Journal of Pharmaceuticals and Natural Sciences

B-CRETA Publisher
Pharma Insight Solutions

Article

Antibacterial Activity of Kersen Leaf Extract (*Muntingia calabura* L.) Against *Salmonella typhi* and *Staphylococcus aureus* Using Bioautography Method

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Citation: Maharani, D.N.F.; Almeida, M.; Arifian, H; Ibrahim, A.; Herman. Antibacterial activity of Kersen leaf extract (*Mutingia calabura* L.) against *Salmonella thypii* and *Staphylococcus aureus* using bioautography method. J Pham Nat Sci 2025, 2(2), 86-94. https://doi.org/10.70392/jpns.v2i2.8694

Academic Editor: Prof. Dr. Abdul Mun'im

Received: May 5, 2025
Revised: May 30, 2025
Accepted: June 1, 2025

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Abstract

Kersen (*Muntingia calabura* L.) is a plant with a large population in Indonesia. Thus, the abundance of this number can be used as a source of raw materials for medicine. Kersen has many benefits, especially in the leaves as an antibacterial. The purpose of this study was to determine the extract yield, compound groups, and antibacterial activity against *Salmonella typhi* and *Staphylococcus aureus*. Antibacterial activity was measured using the well method and TLC Bioautography. The results showed an extract yield of 19.77%. The TLC profile identified flavonoid, alkaloid, tannin, and steroid compound groups. Kersen leaf extract showed antibacterial activity against *Salmonella typhi* and *Staphylococcus aureus* with moderate to strong antibacterial power at concentrations of 40%, 50%, and 60%. The groups of compounds suspected of playing a role in antibacterial activity are flavonoids, alkaloids, tannins, and steroids. This study shows that kersen leaf extract has the potential as an antibacterial agent against *Salmonella typhi* and *Staphylococcus aureus* bacteria.

Keywords: *Muntingia calabura* L; antibacterial activity; TLC profile; bioautography

1. INTRODUCTION

Infectious diseases are health problems that are often suffered by people in developing countries, including Indonesia. Infections are caused by the invasion of pathogenic microorganisms into the human body [12]. *Salmonella typhi* is a gram-negative bacteria that causes typhoid fever infections, while *Staphylococcus aureus* is a gram-positive bacteria that causes infections of the skin, respiratory tract, and digestive tract [5].

Treatment of bacterial infections is generally done using antibiotics. However, excessive use of antibiotics has the potential to cause the risk of bacterial resistance. Bacterial resistance causes difficulty in healing, increased treatment costs and increased risk of death. Increased bacterial resistance to antibiotics is an opportunity for the use of traditional plants as antibacterials [18].

One of the plants used as an antibacterial is kersen (*Muntingia calabura* L.). Kersen has many benefits, especially in the leaves. kersen leaves have been used empirically by the community to treat diseases such as jaundice, gout, coughs, and act as antiseptics [19]. The results of research conducted by Alouw *et al.* (2022) [2] shows that the ethanol extract of kersen leaves is known to contain flavonoid, saponin, and tannin compounds which are useful as antibacterial. Then the results of research conducted by Juariah *et al.* (2020) [5] showed that ethanol extract of kersen leaves has bacterial inhibitory activity against *Salmonella typhi* and *Staphylococcus aureus* using the disc diffusion method. Based on this description, a study was conducted on antibacterial activity and to determine the active compounds of ethanol extract of kersen leaves against *Salmonella typhi* and *Staphylococcus aureus* by well diffusion and bioautography.

2. MATERIALS AND METHODS

2.1. Materials

The materials used in this study include kersen leaves (*Muntingia calabura* L.), 96% ethanol, nutrient agar (NA) medium, 0.9% NaCl physiological solution, *Salmonella typhi* and *Staphylococcus aureus*, chloramphenicol, Dragendorff reagent, Lieberman-Bouchard, $FeCl_3$, and 3% $AlCl_3$, n-hexane, ethyl acetate, chloroform, methanol.

2.2. Apparatus

The equipment used in this study included an oven, rotary evaporator (Butchi), analytical scales, autoclave, vernier calipers, petri dishes (Pyrex/Iwaki), loop needles, LAF (Laminar Air Flow), McFarland Densitometer (Biosan), incubator, GF_{254} silica plate, capillary tube, chamber, UV lamp.

2.3. Prosedur

2.3.1. Extract yield

1. Preparation of dry samples of kersen leaves
   Fresh samples of kersen leaves were collected and cleaned from dirt, then dried using an oven at 40°C for 6-8 hours.
2. Kersen Leaf Extraction
   The refined sample of 541 g was put into a container and given 5 L of 96% ethanol solvent. The sample was soaked for 3 days while stirring until the liquid ethanol extract was obtained. Soaking and replacing the solvent was done 5 times until the soaking solution became clear. The sample was filtered and the liquid ethanol extract obtained was then concentrated with a rotary evaporator at a temperature of 50°C. Furthermore, the ethanol extract obtained was collected and evaporated in a desiccator.

2.3.2. Antibacterial Activity

1. NA media Preparation and Equipment Sterilization
   5 grams of NA powder Dissolved in 250 mL of distilled water then heated until homogeneous. NA media and the tools to be used are sterilized in an autoclave for 15 minutes at a temperature of 121°C.

2. Bacterial rejuvenation *S. Typhi* and *S.aureus*
   One loop of pure culture of test bacteria was inoculated on each surface of sterilized NA slant agar in a test tube. Then incubated for 1×24 hours at 37°C.
3. Bacterial Suspension Preparation
   The test bacteria were taken as much as 1 ose then put into 10 mL of 0.9% NaCl physiological solution. The test bacterial suspension was homogenized and then measured using a McFarland Densitometer to determine whether the bacterial turbidity had met the standard of 0.5.
4. Determination of effective concentration
   Antibacterial activity testing was carried out using the well diffusion method by inserting 20 microliters of test bacterial suspension into a petri dish and adding 15 mL of NA media, then homogenizing and waiting until it solidifies. The existing media was perforated with 5 holes. In this well hole, 20 microliters of extract solution with concentration variations of 40%, 50%, and 60% were filled as well as a positive control in the form of 1% chloramphenicol and a negative control in the form of ethanol. The petri dish was incubated for 1×24 hours at a temperature of 37°C, then the inhibition zone formed around the well hole was observed and measured using a vernier caliper..

2.3.3. Thin Layer Chromatography Profile

1. Determination of mobile phase
   The extract was dissolved using a mixture of chloroform and methanol solvents (1:1) then spotted using a capillary tube on a TLC plate that had been activated using an oven. Then eluted using n-hexane and ethyl acetate eluents with various ratios, namely 5:5, 6:4, 7:3, 8:2, 9:1 which had been saturated in the chamber and waited until the eluent moved to the limit mark on the upper limit on the TLC plate. The TLC plate was lifted and aired then the spots were observed using visible light and UV lamps 254 nm and 366 nm.
2. Metabolite secondary identification
   The extract was dissolved using a mixture of chloroform and methanol solvents (1:1) then spotted using a capillary tube on a TLC plate that had been activated using an oven. Then eluted using n-hexane and ethyl acetate (7:3) eluents that had been saturated in the chamber and waited until the eluent moved to the boundary mark on the upper limit of the TLC plate. The TLC plate was lifted and aired and then sprayed using several reagents, namely 3% AlCl3 to detect flavonoids, 10% FeCl3 to detect tannins, Dragendorf to detect alkaloids, and Lieberman-Bouchard to detect steroids. The results of the spots were observed for the resulting color changes and then the Rf value was calculated..

2.3.4. Thin Layer Chromatography Bioautography

A 20 microliter suspension of test bacteria was put into a petri dish and 15 mL of NA media was added, then this mixture was homogenized by shaking and the media was allowed to solidify. The chromatogram of the results of the separation of compounds by TLC was placed on the surface of the solidified media and left for 30-60 minutes, then the TLC plate was removed from the media. Furthermore, the media was incubated at 37°C for 24 hours. The inhibition zone formed was observed and the Rf value was calculated.

3. RESULT AND DISCUSSION

3.1. Extract Yield

Kersen leaf extract (*Muntingia calabura* L.) is obtained by maceration method extraction. Maceration is one of the extraction methods carried out by soaking samples or simple drugs using certain organic solvents. This method is used because the procedure and equipment are simple, the process is easy, it can extract the sample compound content optimally and can avoid damage to the compound content in samples that are thermolabile because it does not use high temperatures. The sample to be macerated is first chopped before being dried to speed up the drying process and facilitate the maceration process, namely when the solvent draws the compound content in the cells. Kersen leaf extraction uses 96% ethanol solvent because it is a universal solvent that can extract polar and less polar compounds, is selective, non-toxic, has good absorption,

and has high extraction capacity. 96% ethanol solvent easily penetrates the sample cell walls compared to low concentration ethanol solvents, resulting in a concentrated extract [20].

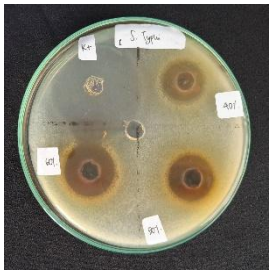
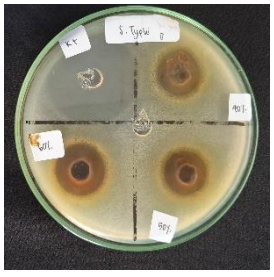
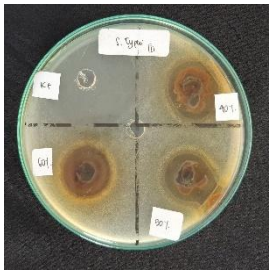
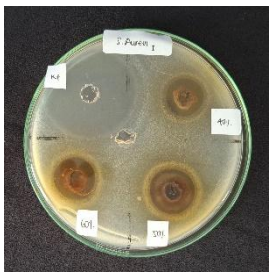
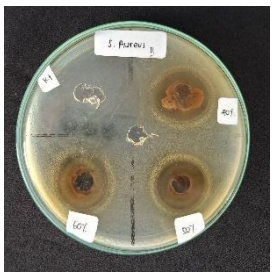
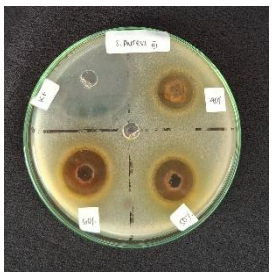
The filtered macerate is then evaporated using a rotary evaporator at a temperature of 50°C to obtain a thick ethanol extract. The use of a temperature of 50°C in the solvent evaporation process makes it easier for ethanol to evaporate. This is related to the working principle of the rotary evaporator, which works by evaporating the solvent below the boiling point, where the boiling point of ethanol is 78°C [9]. The evaporated solvent will condense and move into the collection flask due to the solvent vapor pressure so that the ethanol solvent is obtained again which will be used for sample remaceration. The thick extract obtained is weighed and calculated using the yield formula so that the ethanol extract yield value is 19.77%.

3.2. Antibacterial Activity

Testing the antibacterial activity of kersen leaf extract by well diffusion began with the preparation of nutrient agar (NA) media and then sterilized with the equipment to be used using an autoclave for 15 minutes at a temperature of 121°C. Then the bacteria were rejuvenated to obtain new and young cultures so that the bacteria could reproduce well. The rejuvenation results were incubated in an incubator at a temperature of 37°C for 24 hours. Then a bacterial suspension was made by taking one loop of bacteria from the results of bacterial rejuvenation and then putting it into 10 mL of 0.9% NaCl physiological solution. The test bacterial suspension was homogenized and then measured using a McFarland Densitometer. The preparation of bacterial suspensions was carried out to obtain the desired number of bacteria [16].

The preparation of the test solution used variations in concentrations of 40%, 50%, and 60% by dissolving the kersen leaf extract using 96% ethanol. In addition, the use of 96% ethanol is also a negative control because it adjusts the solvent used in the extract. After that, a positive control was made, namely 1% chloramphenicol by weighing 0.1 grams then putting it into a 10 mL measuring flask and adjusting it to the limit mark with ethanol. Chloramphenicol is a broad-spectrum antibiotic that is active against gram-negative and gram-positive bacteria, both aerobic and anaerobic, so it can inhibit the growth of the test bacteria used [3].

Tabel 1. Kersen leaf extract antibacterial activity

No	Microorganism	Picture		
		Replication 1	Replication 2	Replication 3
1	*Salmonella typhi*			
2	*Staphylococcus aureus*			

Testing the antibacterial activity of kersen leaf extract was carried out using the well diffusion method. The well diffusion method is a method carried out by making holes in the solid agar media that has been inoculated with test bacteria with the number and location of the holes adjusted to the research objectives. This method was chosen because it has the advantage of being easier to measure the area of the inhibition zone formed because the test sample is active on the top surface to the bottom of the nutrient agar [2].

The results of the research on the antibacterial activity of kersen leaf extract against *Salmonella typhi* and *Staphylococcus aureus* bacteria obtained can be seen in tables 1 and 2.

Table 2. Average Results of Inhibition Zone of Antibacterial Activity of Kersen Leaf Extract

No	Microorganism	Test Group	Average (mm)±SD	Category
1	*Salmonella typhi*	Ethanol extract 40%	10.4 ± 0.4	Moderate
		Ethanol extract 50%	11.2 ± 0.529	Strong
		Ethanol extract 60%	13.1 ± 0.503	Strong
		Positive control	29.5 ± 0.872	Very Strong
		Negative control	0	No Activity
2	*Staphylococcus aureus*	Ethanol extract 40%	8.8 ± 0.322	Moderate
		Ethanol extract 50%	11.5 ± 0.3	Strong
		Ethanol extract 60%	12.5 ± 0.252	Strong
		Positive control	30.1 ± 1.498	Very Strong
		Negative control	0	No Activity

Based on the results of the antibacterial activity test of kersen leaf extract at concentrations of 40%; 50%; and 60%, it is known that the inhibitory activity against *Salmonella typhi* bacteria is 10.4; 11.2; and 13.1 (mm), respectively. The inhibitory activity of the positive control (Chloramphenicol) is 29.5 mm and the negative control (96% ethanol) is 0 mm. While on *Staphylococcus aureus* bacteria, it is 8.8; 11.5; and 12.5 (mm), respectively. The inhibitory activity of the positive control (Chloramphenicol) is 30.1 mm and the negative control (96% ethanol) is 0 mm.

According to Davis and Stout (1971), the antibacterial power category is divided into 4 categories based on the diameter of the inhibition zone, namely weak (inhibition zone less than 5 mm), medium (inhibition zone 6-10 mm), strong (inhibition zone 11-20 mm), and very strong (more than 20 mm) [2]. Based on the diameter of the inhibition zone produced against *Salmonella typhi* and *Staphylococcus aureus* bacteria, the antibacterial power of ethanol extract at a concentration of 40% was categorized as moderate, concentrations of 50% and 60% were categorized as strong, and in the positive control of chloramphenicol were categorized as very strong..

The observation results show that with the increase in the concentration of the test sample used, the diameter of the inhibition zone produced also increases. This is suspected because the higher the concentration used, the greater the amount of antibacterial compounds released and penetrated into bacterial cells based on their respective working mechanisms [6]. The best concentration resulting from the antibacterial activity test on ethanol extract of kersen leaves was 60% because this concentration can produce maximum antibacterial strength.

The results of the antibacterial activity test of ethanol extract of kersen leaves have greater inhibitory activity against *Salmonella typhi* bacteria than *Staphylococcus aureus*. This is because the content of compounds in the ethanol extract is still universal so that it includes all compounds with different levels of polarity. *Salmonella typhi* is a gram-negative bacteria that contains more lipids, little peptidoglycan, and an outer membrane in the form of a bilayer (functions as a defense against compounds that enter or leave the cell and cause toxic effects). The outer membrane consists of phospholipids (inner layer) and lipoproteins (outer layer) composed of lipids. Compounds that are thought to work on ethanol extract include flavonoids, polyphenols and tannins, alkaloids, steroids, and saponins that have different levels of polarity can enter the bacterial cell wall and penetrate the lipoprotein layer in bacteria [17].

3.3. Thin Layer Chromatography Profile

Identification of the group of compounds of kersen leaf extract was carried out using the TLC method to obtain the results of the separation of compounds based on their polarity properties by involving two phases, namely the stationary phase (adsorbent) and the mobile phase (eluent). This test begins by determining the best eluent ratio to be used. The ethanol extract was dissolved with a mixture of chloroform and methanol solvents (1:1) to obtain perfect separation results and then spotted on a 7 × 1 cm TLC plate with an upper line of 0.5 cm and a lower line of 1 cm. After that, it was eluted in a chamber with various variations in the ratio of n-hexane and ethyl acetate eluents with a ratio of 5:5, 6:4, 7:3, 8:2, and 9:1. Determination of the eluent system in this study was carried out by the trial and error method. The best eluent chosen was an eluent that could separate the compounds well in the form of a large number of spots and Rf values that were not close together [8]. The results of the spots from each comparison were observed under UV light at 254 nm and 366 nm. The results of determining the best eluent that showed good spot separation were obtained from eluents with a ratio of n-hexane: ethyl acetate (7:3). Then continued spraying with 3% $AlCl_3$ reagent to detect flavonoids, Dragendorff to detect alkaloids, 10% $FeCl_3$ to detect polyphenols and tannins, and Lieberman-Bouchard to detect terpenoids and steroids.

Table 3. Identification of Compound Groups of Kersen Leaf Extract

No	Compound Class	Reagent	Positive Result	Rf Value(cm)
1	Flavonoid	$AlCl_3$ 3%	Yellow	0.56
2	Alkaloid	Dragendorf	Orange	0.49
3	Tanin	$FeCl_3$ 10%	Black	0.44
4	Steroid	Lieberman-Bouchard	Blue	0.27 ; 0.4 ; 0.89

The results of the detection of the flavonoid compound group showed positive results with a color change forming a greenish yellow stain at UV 366 nm with an Rf value of 0.56. The reaction mechanism of $AlCl_3$ with flavonoids is that $AlCl_3$ will react with flavonoid compounds to form a complex between neighboring hydroxyl and ketone groups or neighboring hydroxyl groups. $AlCl_3$ will react with ketones at C4 and OH groups at C3 or C5 in flavone or flavonol compounds to form a yellow complex compound [7]. The results of the detection of alkaloid compound groups using Dragendorff reagent showed positive results of color changes forming orange spots with an Rf value of 0.49. The reaction mechanism between alkaloids and Dragendorff causes a change in ligands where nitrogen which has a free electron pair in the alkaloid forms a coordinate covalent bond with the K^+ ion from potassium tetraiodobismuthate forming a potassium-alkaloid complex [15].

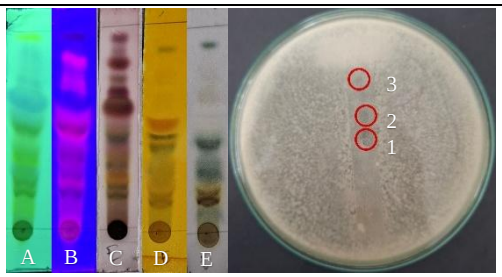
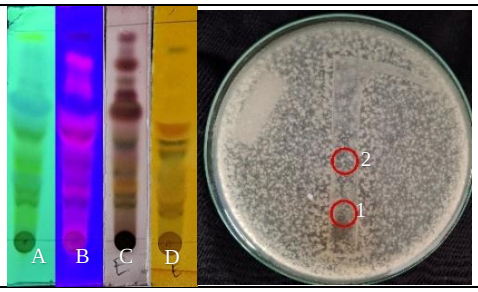
The results of the detection of polyphenol and tannin compound groups showed positive results with a color change to blackish green with an Rf value of 0.44. Polyphenols can release H^+ ions and form phenoxy ions which will react with FeCl3 to form a complex compound of iron (III) hexaphenolate [14]. Meanwhile, tannin will react with $FeCl_3$ with Fe_3^+ ions and will form a complex compound [4]. The results of the detection of terpenoid and steroid compound groups using the lieberman-bouchard reagent showed positive results for blue-green steroids with Rf values of 0.27; 0.4; and 0.89. The reaction between steroid compounds and lieberman-bouchard is condensation or release of H_2O and the combination of carbocations. The reaction begins with the release of hydrogen groups and their electrons, so that the double bond moves. This compound undergoes resonance acting as an electrophile and carbocation. The carbocation attack results in electrophilic addition, followed by the release of hydrogen. Then the hydrogen group and its electrons are released so that the compound undergoes conjugation extension which shows a color change [11].

3.4. Thin Layer Chromatography Bioautography

Antibacterial assay with Thin Layer Chromatography Bioautography was carried out using the contact bioautography method because the work is simple and the results can be seen clearly [10]. This test is a follow-up test to determine what chemical components provide antibacterial activity from ethanol extract of kersen leaves with a concentration of 60%. This

test begins with the test sample being spotted on a TLC plate and then eluted using n-hexane: ethyl acetate (7:3) eluent to produce a separation of compounds based on their polarity which will then be contacted with the media that has been inoculated with test bacteria. Based on the results of the inhibition zone formed, the Rf value can be calculated so that the polarity of the compound that diffuses from the TLC plate into the media that has been inoculated with test bacteria can be determined. Compounds with high Rf values indicate low polarity, while compounds with low Rf values indicate high polarity.

Table 4. Bioautography Thin Layer Chromatography Test Results

No	Microorganism	Documentation	Rf Value
1	*Salmonella typhi*		(1) 0.5 (Alkaloid) (2) 0.64 (Not Detected) (3) 0.89 (Steroid)
2	*Staphylococcus aureus*		(1) 0.05 (Not Detected) (2) 0.49 (Alkaloid)

Description of the appearance of the stain:
(A) UV Ray 254 nm
(B) UV Ray 366 nm
(C) H_2SO_4 10%
(D) Dragendorf (Alkaloid)
(E) Lieberman-Bouchard (Steroid)

The results of the TLC bioautography test on *Salmonella typhi* bacteria showed an inhibition zone at Rf values of 0.5; 0.64 and 0.89. The group of compounds with an Rf value of 0.5 showed higher polarity or tended to be polar, while the group of compounds with Rf values of 0.64 and 0.89 showed lower polarity or tended to be non-polar. When compared with the results of secondary metabolite identification, it is suspected that the group of compounds that play a role is alkaloids at an Rf value of 0.5 where the Rf value is 80 close to the TLC test on the compound, namely 0.49 and steroids at an Rf value of 0.89. Meanwhile, the group of compounds with an Rf value of 0.64 did not show positive results from the identification of secondary metabolites from the group of compounds. This can occur allegedly because the reagent used is not the right reagent for identifying the group of compounds so that it does not produce a color change.

The results of the TLC bioautography test on *Staphylococcus aureus* bacteria showed an inhibition zone at Rf values of 0.05 and 0.49. The group of compounds with these Rf values showed higher polarity or tended to be polar. When compared with the results of the identification of secondary metabolite compound groups, it is suspected that the group of compounds that plays a role in the Rf value of 0.49 is alkaloids. Meanwhile, the group of compounds with an Rf value of 0.05 did not show positive results from the identification of secondary metabolites from this group of compounds.

Based on the results of the TLC bioautography test, the group of compounds suspected of having antibacterial activity from ethanol extract against *Salmonella typhi* and *Staphylococcus aureus* bacteria are alkaloids and steroids. Alkaloids as antibacterials have a working mechanism by inhibiting the components of the peptidoglycan of bacterial cells so that the

cell wall is not formed completely and even causes cell death [12]. Meanwhile, the working mechanism of steroids as antibacterials is related to sensitivity to steroid components and lipid membranes which cause leakage in bacterial liposomes [1].

4. CONCLUSION

Based on the research results obtained, it can be concluded that the yield value of kersen leaf extract is 19.77%. The antibacterial activity of kersen leaf extract has the best inhibitory activity at a concentration of 60%. This is also possible because of the secondary metabolite content contained therein, namely flavonoids, alkaloids, tannins, and steroids which are useful as antibacterials. The group of compounds suspected of being active as antibacterials by TLC bioautography are alkaloids and steroids.

Author Contributions: **Conceptualization**, Herman and Arsyik Ibrahim; **Methodology**, Arsyik Ibrahim; **Validation**, Arsyik Ibrahim and Herman; **Formal Analysis**, Dhea Nur Fadillah Maharani; **Data Curation**, Herman and Arsyik Ibrahim; **Writing–Preparation Original Draft**, Dhea Nur Fadillah Maharani; **Writing–Review Editing**, Herman, Hanggara Arifian, and Maria Almeida; **Visualization**, Hanggara Arifian and Maria Almeida

FUNDING: This research did not receive any funding from any party

ACKNOWLEDGEMENT: -

CONFLICT OF INTEREST: The authors declare no conflict of interest.

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