
Determination of ethyl para methoxycinnamate content and anti-inflammatory test of *Kaempferia galangal*, L rhizome extract by inhibition of protein denaturation method

Laela Hayu Nurani* , Aris Asahi, Hari Susanti

Postgraduate Pharmacy Study Program, Faculty of Pharmacy, Universitas Ahmad Dahlan

Jl. Prof. Dr. Soepomo, Janturan, Yogyakarta, Indonesia

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ABSTRACT

Kencur (Kempferia galanga, L) is one of the rhizomes that is often used as a constituent component in traditional medicinal formulas. One of the *kencur*'s pharmacological activities is anti-inflammatory. The active compound as an anti-inflammatory is ethyl p-methoxycinnamate (EPMC). This study aims to determine the content and ascertainment of EPMC compounds and the anti-inflammatory activity of *kencur* rhizome extract. *Kencur* extract can be obtained by the maceration method using 96% ethanol solvent. The EPMC content determination was held using the TLC-densitometry method, while the EPMC compounds were confirmed using the GC MS method. The anti-inflammatory activity test was done using inhibiting protein denaturation methods. The extract results obtained 12.67% yield. The result of EPMC content in *kencur* extract was 10.05 ± 4.57 . The GCMS *kencur* extract results showed an abundance of EPMC compounds at a retention time of 7.088 with a peak area of 72290779. The results of IC_{50} anti-inflammatory for the standard substance (EPMC) were 5.306 ± 5.028 . The results of IC_{50} anti-inflammatory the sample (*kencur* extract) was 303.487 ± 1.201 . Ethanol extract of *kencur* contains ethyl p-methoxycinnamate (EPMC) and has anti-inflammatory activity by inhibiting protein denaturation.

Keywords: *kencur* extract, EPMC, determination of content, inhibition of protein denaturation

***Corresponding author:**

Laela Hayu Nurani

Postgraduate Pharmacy Study Program, Faculty of Pharmacy

Universitas Ahmad Dahlan, Indonesia

Jl. Prof. Dr. Soepomo, Janturan, Yogyakarta, Indonesia

Email: laela.farmasi@pharm.uad.ac.id

INTRODUCTION

Indonesia is one of the countries with the largest tropical forests globally and has various traditional medicinal plants. One of the plants used for traditional medicine is *kencur*. *Kencur* is one of the commodities that has the biggest contribution to the total production of biopharma plants in Indonesia. The utilization of *kencur* reached 6.33%. *Kencur*'s total production in 2014 was 37.7 million kilograms (BPPP, 2017).

One of them is the pharmacological activity of *kencur* is anti-inflammatory (Samodra and Dina., 2020). Ethyl p-methoxycinnamate (EPMC) compounds are thought to play an active role as an anti-inflammatory (Umar et al., 2014). The content of EPMC in *kencur* is 80.05 % (Umar et al., 2012). Hudha et al. (2015) reported that EPMC compounds could be extracted by the maceration method using 96% ethanol solvent. (Komala et al., 2017) reported that EPMC has anti-inflammatory activity with a pathway inhibition of protein denaturation.

Denaturation of proteins in tissues is a cause of inflammation (In et al., 2016), especially in conditions of arthritis. Protein denaturation is a state of loss of protein's structure and function through several triggers such as temperature, pH, pressure, electricity, a mixture of chemicals, and reducing genes (Farida et al., 2018). The inhibition mechanism of protein denaturation is related to arachidonic acid metabolites as they are protein biomolecules (Umar et al., 2012).

The ester function groups in EPMC compounds contribute to anti-inflammatory activity. Komala et al. (2018) reported that the more C atoms in the ester group, the better the small concentration. EPMC, as an anti-inflammatory drug, has the same effect as a non-steroidal anti-inflammatory drug (NSAID)(Syahrudin et al., 2017).

Its mechanism of action is on arachidonic acid metabolism, with decreased prostaglandin (PG) synthesis. This prostaglandin is an inflammatory mediator (Riasari et al., 2019). Besides inhibiting PG synthesis, EPMC also inhibits several inflammatory mediators such as TNF- α , IL1 β , and Nitric Oxide (NO), cytochrome P450 and CYP2E1 (Sirisangtragul and Sripaidkulchai.,2011; Umar et al., 2014). Therefore, inhibition of protein denaturation causes COX synthesis not to occur, thereby reducing inflammatory symptoms such as osteoarthritis (OA) (Umar et al., 2012; Komala et al., 2017). Based on the foregoing, the researcher wants to test EPMC and *kencur* extract's anti-inflammatory activity by inhibiting the protein denaturation method.

MATERIALS AND METHOD

Materials

Materials used during the study included *kencur* rhizome, ethanol p.a. (Merck KGaA), methanol p.a. (Merck KGaA), ethyl acetate p.a. (Merck KGaA), Silica Gel 60 F₂₅₄ (Merck KGaA), ethyl p-methoxycinnamate (Santa Cruz Biotechnology), NaCl (Merck KGaA), Tris Base (Merck KGaA), Albumin fraction V (Merck KGaA), Aquadest (Bratachem).

Methods

Determination of *Kaempferia galanga*

Determination of *Kaempferia galanga* was carried out in the Biology Laboratory, Faculty of Applied Science and Technology, Ahmad Dahlan University, Yogyakarta.

Preparation of *kencur* rhizome extract

Kencur rhizome extract was made by the maceration method using 96% ethanol solvent. A total of 250 grams of *kencur* powder was put into a container made of glass, then 96 mL of ethanol was added much as 750 ml and stirred with a stirring machine for 3 hours. It was allowed to stand for 24 hours, then filtered with a vacuum pump, and the first macerate was obtained. Furthermore, the waste from the rest of the maceration process was re-macerated twice with the same treatment. The re-maceration process aims to obtain optimal results on the powder performed by the sealing. Maceration results were then evaporated with a rotary evaporator at $\leq 50^{\circ}\text{C}$ until a thick liquid period was obtained.

The evaporation results were evaporated above the water bath to form a thick extract period (Hudha et al., 2015 ; Kochuthressia et al., 2012).

Qualitative test of EPMC compounds in samples (kencur extract)

Thin Layer Chromatography Test (TLC)

Sample solutions (*kencur* extract) were prepared by dissolving 151.5 mg in ethanol p.a. until dissolved, then were filtered and put in 10 ml measuring flask, then the ethanol p.a. was added to the limit of the flask.

Standard solution (ethyl p-methoxycinnamate) was prepared by dissolving 20 mg in ethanol p.a. until dissolved. It was then filtered and put in a 10 mL flask, then ethanol p.a. was added to the flask's limit.

The stationary phase used is Silica Gel 60 F₂₅₄ with plate size (10 x 20 cm). Silica Gel 60 F₂₅₄ is activated in an oven at 100° C for 30 minutes. The mobile phase used 2 types of solvents, namely ethyl acetate and methanol (9:1), then was saturated in the chamber. The standard solution was tested with 5 points tests: the samples were taken as many as 5 points on the plate and eluted to the limit. The Rf value was calculated from the elution result by comparing the length of the spotting distance and the elution limit.

GCMS method

Preparation of standard substance solution (EPMC) began with weighing as much as 50 mg was in a flask with ethanol p.a. to 10 ml flask. Preparation of sample solution (*kencur* extract), weighed as much as 200 mg was in a flask with ethanol p.a. to 10 ml flask, then filtered and a 5 mg/ml concentration was made.

Observation of the EPMC compound in the extract of the rhizome was done using GC-MS in the following conditions: Capillary Column HP-5MS (30 m × 0.25 mm ID × 0.25 μm thickness of film). The separation was run at temperature of 105° C for 2 minutes, then raised to 300° C at a speed of 20 °C/minute and held for 20 minutes, Heater Transfer line MSD, The default gas is helium at a flow rate of 0.7 mL/min, Split ratio 100, solution 1 μL (concentration 5 mg/ml) injected automatically, low mass scanning parameters 35 and a higher mass of 550.

Quantitative test of EPMC compounds in samples (kencur extract)

The result of thin-layer chromatography (TLC) containing standard substance spots and samples on a plate was then read on the TLC-Scanner Densitometer to determine the AUC (area under the curve) and the maximum absorption wavelength. The formula for calculating the was is as follows :

$$\% = \frac{As (1) \times Cp (2) \times f \times 100}{As (2) \times Cs (1)}$$

Keterangan :

$As (1)$	=	Sample area	$Cs (2)$	=	Standard concentration
$As (2)$	=	Standard area	f	=	Dilution factor
$Cs (1)$	=	Sample concentration			

Protein denaturation inhibition assay

Anti-inflammatory activity assay was carried out using the method of inhibiting protein denaturation (Chandra et al., 2012).

Preparation for tris buffer saline (TBS)

As much as 4.35 g of NaCl was dissolved in 200 mL of aqua dest. 605 mg of Tris Base was added and added to 400 mL of distilled water. Then the was is adjusted by adding glacial acetic acid to pH 6.3, Then added aqua dest up to 500 mL.

Preparation of positive control solutions

A total of 20 mg of EPMC was dissolved in a 10 mL volumetric flask with sufficient ethanol p.a to a volume of 10 mL so that a concentration of 2000 µg/ml was obtained. The solution was made in 6 series: 3, 3.5, 4, 4.5, 5, 5.5 µg/mL, and a 0.1% BSA solution was added to the limit of the flask. Furthermore, it was incubated at ± 25 ° C for 30 minutes, then was heated in a closed water bath at 72° C for 5 minutes and cooled at room temperature.

Preparation of negative control solutions

A total of 0.1 g of Bovine Serum Albumin (BSA) were added put into a 100 mL volumetric flask, then added with a TBS solution to a volume of 100 mL. The solution was made in 6 series concentrations of distilled water: 3, 3.5, 4, 4.5, 5, 5.5 µg/mL, with a 0.1% BSA solution added to the flask's limit. Furthermore, it was incubated at ± 25° C for 30 minutes, then heated in a closed water bath at 72° C for 5 minutes and then at room temperature.

Preparation of sample solutions

A total of 100 mg of extracts were dissolved in a 10 mL measuring flask with ethanol PA added up to 10 mL volume, so that a concentration namely 10,000 µg /mL was obtained. The next sample solution was made 6 series of concentrations of 150, 200, 250, 300, 350, 400 µg/mL, and 0.1% BSA solution were added to the limit of the measuring flask. Furthermore, it was then incubated at ± 25° C for 30 minutes, heated in a closed water bath at 72° C for 5 minutes and cooled at room temperature for 25 minutes.

Absorbance Measurement.

The measurement of the solution's absorbance was held using a UV-Vis spectrophotometer at a wavelength of 660 nm.

Calculation of Percentage of Inhibition of Protein Denaturation.

The percentage of protein denaturation inhibition was measured using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{Abs control negative} - \text{Abs Sample})}{\text{Abs control negative}} \times 100$$

Data Analysis

The percent inhibition data was tested partially using SPSS with a 95% confidence level. The anti-inflammatory IC₅₀ value was obtained by determining the linear regression between the percent inhibition (Y) and the concentration (X).

RESULT AND DISCUSSION

Determination of *Kaempferia galanga*

Kaempferia galanga's determination was carried out in the Biology Laboratory of the Faculty of Applied Science and Technology Ahmad Dahlan University. The results of determination show that the *kencur* rhizome plant used in this study was correct; the key of determination is as follows:

1b – 2b – 3b – 4b – 12b – 13b – 14b – 17b – 18b – 19b – 20b – 21b – 22b – 23b –
 24b – 25b – 26b – 27a – 28b – 29b – 30b – 31a – 32a – 33a – 34b – 333b – 334b – 335a – 336a –
 337b – 338a – 339b – 340a – Zingiberaceae
 1a – 2a – 3b – 4a – 5b *Kaempferia*
 1a *Kaempferia galangal L*

Qualitative test of EPMC in samples (kencur extract)

Thin layer chromatography (TLC)

TLC sample test (*kencur* extract) were carried out in Silica Gel 60 F₂₅₄ stationary phase. The mobile phase that used was the ratio between ethyl acetate and methanol (9:1). The appearance of thin layer chromatography under UV₂₅₄ can be seen in Figure 1 with an R_f value of 0.75.

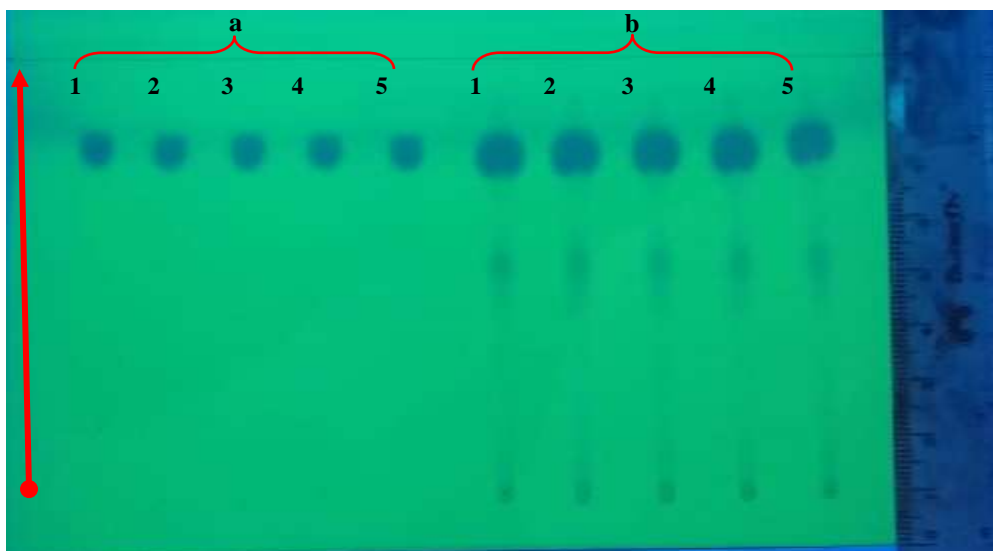


Figure 1. Thin layer chromatography results (a 1-5) EPMC (b 1-5) kencur extract, silica gGel 60 F₂₅₄, mobile phase; ethyl acetate and methanol (9: 1), detection; UV₂₅₄, elution direction

GCMS

The observation of EPMC in *kencur* extract was carried out using the method of Gas Chromatography-Mass Spectrometry (GCMS) method. The standard chromatogram substance at 7.045 retention time was 112851970, and *kencur* extract chromatogram abundant at 7.088 retention time was 72290779. The abundance of compounds based on retention times was presented in Table 1. Results of standard substance chromatograms Figure 2.

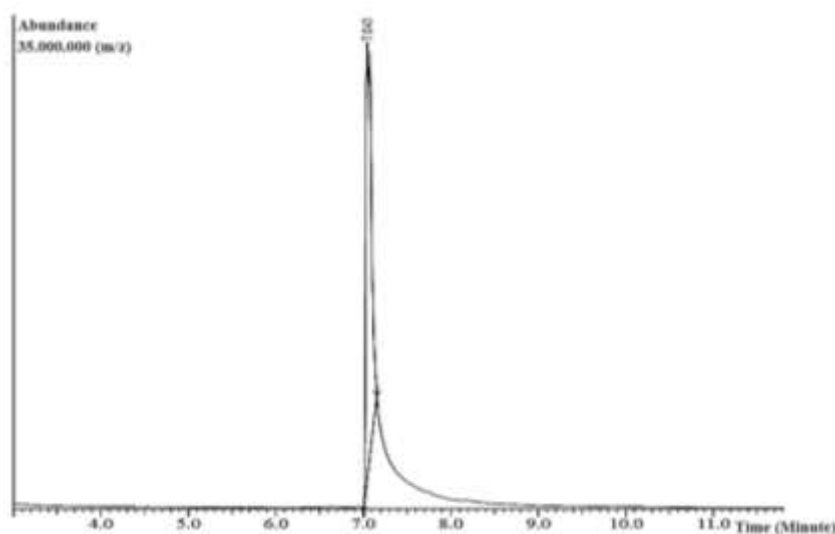


Figure 2. Standard substance chromatogram (EPMC)

Table 1. Abundance of compounds based on retention time

Retention Time	Compound	Abundance
5.583	Pentadecan	10551694
6.207	Pentadecan	110952
6.614	Oktadecan	2343457
6.691	2-Propenoic acid	1909661
6.872	Limonene oxide	1895806
7.088	Ethyl p-methoxycinnamate	72290779
8.161	Hexadecanoic acid	5465394
9.773	9-Oktadecenoic acid	1535728

Quantitative Test of EPMC compounds in samples (Kencur Extract)

Maximum absorption wavelength screening from *kencur* extract was 307 ± 0.46 and EPMC 308 ± 0.32 . TLC results were screened on a TLC-Scanner densitometer tool to determine the peak area of each spot. Area under curve (AUC) values for Figure 1 were presented in Table 2. The result of the calculation of the average content was 10.05 ± 4.57 . Indonesian herbal pharmacopeia guidelines state that the minimum value in determining EPMC levels by the TLC-Densitometry method was not less than 4.30%, meaning that the test was appropriate.

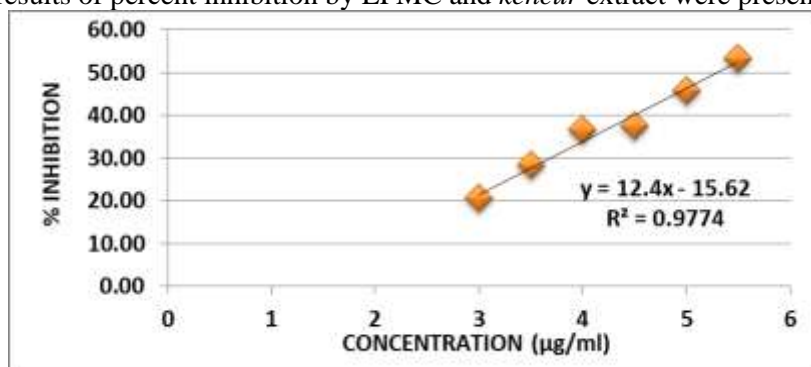
Table 2. AUC Values of standard substances (EPMC) and AUC samples (Kencur extract)

AUC Standard	AUC Sample	% Content
106007.1	82101.9	10.44
100510.2	82713.8	10.51
104032.3	78582.3	9.99
104930.7	78192.5	9.94
103798.0	73712.7	9.37

Information : Concentration Standard : 2 mg/mL ; Concentration Sample : 15.15 mg/mL

Inhibition of protein denaturation

The anti-inflammatory activity test with protein denaturation inhibition was carried out with Bovine Serum Albumin (BSA) 0.1%. BSA as a negative control denatured due to heating in in-vitro testing. The standard substance (EPMC) is a positive control that inhibits denatured BSA, while the inhibition by *kencur* rhizome extract in a variety of concentrations will then be observed. If the inhibitory percentage result $> 20\%$, it was considered to have anti-inflammatory potential (Farida et al., 2018). The results of percent inhibition by EPMC and *kencur* extract were presented in Figure 3.

**Figure 3. Percent inhibition of protein denaturation by EPMC**

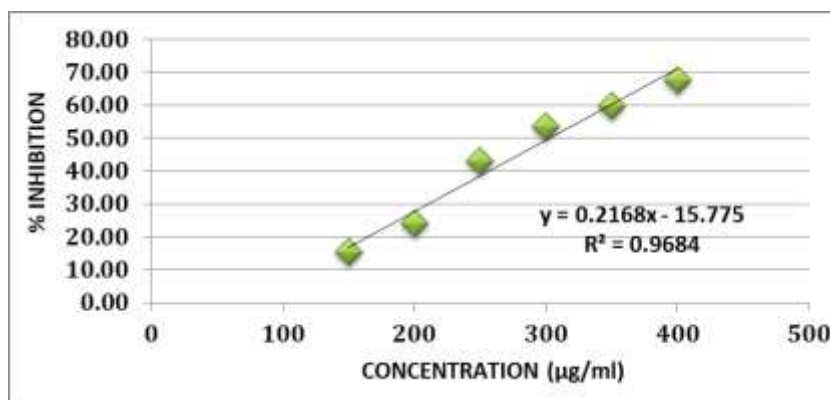


Figure 4. Percent inhibition of protein denaturation by kencur extract

Figure 3 and Figure 4 shows the average of denaturation inhibition. The increased inhibition of protein denaturation accompanied increased concentration. It means *kencur* extract has the same potential as ethyl para methoxycinnamate in inhibiting protein denaturation. Determination of IC_{50} value of standard anti-inflammatory substance (EPMC) and samples (*kencur* extract) in inhibiting protein denaturation began with determining a linear regression between concentration (X) and percentage of inhibition (Y). IC_{50} values of standard anti-inflammatory substances (EPMC) were presented in Table 3, and samples (*kencur* extract) were presented in Table 4.

Table 3. IC_{50} Anti-inflammation standard substance (Ethyl Para Methoxy Cinnamate)

Concentration (µg/ml)	% Inhibition Of Proteiin Denaturation				
	Replication 1	Replication 2	Replication 3	Replication 4	Replication 5
3	24.261	19.581	20.074	19.581	20.197
3.5	29.461	33.174	30.299	24.192	24.311
4	38.056	36.300	42.740	33.021	32.904
4.5	41.829	40.229	32.686	36.229	36.800
5	46.416	44.596	48.350	43.345	46.871
5.5	56.285	53.504	61.290	47.497	48.276

Table 4. IC_{50} Anti-inflammation sample (Kencur Extract)

Concentration (µg/ml)	% Inhibition Of Protein Denaturation				
	Replication 1	Replication 2	Replication 3	Replication 4	Replication 5
150	15.079	15.079	15.193	15.420	15.420
200	23.208	25.939	22.753	25.028	22.753
250	41.133	43227	45.074	44.335	41.379
300	52.798	55.961	51.582	53.771	53.528
350	61.758	60.689	59.501	56.651	60.570
400	65.192	66.589	67.520	72759	65.308

The percent denaturation of protein inhibition was tested using Statistic Product and Service Solutions (SPSS) with a 95% confidence level. T-test results on standard substances (EPMC) showed that the value of T Count > T Table (14.964 > 2.13185); it was concluded that the increase in

concentration had a significant effect on the percent inhibition. T-test results on the sample (*kencur* extract) showed the value of T Count > T Table ($26.165 > 2.13185$); it was concluded that the increase in concentration had a significant effect on the percent inhibition.

Komala et al. (2018) reported that isolates (EPMC) at a $100 \mu\text{g} / \text{mL}$ concentration could inhibit 51.6 ± 1.2 . In this study, *kencur* extract showed a non-strong anti-inflammatory potential at a concentration of 303.487 ± 1.201 , because the EPMC content of the isolated product was greater. Based on this, the sample (*kencur* extract) has the potential to inhibit protein denaturation $\pm 32.95\%$. The standard substance (EPMC) shows a strong anti-inflammatory potential at a concentration of 5.306 ± 5.028 , due to the pure substance (EPMC) used in the test.

The mechanism of inhibition of protein denaturation by the EPMC compound is closely related to the inhibition of arachidonic acid metabolites. Arachidonic acid is a biomolecule in proteins, such as cyclooxygenase and lipoxygenase, that cause inflammation. Umar et al. (2012) reported that EPMC has a mechanism to inhibit COX1 and COX2. In the treatment of inflammation with *kencur* extract in the medical world, it was reported that *kencur* extract has the same effect as one of the non-steroidal anti-inflammatory drugs (NSAIDs) namely meloxicam (Ekowati et al., 2015 ; Syahrudin et al., 2017).

The functional groups of ethyl esters in EPMC compounds contribute the most to anti-inflammatory activity. Komala et al. (2018) reported that the more C atoms in the ester group, the better the small concentration. The potential of EPMC as an anti-inflammatory drug is similar to meloxicam. Its action mechanism is to inhibit the enzyme cyclooxygenase (COX) in arachidonic acid metabolism, resulting in decreased prostaglandin (PG) synthesis arising from tissue injury. This prostaglandin is an inflammatory mediator that can cause tumors (swelling) and pain onset (dolor). Therefore, inhibiting COX synthesis can reduce inflammatory symptoms such as osteoarthritis (OA). In addition to inhibiting PG synthesis, EPMC also inhibits several inflammatory mediators TNF- α , IL1 β , and Nitric Oxide (NO), cytochrome p450 and CYP2E1 (Ekowati et al., 2015 ; Sirisangtragul and Sripanidkulchai., 2011; Umar et al., 2014).

CONCLUSION

Ethanol extract of *Kaempferia galanga* has an EPMC content of 10.05 ± 4.57 . The IC_{50} value of standard anti-inflammatory substances (EPMC) was 5.306 ± 5.028 , and the IC_{50} value of the ethanol extract of *kencur* extract was 303.487 ± 1.201 by inhibition of protein denaturation mechanism.

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