 post 24, 48 and 72 h exposure, respectively. The obtained data showed also that the LD_{50} values were 166.5, 92.59 and 70.63 µg/g b.w for 24, 48 and 72 h, respectively.

Enzymes assays

The *in vivo* impacts of 0.25 and 0.5 of $CuSO_4$-LD_{50} values post 24, 48 and 72 h on AChE, ALP, AST, and ALT of *T. pisana* snail are illustrated in Figures 1–4.

AChE activity

As shown in Figure 1, AChE activities in treated snails with 0.25 and 0.5 of LD_{50} values were remarkably higher than those in control snails after 24, 48 and 72 h, where $CuSO_4$ increased AChE activities by 1.51, 1.14 and 1.73-folds of control in 0.25 LD_{50} group but increased the enzyme activities by 1.37, 1.31 and 1.38-folds of control in 0.5 LD_{50} group after 24, 48 and 72 h, respectively.

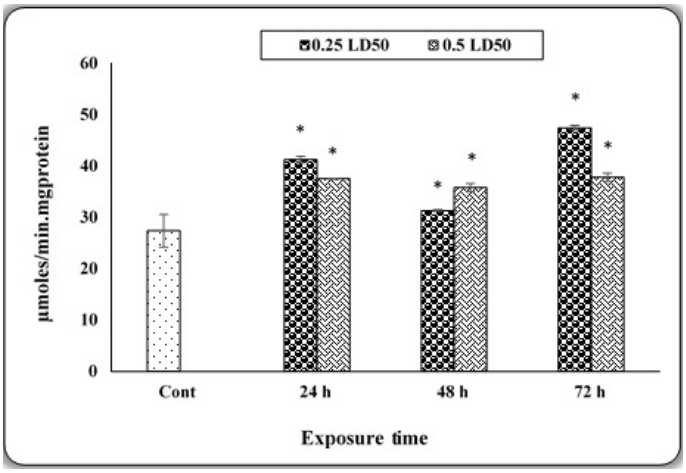

Figure 1: Effect of copper sulfate on the AChE activity in *Theba pisana* after 24, 48 and 72 h. Each value represents the means ± SE of five animals, *significantly different from control value, (n=3, p<0.05).

Table 1: Lethal effect of copper sulfate against *Theba pisana* snail after 24, 48 and 72 h of exposure.

Exposure time/h	Mortality %							LD_{50} (µg/g b. w)	Confidence limit 95%		Slope	X^2
	64*	80	96	112	128	144	160		Lower	Upper		
24	7.14	21.43	23.81	28.57	35.17	38.09	57.14	166.5	147.73	202.71	2.65	6.58
48	14.28	47.61	57.14	66.66	71.42	76.19	90.47	92.59	81.62	101.71	4.78	12.14
72	38.09	64.28	78.57	85.71	90.47	92.85	100	70.63	64.77	75.26	5.41	1.18

*Each bold figure represents as a test dose. X^2 = chi square.

ALP activity

The data indicated that 0.25 and 0.5 of LD_{50} of examined compound markedly reduced the ALP activity after all exposure times. The reduction percent of the enzyme activities were 61.04, 68.52 and 81.64 % at 0.25 of LD_{50}, while the percent were 72.16, 51.3 and 60.59 % at 0.5 of LD_{50} after 24, 48 and 72 h exposure, respectively (Figure 2).

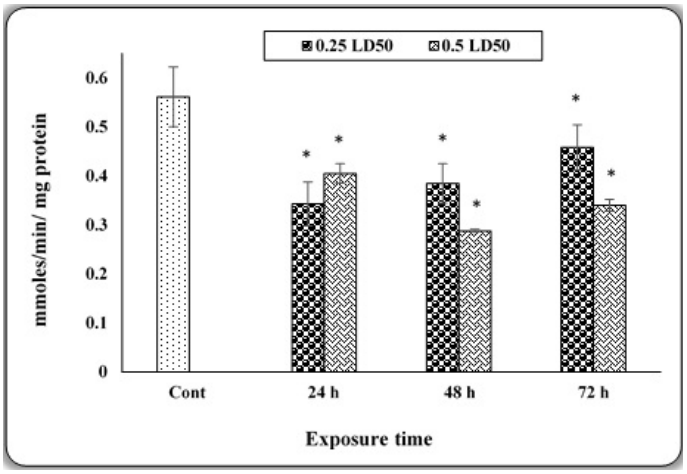

Figure 2: Effect of copper sulfate on the ALP activity in *Theba pisana* after 24, 48 and 72 h. For statistical details, see Figure 1.

AST activity

In treated snails, both 0.25 and 0.5 of LD_{50} significantly declined the activities of AST in the tissue after three tested times compared with the untreated snails (Figure 3). AST activities in the soft tissues of untreated snails was 0.513 mmoles/min/mg protein, however, the activities were 0.448, 0.450 and 0.491 mmoles/min/mg protein; in the treated snails with 0.25, as well as 0.497, 0.379 and 0.311 mmoles/min/mg protein; in the treated snails with 0.5 LD_{50} after 24, 48 and 72 h, respectively.

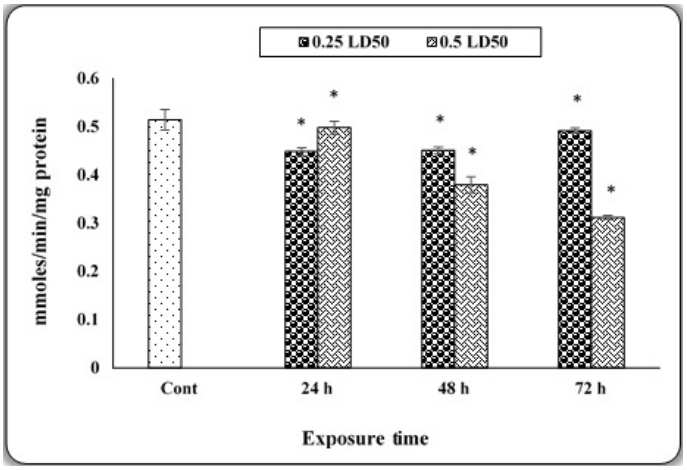

Figure 3: Effect of copper sulfate on the AST activity in *Theba pisana* after 24, 48 and 72 h. For statistical details, see Figure 1.

ALT activity

The ALT activities of treated snails with 0.25 and 0.5 of LD_{50} after 24 h were markedly higher than those of untreated control, however, the enzyme activities were significantly lower in treated snails with 0.25 and 0.5 of LD_{50} for 48 h. After 72 h of exposure the activity of ALT was significantly increased in case of snails intoxicated with 0.25 of LD_{50}, while the activity significantly decreased in case of snails intoxicated with 0.5 of LD_{50} than those of untreated snails (Figure 4).

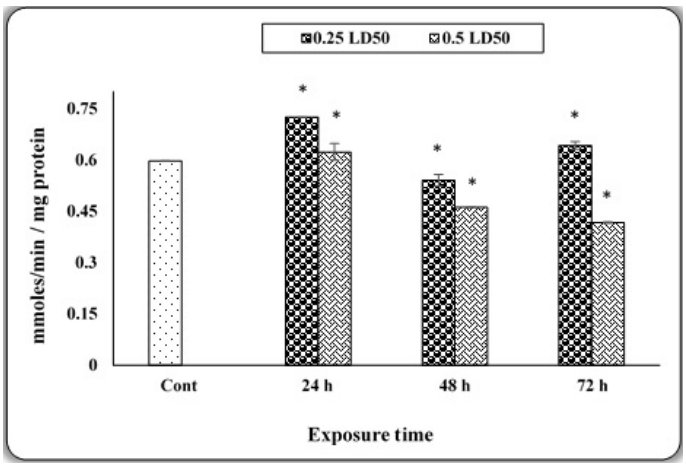

Figure 4: Effect of copper sulfate on the ALT activity in *Theba pisana* after 24, 48 and 72 h. For statistical details, see Figure 1.

In the present study, *Theba pisana* snail was utilized as a target pest to assess the toxic impact of copper sulfate. Our results demonstrate that, $CuSO_4$ has molluscicidal properties against adult *T. pisana*. The current data are very coincidence with the research of El-Gendy *et al.* (2009) who reported that, LD_{50} of $CuSO_4$ after 48 h using topical application technique on *T. pisana* was 26.54 µg/snail. Radwan (1998) noted that $CuSO_4$ was effective against *Limax flavus* slug after 1 h exposure to $CuSO_4$ residual film. Bioassay results of Shaker *et al.* (2015) showed that LC_{50} values of $CuSO_4$ against *Eobania vermiculata* were 1.621, 1.139, 0.734, and 0.672 % after 24, 48, 72, and 96 h, respectively. $CuSO_4$ has a lethal toxic action on *Archachatina marginata* snails with LC_{50} value of 2.35 mM after feeding on sprinkled food with 2.00–3.20 mM Cu solution for 168 h (Otitoloju *et al.*, 2009). In addition, $CuSO_4$ spraying has been shown to be very efficient in decreasing the infestation of *T. pisana* in *Vicia faba* and citrus trees (El-Wakil and Mesbah, 1995; El-Wakil, 1999). In the case of freshwater snails, the molluscicidal efficacy of $CuSO_4$ (LC_{50} = 1.79 ppm) against *Biomphalaria alexandrina* post 24 h exposure was demonstrated by Rawi *et al.* (2011). Moreover, the LC_{50} of $CuSO_4$ against *Pomacea canaliculata* snails at 24, 48, 72 and 96 h of exposure were 330, 223, 177 and 146 mg/L, respectively (Dummee *et al.*, 2015).

The important enzyme in the cholinergic nervous system of vertebrates and invertebrate is AChE. It is

mainly found at neuromuscular junctions and in chemical synapses of the cholinergic type, where its activity terminates the synaptic transmission (Lionetto *et al.*, 2013). Moreover, AChE is a sensitive target for organophosphate and carbamate pesticides that cause its inhibition (Radwan *et al.*, 1992).

In current investigation, it was observed that AChE activity elevated in $CuSO_4$-intoxicated snails. Based on these considerations, we can suggest that the copper metal, under our exposure conditions, could interfere with acetylcholine receptor and thereby affecting its binding efficacy, resulting in an increment of AChE synthesis, for decomposing the higher neurotransmitter levels, as an acute response. In addition, the augmentation of AChE activity may be due to the up-regulation of AChE gene, induced via the primary inhibitory impact of metal. However, this explanation needs some further work. The obtained data are in line with the results of Romani *et al.* (2003) who observed an augmentation in AChE activity of both brain and muscle of fish, *Sparus auratus* treated with sublethal concentration of $CuSO_4$ over 20 days. In a laboratory experiment carried out by (Padrilah *et al.*, 2017) who found that ChE activity of the *Clarias gariepinus* liver was affected by the sublethal concentrations of $CuSO_4$ (from 0.2 to 20.0 mg/L), 100% inhibition was found at 20.0 mg/L after 96 h. On the other hand, *Perna perna* mussels treated with 40 mg/L of $CuSO_4$ for 12, 24, 72 and 120 h exhibited no change in AChE activity during the whole periods of exposure (Bainy *et al.*, 2006).

ALP is a homodimer protein enzyme of 86 kd that help in the synthesis and transport of metabolites across the membrane, secretory activity, and protein synthesis and glycogen metabolism. ALP is also enzyme involved in the biosynthesis of mucopolysaccharides (Kroon, 1952) and fibrous proteins (Johnson and McMinn, 1958), or it might benefit regulators of intra-cellular phosphate level (Gutman, 1959).

In this research, the tested compound decreased the activity of ALP in all treated snails groups. The decrease in ALP activity may be directly associated to the effect of the Cu ion on the enzymatic system and the lysosomal metabolism disruption in tissue (Sanisa *et al.*, 1982), binding of Cu to SH group in the enzyme or to interfere of metal with the enzyme cofactors and regulators. Our results are consistent with the findings of previous authors; Sonawane (2017) who revealed a constant decrease of ALP activity in foot, mantle and digestive gland of *Lamellidens Marginalis* bivalve after the acute and chronic $CuSO_4$ stress. In perialveolar region and perilobular margin of *Lymnaea luteola* digestive gland, ALP showed average activity during 24 and 48 and 72 h of $CuSO_4$ exposure (Mathur and Gupta, 2008). Moreover, ALP activity was reduced in earthworm, *Metaphire posthuma* by all tested concentrations of 100, 500, 1000 mg of *CuO* nanoparticles and $CuSO_4$ per kg soil for 7 and 14 d (Gautam *et al.*, 2018).

Aminotransferases or transaminases are a class of enzymes that act a considerable part in aminoacids and proteins metabolism in various body organs (Gómez-Milán *et al.*, 2007). AST and ALT, are the two vital aminotransferase enzymes and they are recognized as susceptible biomarkers and altered the activity of these enzymes reflect hepatic dysfunction.

Our data demonstrated that the significant alteration of AST and ALT activities in *T. pisana* by low metal levels enable them to being good biomarkers of sublethal copper contamination. So far, the literature information about the impact of $CuSO_4$ on AST and ALT activity in land gastropods is still very scarce. The data from an existing study parallel to Radwan (1998) who found that 1/10 LC_{50} of $CuSO_4$ caused a significant elevation in ALT and AST activities in soft tissue of the slug *L. flavus* using contact action toxicity test. Further, the metabolic enzymes (AST and ALT) activities in the homogenate, cytosolic and mitochondrial fractions of *Helisoma duryi* and *Lymnaea natalensis* freshwater snails were elevated with Cu ion concentration up to 0.21 mg/l, but inhibited at Cu concentration reached to 1 mg/l in breeding waters (Masola *et al.*, 2003).

The stimulation of the transaminases activities in $CuSO_4$–treated animals may be due to the damage of cell membrane system, resulting in alterations of intercellular metabolism and membrane permeability as recorded by Pelgrom *et al.* (1995). It has also been suggested that increased of these enzyme levels in tissues are associated with elevated energy requirement as organisms try to cope the toxic impacts of xenobiotics (Reddy and Yellamma, 1991), and elicit a probable alteration on protein metabolism in the tissues. Additionally, it is probable that the elevations in these enzymes activity were due in part to the need for deamination of amino acids as a result of potential increased tissue destruction as a consequence of the toxic impacts of contaminants. The reduction in AST and ALT might indicate damage of snails' tissue due to high copper levels. ALT is also notoriously unstable (Mukorah *et al.*, 1998) and its instability increases in the existence of high Cu levels.

Conclusions and Recommendations

This investigation showed that $CuSO_4$ has a molluscicidal efficiency against the white land snail, *T. pisana* through a contact toxicity testing. Furthermore, results exhibited that $CuSO_4$ at two tested sublethal doses caused alterations in the activities of AChE, ALP, AST and ALT of *T. pisana* snail and consequently can be

utilized as effective biomarkers in land snails to reveal the biochemical mechanisms of $CuSO_4$ toxicity.

Conflict of interest

The authors have declared no conflict of interest.

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