

CHARACTERIZATION OF NANOPARTICLES PRODUCED BY CHLOROFORM FRACTION OF *KAEMPFERIA ROTUNDA* RHIZOME LOADED WITH ALGINIC ACID AND CHITOSAN AND ITS BIOLOGICAL ACTIVITY TEST

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ABSTRACT

Objective: The main objectives of this research are to characterize the nanoparticles produced by chloroform fraction of *Kaempferia rotunda* loaded with alginic acid and combination of alginic acid-chitosan, and its biological activity test.

Methods: Chloroform fraction of *K. rotunda* was loaded on alginic acid and combination of alginic acid-chitosan nanoparticles by ionic gelation method in various compositions. Characterizations of the products were investigated in particle size, zeta potential, and morphology by scanning electron microscopy (SEM). The biological activity of the products as an antioxidant was tested by the 2,2-diphenyl-1-picrylhydrazyl method. The cytotoxic effect was analyzed using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Result: The alginic acid nanoparticles can be synthesized at the optimal mass ratio range of alginic acid:CaCl₂ of 10:1 (% w/v), while the combination of alginic acid-chitosan nanoparticle at the optimal mass ratio range of alginic acid:chitosan of 10:1 (% w/v) and added calcium ion at 0.015% w/v. Antioxidant activity from alginic acid nanoparticle; combination of alginic acid-chitosan nanoparticle; and chloroform fraction from *K. rotunda* showed inhibition activity (IC₅₀): 225.63±3.5, 237.12±2.1, and 25.20±1.3 mg/mL, respectively, whereas the cytotoxic effect against human breast cancer T47D from these compounds showed IC₅₀: 145.25±2.2, 281.21±2.3, and 41.72±0.47 µg/mL, respectively.

Conclusion: The nanoparticle products of chloroform fraction from *K. rotunda* loaded alginic acid and combination alginic acid-chitosan were successfully obtained by ionic gelation method. The nanoparticle products show lower activity in antioxidant and cytotoxic effects against human breast cancer T47D cell lines than the starting material chloroform fraction of *K. rotunda*.

Keywords: Alginic acid, Chitosan, Nanoparticles, *Kaempferia rotunda*, Antioxidant, Cytotoxic effect, Human breast cancer T47D cell lines.

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INTRODUCTION

Kaempferia is a genus, belonging to the family of Zingiberaceae. This plant grows in Southeast Asia, India, Sri Lanka, Indonesia, and southern China. Economically important species among the plant families, the Zingiberaceae, which are perennial rhizomatous herbs, contain volatile oil and other important compounds of enormous medicinal values [1]. Many researchers reported that the chemical constituents of *Kaempferia pandurata* have anti-HIV, antibacterial, anti-inflamasi, analgesic, antipyretic, antitumor, and antioxidant activities. Some compounds such as panduratin isolated from *Boesenbergia pandurata* showed cytotoxic against pancreatic PANC-1 cancer cell under nutrient-deprived condition [2,3]. *K. pandurata* showed a synergistic effect on all antibiotics tested against MSSA (methicillin-sensitive *Staphylococcus aureus*) [4]. *Kaempferia rotunda*, known as kunci pepet or kunir putih in Indonesia, has been traditionally used for treating abdominal pain, sputum laxative, wounds, and diarrhea colic disorder. Many researchers reported that this plant has biological activities such as antioxidant [5], antimutagenic [6], and anticancer [7,8]. Phytochemical investigation on rhizomes of *K. rotunda* has afforded three flavanones, namely 5-hydroxy-7-methoxyflavanone (pinostrobin), 7-hydroxy-5-methoxyflavanone, and 5, 7- dihydroxyflavanone and one lactone is crotepoxide [8].

In recent years, nanotechnology has begun to grow in the fields of engineering, medicine, electronics, optics, and biomedicine [9]. Application of nanotechnology in the pharmaceutical field has many advantages, among others are increasing the solubility of the compound, reducing the dose of medication, and increasing absorption. Nanotechnology has been enabling to manipulate drugs to reach

a target with a right dose. Some researchers have used it to cure serious diseases such as tumors, cancer, and HIV [10]. Nanoparticles used as drug carriers in the form of colloidal solid have a diameter of 10-1000 nm and are composed of synthetic polymers, natural, semi-synthetic, or encapsulating drug molecules [11]. However, the polymers used for drug delivery should be easy to synthesize, in-expensive, biocompatible, biodegradable, non-immunogenic, non-toxic, and water soluble. Previous study showed that the preparation of phospholipid complex of *K. galanga* rhizome extract using phosphatidylcholine can increase the bioavailability and analgesic activity compared to the free of extract [12].

Biodegradable polymers generally have favorable properties because they can be degraded in the body, the surface structure can be easily modified and adjusted as required. These advantages prove that the polymer nanoparticles are effective system to entrap or encapsulate drugs that are usually sensitive to environmental changes. Polymeric nanoparticles that bind to peptides can be used for oral delivery and also to increase the absorption and bioavailability. In addition, the method of production in this case can be performed on a large scale [13]. The most commonly used natural polymers in the preparation of polymeric nanoparticles are chitosan, gelatin, sodium alginate, and albumin [14].

Alginate is a natural polysaccharide which can be found on the species of brown algae such as *Sargassum* sp., *Laminaria* sp., *Ascophyllum* sp., and *Macrocystis pyrifera*. Alginate is a water-soluble linear polysaccharide extracted from brown sea weed and is composed of alternating blocks of 1-4 linked α -L-guluronic and β -D-mannuronic acid residues.

Alginate has numerous potential for pharmaceutical and biomedical applications such as drug delivery system and cell encapsulation because it is mucoadhesive, biodegradable, and biocompatible [15]. Chitosan, a linear polysaccharide consisting of glucosamine and *N*-acetylglucosamine units, is biocompatible, biodegradable, and nontoxic in the application of per-oral delivery of drugs. Chitosan has further demonstrated the capacity to enhance the epithelial permeation of macromolecules through transient opening of epithelial tight junctions. In addition, chitosan is known to be biocompatible and exhibits very low toxicity. Compared to many other natural polymers, chitosan has a positive charge and it is mucoadhesive [16].

Alginate is polyanion, while chitosan is polycation, so mixing alginate and chitosan in normal conditions can form polyelectrolyte complexes that are capable of trapping the drug in it. Previous study showed that nanoparticle produced by chloroform fraction of *K. rotunda*-loaded chitosan can be synthesized at the concentration ratio of 10:1 for chitosan/sodium tripolyphosphate (Na-TPP). The products have the size in the range of 172-877 nm, with a zeta potential of +28.06 to +38.03 mV. The antioxidant activity of chitosan nanoparticles of chloroform fraction of *K. rotunda* showed less activity compared with the previous fraction [17]. In this study, we report the synthesis of nanoparticles produced by chloroform fraction of *K. rotunda*-loaded alginic acid (product 1) and the combination of alginate-chitosan acid (product 2). As well, analysis of the characteristics of the products which include particle size, zeta potential, and morphology of nanoparticles is recorded. The results of biological activity as antioxidants and cytotoxic effects of breast cancer against human T47D cell lines were also reported.

METHODS

Apparatus and reagent

Glassware, analytical balance, evaporator Buchi Rotavapor R-114, magnetic stirrer, centrifuge, scanning electron microscopy (SEM, Jeol T-300), particle size analysis (PSA, Horiba 550), zeta potential, refrigerator, freeze dryer, Spectronic 20 (Genesys), water bath, shaker bath, microscope, camera, counter, desk glass, Eppendorf, object glass, and analytical balance were commonly used in this work.

Ethanol, aqua bidest, chitosan (low molecular weight, Sigma), Na-TPP, (Sigma-Aldrich), acetic acid (p.a. Sigma), chloroform (p.a. Sigma), rhizome of *K. rotunda*, 2,2-diphenyl-1-picrylhydrazyl (DPPH, Aldrich), and ascorbic acid (Aldrich) were purchased and used without further purification. Human breast cancer cells of T47D cell-line were obtained from the collection of the Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada (UGM). The cells were grown in medium culture of Dulbecco's modified eagle's medium from Gibco, containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco), and was incubated at 37°C at a humidified atmosphere of 5% CO₂-95% air. Trypsin-ethylenediaminetetraacetic 0.025% (Gibco) solution was used to detach cells on the flask.

Preparation of chloroform fraction of *K. rotunda*

The *K. rotunda* rhizome used in this study was obtained from Beringharjo market, Yogyakarta, Indonesia. The plant was identified by the staff at the Faculty Biology of UGM, Indonesia, and a voucher specimen had been deposited at the herbarium. The milled dried rhizome of *K. rotunda* (3 kg) was extracted exhaustively with ethanol. The ethanol extract was partitioned 3 times with *n*-hexane, chloroform, and ethyl acetate. The chloroform fraction was evaporated using a vacuum evaporator to dry to yield the brown residue of about 230 g.

Preparation of chloroform fraction of *K. rotunda*-loaded alginic acid nanoparticles (product 1) and combination of alginic acid-chitosan nanoparticles (product 2)

Products 1 and 2 were prepared using the same method as has been reported in previous studies [18]. Product 1 is made in the following manner: chloroform fraction of *K. rotunda* was dissolved in 35 ml of ethanol and 15 mL of distilled water. Furthermore, the solution was

added 100 ml of alginic acid (0.1% w/v dissolved in 0.1 M NaOH) and stirred using a magnetic stirrer to obtain a homogeneous mixture. The resulting solution was further added with 350 mL calcium chloride (in aquadest) at various concentrations (0.01-0.05% w/v) slowly while stirring using a magnetic stirrer at low speed, and the mixture was kept till complete dissolution with a magnetic stirrer at medium speed for about 2 hrs. The mixture was then stabilized overnight at refrigerator. The nanoparticles were collected by centrifugation at 12,000 rpm for 15 minutes. The precipitate was washed with distilled water repeatedly and was dried by a freeze dryer. While the second product is made in the same way by adding 50 mL alginic acid (dissolved in NaOH 0.1 M) at various concentrations (0.01-0.1% w/v) and then 50 mL chitosan (dissolved in acetic acid 1%) at various concentrations (0.01-0.1% w/v), and the mixture was allowed to stand until homogeneity. The resulting solution was further added with 350 mL calcium chloride (0.015% w/v in aquadest), and was kept for complete dissolution by magnetic stirrer for about 2 hours. All nanoparticles produced were stored in the refrigerator that can be used for the next test. The characterization of this product was analyzed in terms of particle size, zeta potential, and SEM.

Antioxidant activity of the nanoparticle product

The antioxidant activity can be done by DPPH as the source of free radicals. The method was adopted with modification by Hanumantharaju *et al.* [19]. The sample product suspended in ethanol was used for the analysis. About 5 mL of the sample was mixed with 5 mL of methanolic solution of DPPH (0.12 mM) and kept in dark at room temperature for 30 minutes. The DPPH scavenging activity in the sample was determined using Spectronic 20 (Genesys) at 516 nm against DPPH solution as control. The samples were tested in triplicates. The antioxidant activity was calculated as percentage of DPPH that was decreased in comparison with the control. The inhibition activity could be calculated to determine IC₅₀.

In vitro cytotoxicity test of the nanoparticle product

The *in vitro* cytotoxicity test was investigated using a plate with 96 wells with cell density of 2×10⁴ cells/mL assay reported by Mosmann [20]. 100 µL cells in culture medium (87.5% RPMI 10.4 g/L; 2% penicillin-streptomycin; and 10% FBS) were added into each well and were then incubated in CO₂ incubator for 12-24 hrs at 37°C. Each sample was dissolved in culture medium containing 0.05% dimethyl sulfoxide (DMSO), and 100 µL of each sample in different concentrations was added into each well in triplicate and was then incubated in CO₂ incubator for 12-24 hrs at 37°C. (3-4, 5-dimethylthiazol-2-yl) -2,5 diphenyltetrazolium bromide solution (10/100 µL medium) was added to all wells of an assay, and the plates were incubated for 4 hours at 37°C in CO₂ incubator. As much as 100 µL formazan (10% sodium dodecyl sulfate and 0.01 N chloride acid) was added into each well and mixed on a shaker for 5 minutes. The wells were incubated in the dark room for 12-24 hrs at room temperature. The corresponding absorbance was measured using multiwell scanning spectrophotometer (ELISA reader) at 595 nm. The intensity of absorbance is directly proportional to the number of living cells. Hence, the dead cell could be calculated to determine lethal concentration 50.

Statistical analysis

The data of all experiments were represented as mean±standard deviation and were analyzed with SPSS 13.0 statistical software. The differences were considered statistically significant at p<0.05.

RESULTS AND DISCUSSION

Nanoparticles produced by chloroform fraction of *K. rotunda* rhizome loaded with alginic acid (product 1) and combination of alginic acid-chitosan (product 2) were synthesized using ionic gelation method which was carried out at room temperature. Data from Tables 1 and 2 show the particle size, zeta potential, yield, and physical properties of these products. In this work, the particle size was analyzed using PSA. The stability of nanoparticle products was analyzed by zeta potential, and it is to determine the surface charge of nanoparticles in solution

Table 1. The characteristics of synthesized nanoparticles produced by chloroform fraction of *K. rotunda*-loaded alginic acid and calcium ion (Ca²⁺) (product 1)

| Formula code | Ext (g) | Alg (% w/v) | Ca ²⁺ (% w/v) | Mean yield (mg) color | % nanoparticle | Size (nm) | % micro-particle | Size (µm) | Mean zeta potential (mV) |
|--------------|---------|-------------|--------------------------|--------------------------|----------------|-----------|------------------|-----------|--------------------------|
| C1 | 1 | 0.1 | 0.01 | 600±2.3; yellow brown | 100 | 87-584 | - | - | -39.0 |
| C2 | 1 | 0.1 | 0.015 | 150±1.5; yellow brown | 98.7 | 115-877 | 1.3 | 1005 | -42.5 |
| C3 | 1 | 0.1 | 0.02 | 270±1.9; yellow brown | 100 | 100-766 | - | - | -46.9 |
| C4 | 1 | 0.1 | 0.03 | 370±1.2, yellow brown | 48.1 | 172-877 | 51.9 | 1005-1318 | -12.0 |
| C5 | 1 | 0.1 | 0.05 | 146±2.5; yellow brown | 18.5 | 197-445 | 81.5 | 1151-2976 | -15.0 |

Table 2: The characteristics of synthesized nanoparticles produced by chloroform fraction of *K. rotunda*-loaded alginic acid-chitosan and calcium ion (Ca²⁺) (product 2)

| Formula code | Ext (g) | Alg (% w/v) | Chi (% w/v) | Ca ²⁺ (% w/v) | Mean yield (mg) color | % nano particle | Size (nm) | % micro particle | Size (µm) | Mean zeta potential (mV) |
|--------------|---------|-------------|-------------|--------------------------|--------------------------|-----------------|-----------|------------------|-----------|--------------------------|
| D1 | 1 | 0.10 | 0.05 | 0.015 | 230±1.2; yellow brown | 26.0 | 172-877 | 74.0 | 1005-3905 | * |
| D2 | 1 | 0.10 | 0.01 | 0.015 | 200±1.5; yellow brown | 100.0 | 87-877 | 0 | - | -27.1 |
| D3 | 1 | 0.10 | 0.03 | 0.015 | 160±2.4; yellow brown | 95.5 | 150-877 | 4.5 | 1005-1151 | -24.4 |
| D4 | 1 | 0.01 | 0.10 | 0.015 | 150±3.5; yellow brown | 72.3 | 339-877 | 27.7 | 1005-1981 | * |
| D5 | 1 | 0.05 | 0.10 | 0.015 | 300±0.8; yellow brown | 4.7 | 259-339 | 93.3 | 2599-6000 | * |

Alg: Alginic acid; Chi: Chitosan,

or colloid. Electrical potential on the second boundary layer called the zeta potential of particles was measured in a typical value of +100 mV to -100 mV. Zeta potential of nanoparticles with a value >+25 mV or <-25 mV has a high degree of stability [21]. Dispersions with low zeta potential values eventually will aggregate due to van der Waal interparticle attractions. A colloidal solution containing a high percentage of nanoparticles will demonstrate the high value of the zeta potential so that a solution will be difficult to form a precipitate. In this work, the nanoparticles produced by the ethanol extract of *K. rotunda* were loaded with alginic acid (product 1), and the combination of chitosan-alginic acid (product 2) shows the negative charge of the zeta potential.

Nanoparticles produced by chloroform fraction of *K. rotunda* rhizome loaded with alginic acid (product 1) can be prepared by adding calcium ions as the cross junction to form a porous gel. The concentration of Ca²⁺ has a significant effect on the stability and pore size of the gel. The nanoparticles of product 1 can be synthesized at a concentration of alginic acid/Ca²⁺ ratio of 5:1 to produce 100% of nanoparticle, with the range size of 100-766 nm and a zeta potential of -46.9 mV. Product 1 can also be obtained on the comparison of alginic acid/Ca²⁺ ratio of 10:1 to produce 100% of nanoparticle, with the range size of 87-584 nm and a zeta potential of -39.0 mV; thus, this result should have a high-degree stability [21]. The data of particle size show that nanoparticle products obtained in comparison with alginic acid/Ca²⁺ of 10:1 indicate a more optimal result because the particle size distribution is lower than that obtained at the ratio of 5:1. When the concentration of calcium ion was high, the gel microparticles were formed. Morphology product 1 identified using an optical microscope with a SEM shows a spherical shape and smooth surface (Fig. 1).

Nanoparticles of product 2 were obtained at a concentration ratio of alginic acid/chitosan 10:1 (formula code D2), with the concentration of calcium ion 0.015% w/v to produce percentage nanoparticle of 100%, and the range size of 87-877 nm, with a zeta potential of -27.1 mV. The results of this study also showed that chitosan with a concentration of more than 0.03% w/v, percentage of nanoparticle was decreased and percentage of microparticles production was increased. Morphology product 2 identified using an optical microscope with a SEM shows a different shape with the first product that forms rectangular beam (Fig. 2). Previous study showed that the nanoparticle can be synthesized at the concentration ratio of 10:1 for chitosan/Na-TPP. The size was in the range of 172-877 nm, with a zeta potential of +28.06 to +38.03 mV. The nanoparticle was cylindrical and smooth in surface [17].

Several studies of nanoparticle products showed an increased biological activity. For example, the work of Basniwal *et al.* [22] showed reducing the particle size of curcumin in the nanometer range not only improves its aqueous phase solubility and cellular uptake but also enhances its activity as an anticancer agent. The results also showed that curcumin nanoparticles have better antibacterial activity. Curcumin nanoparticles are easier to get into the bacterial cell wall and can cause cell death [23]. The research of curcumin encapsulated that poly lactic-co-glycolic acid (PLGA) nanoparticles can destroy amyloid aggregates, exhibit antioxidative property, and are non-cytotoxic. The encapsulation of the curcumin in PLGA does not destroy its inherent properties and so, the PLGA-curcumin nanoparticles can be used as a drug with multiple functions in treating Alzheimer's disease [24].

The biological activity test of nanoparticles produced by chloroform fraction of *K. rotunda* loaded alginic acid and the combination of

Table 3: The IC₅₀ as antioxidant and cytotoxic effects on T47D breast cancer cell of nanoparticles produced by chloroform fraction of *K. rotunda*-loaded alginate and chitosan

| Code | Sample (%) | Inhibition activity (IC ₅₀ ±SD (µg/mL)) | Cytotoxic effects on T47D breast cancer cell IC ₅₀ ±SD µg/mL |
|--|---|--|---|
| B1 | Chitosan nanoparticles (100) | 200.00±3.2 | 130.13±1.3 |
| C2 | Alginate acid nanoparticle (98.7) | 357.78±4.5 | 255.10±3.5 |
| C3 | Alginate acid nanoparticle (100) | 225.63±3.5 | 145.25±2.2 |
| D2 | Chitosan-alginate acid nanoparticle (100) | 237.12±2.1 | 281.21±2.3 |
| Chloroform fraction of <i>K. rotunda</i> | - | 25.20±1.3 | 41.72±0.47 |
| Positive control (Ascorbic acid) | - | 3.77±0.2 | - |

IC₅₀: Inhibition activity, SD: Standard deviation, *K. rotunda*: *Kaempferia rotunda*

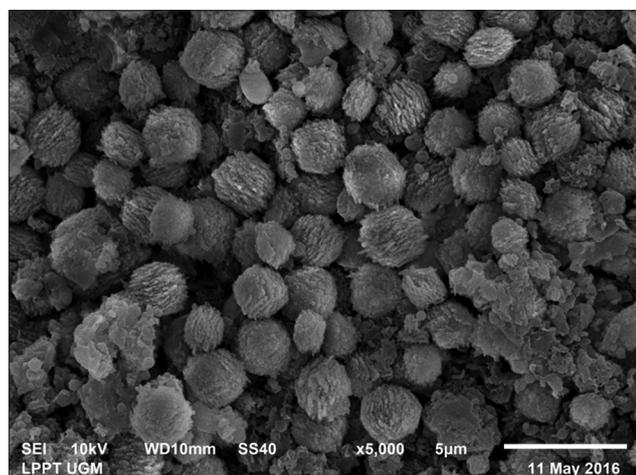


Fig. 1: Morphology of nanoparticles produced by chloroform fraction of *Kaempferia rotunda* loaded by alginate acid-Ca²⁺ (code C3) with scanning electron microscopy (JSM 6510) magnifications of ×5000

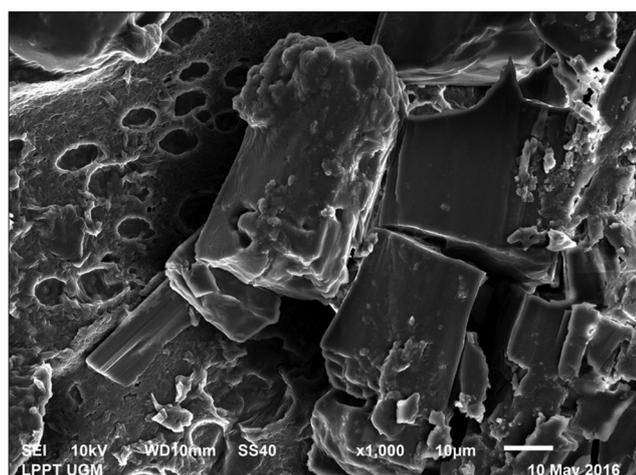


Fig. 2: Morphology of nanoparticles produced by chloroform fraction of *Kaempferia rotunda* loaded by alginate acid-chitosan-Ca²⁺ (code D2) with scanning electron microscopy (JSM 6510) magnifications of ×5000

alginate-chitosan acids were studied as antioxidants on DPPH radicals and cytotoxicity assay against breast cancer cell line T47D. The activity test results are in Table 3. The results show that the nanoparticle products have lower biological activity than the starting material chloroform fraction *K. rotunda*. This is likely because in the synthesis of nanoparticles not all active compounds contained in the chloroform fraction *K. rotunda* can be absorbed into the matrix nanoparticle product.

CONCLUSION

The nanoparticle products of chloroform fraction of *K. rotunda*-loaded alginate acid and combination of alginate acid-chitosan were successfully obtained by ionic gelation method. The alginate acid nanoparticles can be synthesized at the optimal mass ratio range of alginate acid:CaCl₂ of 10:1 (% w/v), and the percentage of nanoparticle was 100%, the size range of the nanoparticles was 87-584 nm, with a zeta potential of -39.0 mV, and the morphology shows a spherical shape and smooth surface. Meanwhile, the nanoparticle products was obtained from a combination of alginate acid-chitosan at mass ratio optimal alginate acid- chitosan of 10: 1 (% w/v), and calcium ions at 0.015% w/v, the percentage of nanoparticles 100%, the size of the nanoparticles 87 -877 nm, with a zeta potential -27.1 mV, and has a morphology such as a rectangular beam. Antioxidant activity from alginate acid nanoparticle; combination of alginate acid-chitosan nanoparticle; and chloroform fraction from *K. rotunda* showed IC₅₀ 225.63±3.5; 237.12±2.1; and 25.20±1.3 µg/mL, respectively, whereas the cytotoxic effect against human breast cancer T47D from these compounds showed IC₅₀ 145.25±2.2; 281.21±2.3; and 41.72±0.47 mg/mL, respectively. The antioxidant and cytotoxic activities of chitosan nanoparticle of chloroform fraction of *K. rotunda* show lower activity compared with the previous fraction before modification.

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