

## CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITIES OF CICADA EXUVIAE EXTRACTS FROM *Cryptotympana mandarina*

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### ABSTRACT

This study explored the chemical constituents and bioactivities of cicada exuviae extracts from *Cryptotympana mandarina*. Gas chromatography–mass spectrometry (GC-MS) analysis identified 23 diverse compounds, including fatty acids and their esters, aromatic compounds, alkanes, and other constituents. Among these, aromatic compounds and fatty acids with their esters were predominant. *In vitro* assays revealed that the cicada exuviae extracts exhibited notable antioxidant activity by DPPH and ABTS radical scavenging with  $IC_{50}$  of 0.069-0.752 mg/mL. These extracts also displayed antidiabetic potential, inhibiting  $\alpha$ -amylase and  $\alpha$ -glucosidase with  $IC_{50}$  of 0.65-3.69 mg/mL. In addition, the cicada exuviae extracts displayed antimicrobial effects against the examined microorganisms with minimum inhibitory concentrations (MICs) ranging from 64 to 512  $\mu$ g/mL. These findings highlight cicada exuviae extracts as a promising natural source of bioactive compounds with antioxidant, antidiabetic, and antimicrobial properties.

**Keywords:** Antioxidant activity, Antidiabetic activity, Antimicrobial activity, Cicada exuviae, *Cryptotympana mandarina*

## THÀNH PHẦN HÓA HỌC VÀ HOẠT TÍNH SINH HỌC CỦA CAO CHIẾT VỎ VE SÀU

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### TÓM TẮT

Nghiên cứu tiến hành khảo sát thành phần hóa học và hoạt tính sinh học của các cao chiết từ vỏ ve sấu (*Cryptotympana mandarina*). Phân tích sắc ký khí khối phổ (GC-MS) đã xác định được 23 hợp chất khác nhau, bao gồm axit béo và este của axit béo, các hợp chất thơm, các ankan và các hợp chất khác. Trong đó, các hợp chất thơm, axit béo và este axit béo chiếm chủ yếu. Các thử nghiệm *in vitro* cho thấy các cao chiết từ vỏ ve sấu có hoạt tính chống oxy hóa tốt thông qua khả năng loại bỏ gốc tự do DPPH và ABTS với  $IC_{50} = 0,069-0,752$  mg/mL. Các cao chiết từ vỏ ve sấu cũng có tiềm năng chống tiêu đường thông qua khả năng ức chế  $\alpha$ -amylase và  $\alpha$ -glucosidase với  $IC_{50} = 0,65-3,69$  mg/mL. Ngoài ra, các chiết xuất từ vỏ ve sấu cũng thể hiện hoạt tính kháng khuẩn đối với các chủng vi sinh vật được thử nghiệm với MIC=64-512  $\mu$ g/mL. Các kết quả này cho thấy rằng các cao chiết từ vỏ ve sấu là một nguồn tiềm năng của các hợp chất hoạt tự nhiên có tác dụng chống oxy hóa, chống tiêu đường và kháng vi sinh vật.

**Từ khóa:** *Cryptotympana mandarina*, Hoạt tính chống oxy hóa, Hoạt tính chống tiêu đường, Hoạt tính kháng vi sinh vật, Vỏ ve sấu

## 1. INTRODUCTION

Cicada exuviae, also referred to as cicada shells, exoskeletons, cicada slough, or *Periostracum cicadae* in traditional Chinese medicine, are byproducts of the molting process in cicadas (e.g., *Cryptotympana* spp.). Cicada exuviae are traditionally used in East Asian medicine to treat fever, sore throat, skin eruptions, and spasms (Xie et al., 2023). Recent pharmacological investigations have revealed that cicada exuviae extracts exhibit anticonvulsive, anti-inflammatory, antitussive, and anticancer activities (Xie et al., 2023), whereas other potential bioactivities, such as antimicrobial, antioxidant, and antidiabetic effects, have not yet been investigated. These bioactivities are associated with their complex composition, which includes chitin, proteins, minerals, fatty acids, and bioactive small molecules (Wu et al., 2013; Xie et al., 2023; Yali et al., 2015).

Although *C. atrata* is the officially recognized source of *Periostracum cicadae*, other species such as *C. mandarina* are widely distributed across Southeast Asia and are frequently used in traditional medicine (Siddiqui et al., 2023). *Cryptotympana mandarina* is an Asian cicada species distributed across China, Vietnam, Laos, Thailand, India, and surrounding regions (Price et al., 2016). Nevertheless, chemical composition and biological properties of *C. mandarina* exuviae remain insufficiently characterized, underscoring the necessity of investigating *C. mandarina* exuviae to evaluate their therapeutic potential.

The present study aims to characterize the chemical constituents of *C. mandarina* exuviae using GC-MS and to assess their antioxidant, antidiabetic, and antimicrobial activities. These results may contribute to a better understanding of the potential value of this underexplored natural resource. To our knowledge, this is the first report on the chemical composition

and biological properties of *C. mandarina* exuviae.

## 2. MATERIALS AND METHODS

### 2.1. Preparation of the cicada exuviae extracts

*C. mandarina* exuviae were collected in Bach Ma National Park in May 2024. Cicada exuviae of *C. mandarina* (100g) were ground into powder and subjected to ultrasonic-assisted extraction with solvents such as *n*-hexane, methanol and dichloromethane (3×1000mL) for 2h at 50-60 °C each time. The *n*-hexane, methanol, and dichloromethane extracts were concentrated under reduced pressure to yield *n*-hexane extract (6.3g), methanol extract (10.2g), and dichloromethane extract (8.9g).

### 2.2. Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

Volatile components of the exuviae extracts were analyzed by GC-MS using an Agilent 7890B gas chromatograph coupled to a 5977A mass spectrometer equipped with an HP-5MS capillary column (30m×0.25mm×0.25µm) (Dat et al., 2021). Samples were diluted 1:10 in *n*-hexane, and 1µL was injected in splitless mode using helium carrier gas at a flow rate of 1 mL/min. The oven temperature was set from 60°C for 2 min to 260°C at 5°C/min, with a final hold of 1 min. The injector and ion source temperatures were set at 260°C and 280°C, respectively, with a 3 min solvent delay. Mass spectra were obtained under electron ionization conditions (70eV), scanning *m/z* 50-550. Compounds were identified by matching spectra (≥90% similarity) against the Wiley and NIST mass spectral libraries, and relative abundances were estimated from GC peak areas.

### 2.3. Antioxidant Assays

#### 2.3.1. DPPH radical scavenging assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the extracts was determined by

measuring the absorbance reduction of the DPPH solution in the presence of the extracts (Dat et al., 2021). Briefly, 10 $\mu$ L of each extract was mixed with 190 $\mu$ L of 0.1 mg/mL DPPH in 96-well plates. The mixtures were incubated at room temperature for 30 min and absorbance was then measured at 517nm using an ELx800 microplate reader (BioTek Instruments, Winooski, VT, USA). The percentage of DPPH radical scavenging activity was determined using the following equation:

$$\text{Scavenging activity (\%)} = 100 \times [\text{Ac} - (\text{As} - \text{Asb}) / \text{Ac}]$$

where Ac is the absorbance of the control, As is the absorbance of the sample, and Asb is the absorbance of the sample blank. Ascorbic acid was used as a reference antioxidant agent.

#### 2.3.2. ABTS radical scavenging assay

ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging effect of the extracts was determined by measuring the absorbance reduction of the ABTS solution in the presence of the extracts (Dat et al., 2021). ABTS radicals were generated by combining 7mM ABTS with 2.45mM potassium persulfate and incubating the mixture in darkness at room temperature for 16 h. The prepared ABTS radical solution was diluted in ethanol to achieve an absorbance of 0.700 $\pm$ 0.02 at 734nm. For the assay, a mixture of 10 $\mu$ L extract and 190 $\mu$ L ABTS solution was incubated for 10 min at room temperature, after which absorbance at 734nm was measured with an ELx800 microplate reader (BioTek Instruments, Winooski, VT, USA). The percentage of ABTS radical scavenging activity was determined using the following equation:

$$\text{Scavenging activity (\%)} = 100 \times [\text{Ac} - (\text{As} - \text{Asb}) / \text{Ac}]$$

where Ac is the absorbance of the control, As is absorbance of sample, and Asb is the absorbance of the sample blank. Ascorbic acid was used as a reference antioxidant agent.

## 2.4. Antidiabetic Assays

### 2.4.1. $\alpha$ -Amylase Inhibitory Assay

The  $\alpha$ -amylase inhibitory activity of extracts was determined using a colorimetric assay with starch azure as a substrate (Nguyen et al., 2023). Briefly, starch azure was suspended in 0.05 M Tris-HCl buffer (pH 6.9, containing 0.01M CaCl<sub>2</sub>), boiled for 5 min, and then preincubated at 37°C. The reaction mixture, composed of 50 $\mu$ L extract solution, 50 $\mu$ L substrate suspension, and 25 $\mu$ L  $\alpha$ -amylase solution (2 U/mL), was incubated in 96-well plates at 37°C for 10 min. After termination of the reaction with 75 $\mu$ L of 50% acetic acid and centrifugation, the absorbance of the supernatant was measured at 650nm. The percentage inhibition was subsequently calculated as follows:

$$\text{Inhibition (\%)} = 100 \times [1 - (\text{As} - \text{Asb}) / (\text{Ac} - \text{Acb})]$$

where Ac and As are the absorbances of the control and sample, and Acb and Asb are the corresponding blanks of the control and sample. Acarbose was used as a reference inhibitor.

### 2.4.2. $\alpha$ -Glucosidase Inhibitory Assay

The  $\alpha$ -glucosidase inhibitory activity was determined using a colorimetric assay with 4-nitrophenyl  $\beta$ -D-glucopyranoside (pNPG) as a substrate (Nguyen et al., 2023). Briefly, a mixture of 50 $\mu$ L extract and 50 $\mu$ L potassium phosphate buffer (0.1M, pH 6.8) with  $\alpha$ -glucosidase (0.5 U/mL) was incubated at 37°C for 10 min. The assay was initiated with 50 $\mu$ L of 5mM pNPG, incubated at 37°C for 30 min, and stopped with 50  $\mu$ L of 0.2M Na<sub>2</sub>CO<sub>3</sub>. Absorbance at 405nm was used to quantify p-nitrophenol release, and percentage inhibition (%) was calculated as follows:

$$\text{Inhibition (\%)} = 100 \times [1 - (\text{As} - \text{Asb}) / (\text{Ac} - \text{Acb})]$$

where Ac and As are the absorbances of the control and sample, and Acb and Asb

are the corresponding blanks of the control and sample. Acarbose was used as a reference inhibitor.

### 2.5. Antimicrobial assay

Antimicrobial activity of the extracts was evaluated using broth microdilution against seven reference strains (*Salmonella enterica* ATCC 13076, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231) (Dat et al., 2022). The microbial strains obtained from the Mien Trung Institute for Scientific Research were grown in MH (Mueller–Hinton) medium for bacterial cultures and in RPMI (Roswell Park Memorial Institute) medium supplemented with 2% glucose for yeast cultures. For microbial assays, 100  $\mu$ L of microbial inoculum ( $10^6$  CFU/mL for bacteria and  $10^5$  CFU/mL for yeast) was added to 100  $\mu$ L of the extracts serially diluted in 96-well microplates. Bacterial cultures were incubated for 24 hours at 37°C, whereas yeast cultures were incubated for 48 hours at 28°C. Microbial growth was assessed by measuring absorbance at 630 nm for bacteria and 530 nm for yeast. The minimum inhibitory concentration (MIC) was the lowest extract concentration that fully suppressed microbial growth. The antibiotics, ciprofloxacin and fluconazole were used as the positive controls for bacteria and yeast, respectively.

### 2.6. Statistical analysis

All experiments were carried out in triplicate, and data are presented as mean  $\pm$  standard deviation. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test ( $\alpha = 0.05$ ) in SPSS v.20.

## 3. RESULTS AND DISCUSSION

### 3.1. Chemical composition of cicada extracts

The GC–MS analysis of cicada exuviae extracts identified a total of 33 compounds, including fatty acids and their esters, aromatic compounds, alkanes, and other constituents such as phenolics, nitrogen-containing compounds, terpenes, naphthalene derivatives, phthalate esters, acetals, and siloxanes (Table 1). Among these, aromatic compounds and fatty acids and their methyl esters represented the most abundant classes. However, the chemical profiles varied among the different solvent extracts. In the *n*-hexane extract, the major constituents were terephthalic acid, 4-octyl octyl ester (28.73%), dodecanoic acid (7.86%), *n*-hexadecanoic acid (6.76%), and 6-octadecenoic acid (6.35%). The methanol extract was dominated by terephthalic acid, di(4-octyl) ester (39.32%), dodecanoic acid (9.75%), and hexadecanoic acid, methyl ester (8.87%). In contrast, the dichloromethane extract contained high levels of terephthalic acid, di(4-octyl) ester (28.51%), terephthalic acid, 2-ethylhexyl octyl ester (11.46%), terephthalic acid, di(2-ethylhexyl) ester (8.99%), and hexadecanoic acid, methyl ester (7.76%).

These findings are consistent with previous reports on edible insect extracts, which are typically rich in fatty acids and esters, particularly palmitic, oleic, and linoleic acids (Yali et al., 2015). Earlier studies on cicada exuviae have also shown that they are composed of predominant  $\alpha$ -chitin, together with proteins, amino acids, inorganic elements, and secondary metabolites (Poerio et al., 2020). Moreover, cicada exuviae are reported to contain abundant derivatives of N-acetyldopamine, as well as nucleosides, phenolic compounds, amino derivatives, and minerals (Xie et al., 2023).

**Table 1.** Chemical constituents identified in the *C. mandarina* exuviae extracts by GC–MS

Compounds	n-HE extract (%)	MeOH extract (%)	DCM extract (%)
<b>Fatty acids and esters</b>			
(9e)-9-Octadecenoic acid	5.32		1.28
6-Octadecenoic acid	6.35	2.47	
Dodecanoic acid	7.86	9.75	2.47
<i>n</i> -Hexadecanoic acid	6.76	4.79	
Octadecanoic acid	3.87	5.09	4.63
Tetradecanoic acid	0.18	1.46	5.15
Hexadecanoic acid, methyl ester	4.24	8.87	7.76
<b>Aromatic compounds</b>			
Terephthalic acid, 2-ethylhexyl octyl ester			11.46
Terephthalic acid, 4-octyl octyl ester	28.73		
Terephthalic acid, di(2-ethylhexyl) ester			8.99
Terephthalic acid, di(4-octyl) ester		39.32	28.51
<b>Alkanes</b>			
Heptacosane	2.84	1.76	1.39
Icosane	1.65	0.98	3.91
Tricosane	2.73	1.08	2.35
Undecane	0.63	1.38	
<b>Phenolic and nitrogen-containing compounds</b>			
3,7-Dimethoxy-11a-methylpterocarpan		0.38	
6-( <i>T</i> -butyl)-2-methyl-4-phenylpyridine-3-carboxamide		0.11	
Benzeneethenylamine, 3,4-dihydroxy- <i>n</i> -isopropyl		2.69	
<b>Naphthalene and phthalate esters</b>			
<i>N</i> -phenyl-2-naphthylamine			0.05
Diethyl phthalate			0.08
<b>Terpene</b>			
(-)-Limonene			0.72
<b>Acetal and ester</b>			
2,2-Dimethoxybutane	0.14	0.49	
<b>Siloxane</b>			
1H,15H-hexadecamethyloctasiloxane	0.21		
<b>Total</b>	<b>71.51</b>	<b>80.62</b>	<b>78.75</b>

**3.2. Antioxidant, antidiabetic and antimicrobial activity of the *C. mandarina* exuviae extracts**

Antioxidant assays demonstrated that cicada exuviae extracts possessed notable DPPH and ABTS radical scavenging activity, with IC<sub>50</sub> values ranging from 0.069 to 0.752 mg/mL, compared to the reference antioxidant, ascorbic acid, which showed IC<sub>50</sub> of 0.012

– 0.014 mg/mL (Table 2). Among them, methanol extract showed the strongest DPPH and ABTS radical scavenging activity with IC<sub>50</sub> of 0.122±0.006 and 0.069±0.004 mg/mL, respectively, followed by dichloromethane extract with IC<sub>50</sub> of 0.544±0.024 mg/mL and 0.143±0.008 mg/mL, respectively, and *n*-hexane extract with IC<sub>50</sub> of 0.752±0.028 mg/mL and 0.243±0.010 mg/mL, respectively.

**Table 2.** DPPH and ABTS radical scavenging activity of the *C. mandarina* exuviae extracts

Sample	IC <sub>50</sub> (mg/mL)	
	DPPH radical scavenging	ABTS radical scavenging
<i>n</i> -Hexane extract	0.752 ± 0.028 <sup>a</sup>	0.243 ± 0.010 <sup>a</sup>
Methanol extract	0.122 ± 0.006 <sup>b</sup>	0.069 ± 0.004 <sup>b</sup>
Dichloromethane extract	0.544 ± 0.024 <sup>c</sup>	0.143 ± 0.008 <sup>c</sup>
Ascorbic acid	0.012 ± 0.004 <sup>d</sup>	0.014 ± 0.004 <sup>d</sup>

Data are presented as mean ± standard deviation. Values labeled with different letters in the same column are significantly different ( $p < 0.05$ , Tukey HSD)

Antidiabetic assays also revealed that cicada exuviae extracts exhibited potential inhibitory activity against  $\alpha$ -amylase and  $\alpha$ -glucosidase with IC<sub>50</sub> of 0.65-3.69 mg/mL, compared to the reference antidiabetic drug, acarbose, which showed IC<sub>50</sub> of 0.11-0.12 mg/mL (Table 3). Among them, *n*-hexane extract showed strongest  $\alpha$ -amylase and  $\alpha$ -

glucosidase inhibitory activity with IC<sub>50</sub> of 2.04±0.05 mg/mL and 0.65±0.02 mg/mL, respectively, followed by methanol extract with IC<sub>50</sub> of 2.59±0.06 mg/mL and 1.58±0.16 mg/mL, respectively, and dichloromethane extract with IC<sub>50</sub> of 3.69±0.15 mg/mL and 1.77±0.06 mg/mL, respectively.

**Table 3.**  $\alpha$ -Amylase and  $\alpha$ -glucosidase inhibitory activity of the *C. mandarina* exuviae extracts

Sample	IC <sub>50</sub> (mg/mL)	
	$\alpha$ -Amylase inhibition	$\alpha$ -Glucosidase inhibition
<i>n</i> -Hexane extract	2.04 ± 0.05 <sup>a</sup>	0.65 ± 0.02 <sup>a</sup>
Methanol extract	2.59 ± 0.06 <sup>a</sup>	1.58 ± 0.16 <sup>b</sup>
Dichloromethane extract	3.69 ± 0.15 <sup>b</sup>	1.77 ± 0.06 <sup>b</sup>
Acarbose	0.11 ± 0.01 <sup>c</sup>	0.12 ± 0.01 <sup>c</sup>

Data are presented as mean ± standard deviation. Values labeled with different letters in the same column are significantly different ( $p < 0.05$ , Tukey HSD)

Furthermore, antimicrobial tests revealed that cicada exuviae extracts exhibited broad-spectrum antimicrobial activity, with MIC values ranging from 64-512  $\mu$ g/mL, compared to reference antibiotics, ciprofloxacin and fluconazole, which showed MIC values in range of 0.5-4  $\mu$ g/mL (Table 4). Notably, the *n*-hexane extract exhibited the strongest activity against all tested microorganisms with MIC=128-256  $\mu$ g/mL, whereas methanol extract showed antimicrobial activity against five tested strains, *E. coli*, *P. aeruginosa*, *E.*

*faecalis*, *S. aureus*, and *C. albicans* with MIC=256-512  $\mu$ g/mL, and dichloromethane extract showed antibacterial activity against four tested bacteria, *E. coli*, *S. enterica*, *E. faecalis*, and *S. aureus* with MIC=256-512  $\mu$ g/mL. The differences in bioactivities observed among the cicada exuviae extracts are likely attributable to variations in the diversity and relative abundance of their chemical constituents. These findings suggest that *C. mandarina* exuviae contain bioactive compounds with potential antimicrobial and antidiabetic properties.

**Table 4.** Antimicrobial activity of the *C. mandarina* exuviae extracts

Microorganisms	MIC ( $\mu$ g/mL)				
	HEX	MET	DCM	CIP	FLU
<i>Escherichia coli</i>	128	256	256	0.5	-
<i>Salmonella enterica</i>	128	-	512	1	-
<i>Pseudomonas aeruginosa</i>	256	512	-	2	-
<i>Enterococcus faecalis</i>	256	256	512	4	-
<i>Staphylococcus aureus</i>	64	256	512	1	-
<i>Bacillus cereus</i>	128	-	-	2	-
<i>Candida albicans</i>	256	256	-	-	4

*n*-hexane extract (HEX), methanol extract (MET), dichloromethane extract (DCM), ciprofloxacin (CIP) and fluconazole (FLU)

Previous investigations have revealed promising biological activity of cicada exuviae. Polysaccharide-rich fractions from the cicada exuviae have demonstrated strong reducing power and significant radical-scavenging activity against DPPH, hydroxyl, and superoxide radicals (Xie et al., 2023). Different cicada exuviae extracts have also shown DPPH and ABTS radical-scavenging effects with IC<sub>50</sub> values of 0.3-1.5 mg/mL (Guo et al., 2022). In addition, methanol extracts were reported to reduce UVB-induced ROS production and suppress downstream inflammatory mediators, including IL-6 and MMP-2/9, in HaCaT keratinocytes (Chang et al., 2017).

Cicada exuviae extracts have further been associated with broad-spectrum antibacterial effects. Both crude extracts and chitooligosaccharides derived from the exuviae inhibited the growth of *B. subtilis*, *S. aureus*, and *E. coli* (Wu et al., 2013; Xie et al., 2023). Several key bioactive small molecules have also been identified, notably N-acetyldopamine (NADA) dimers and tetramers (e.g., cicadamides A and B), which display antioxidant and anti-inflammatory activities (Liu et al., 2019; Xu et al., 2006; Yang et al., 2016). Moreover, cicada exuviae are rich in minerals and amino acids, which may contribute to their traditional use in managing anemia and supporting immune health (Xie et al., 2023).

Some compounds detected in this work have earlier been reported to show notable biological activities. Limonene has been shown to exhibit antitumor, antiviral, anti-inflammatory, and antibacterial properties (Anandakumar et al., 2021). Fatty acids such as dodecanoic acid, *n*-

hexadecanoic acid, octadecanoic acid, and (9E)-9-octadecenoic acid are well known for their antimicrobial effects, primarily through disruption of microbial membranes and inhibition of biofilm formation (Casillas-Vargas et al., 2021; Jin et al., 2021). In addition, natural phthalate derivatives and terephthalate esters have been reported to display a wide range of biological activities, including antibacterial, antifungal, antioxidant, anti-inflammatory, antitumor, larvicidal, and antifouling effects (Huang et al., 2021; Roy, 2020).

#### 4. CONCLUSION

This study explored the chemical profile and biological activity of cicada exuviae extracts derived from *C. mandarina*. Gas chromatography–mass spectrometry (GC-MS) analysis identified 23 compounds with aromatic compounds and fatty acids and their esters as the predominant constituents. *In vitro* assays demonstrated that the cicada exuviae extracts exhibited notable antioxidant activity via DPPH and ABTS radical scavenging effects with IC<sub>50</sub> of 0.069 – 0.752 mg/mL. These extracts also exhibited antidiabetic potential, inhibiting  $\alpha$ -amylase and  $\alpha$ -glucosidase with IC<sub>50</sub> of 0.65 – 3.69 mg/mL. Furthermore, the exuviae extracts showed antimicrobial activity against the examined microorganisms with MICs ranging from 64 to 512  $\mu$ g/mL. These results highlight the potential of the cicada exuviae extracts as a natural source of antioxidant, antidiabetic, and antimicrobial agents.

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