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Evaluation Of Toxicity Symptoms And Profile Semiquantitative Histology Of Kidney And Hepar Organs Of Nanosilver Inulin

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Nanosilver has been widely used in the medical field. Gembili tuber inulin has succeeded in forming nanosilver through a bioreduction mechanism. Nanosilver inulin, with a size of 480 nm, is active as a broad-spectrum antibacterial and has immunostimulatory activity at a dose of 4 mg/kg BW. However, the acute toxicity effects of nanosilver inulin are not yet known. This research aims to find the dose of nanosilver inulin that produces LD_{50}, observe toxicity symptoms after exposure, and profile semiquantitative histology of kidney and liver organs in Balb-C mice. The research stage is the application for ethical clearance based on the regulations of the Indonesian Food and Drug Supervisory Agency, isolation of gembili's inulin, biosynthesis process, characterization, determination of test animal groups (water, inulin, silver, nanosilver at dose 4, 16, 64, 254 mg/kg BW), conditioning of test animals, acute toxicity test, physical observation of toxic symptoms, and observation of the profile semiquantitative histology organs. Statistical data analysis is using SPSS 21 with the One-way ANOVA. The results show a significant change (Sig.<0.05) in body weight profile, feed intake, and toxic symptoms such as weakness, decreased activity, appetite, and dyspepsia. The highest 256 mg/KgBW dose does not yet produce an LD50 value. Profile semiquantitative histology of the liver organ causes liver necrosis, sinusoid dilation, and fibrosis in mice as the treatment dose increases. Observation of kidney organs showed significant changes (sig.<0.05) in the form of intratubular hemorrhage, pyknosis, karyoexis, and karyolysis, but not significant in the form of congestion.

Keywords: Nanosilver inulin, Acute Toxicity, LD50, organ histology, toxicity symptoms.

Introduction

Nanosilver is used as an antiseptic and broad spectrum biocidal activity [1]. Nanosilver was developed mainly in the medical field because it has broad-spectrum antibacterial activity and can overcome resistance [2]. Nanosilver penetrates bacterial cell walls and changes the structure and permeability of the cell membrane so that it can kill bacteria [3]. Silver can increase the body's immunity when in nanoparticle form, and nanosilver can act as a catalyst for the immune system to eradicate viruses, pathogens, and bacteria that reside in the human body [4].

Nanosilver preparation uses a bottom-up chemical synthesis technique by mixing silver salts with reducing agents and stabilizers in the form of inorganic chemicals that are toxic and harmful to health and the environment [5]. Therefore, nanosilver synthesis continues to be environmentally friendly and economical using plant media [6]. One environmentally friendly synthesis method is biosynthesis with the bioreductor inulin. The local raw material that has potential as a source of inulin is gembili (Dioscorea esculenta L.). Research conducted by Winarti [7] showed that gembili tuber inulin generally has prebiotic activity for the growth of Bifidobacteria and Lactobacillus. The inulin content in gembili is 14.77% [8]. The inulin content in gembili is similar to that in chicory tubers, which is 15-20% [9]. Research by Ermawati et al [10] stated that the results of nanosilver biosynthesis using gambili tuber inulin showed that the solubility of inulin at temperatures of 25 was in the nanometer range (<50 nm) and has broad-spectrum antibacterial activity. In research conducted by Ermawati [4], who examined the effectiveness of the immunomodulatory dose of nanosilver inulin based on vaccine-induced immunoglobin G levels of mice compared with herbal immunostimulants, an effective dose of 4 mg/KgBW was obtained. However, the safety use of nanosilver inulin needs acute toxicity data so that the safety limits of its use are known especially when it will be developed as an oral health supplement.

Research results of acute toxicity tests showed that nanosilver at doses of more than 8 mg/kg BW, namely doses 9 and 10, had a toxic effect on mice using synthetic chemical reductants [11]. This is supported by research conducted by Tiwari et al [12] that nanosilver at a dose of more than 10 mg/kg BW causes liver toxicity. Therefore, in this study, various doses of 4, 16, 64, and 256 mg/kgBW were used using a biological reductant. The toxicity test used is an oral acute toxicity test, which can measure the toxic effect in a short time with a single dose and determine the level of toxic dose by determining the LD50 value of a substance and symptoms of toxicity [13].

The liver is a vital excretory organ that functions for filtration and excretion when in direct contact with active drug substances. This organ is essential for the systemic effects of a substance in toxicity tests [14]. As a result of exposure to dangerous active substances, the liver organ will be damaged directly or indirectly through the blood vessels or central nervous system [15]. Signs of abnormalities in the liver organ include dilatation (widening) of the liver sinusoids, necrosis begins with changes in the morphology of the cell nuclei, which shrink, and the borders are irregular and dark in color, which is a characteristic of pyknotic nuclei [16][17] which can be destroyed in cells, this process is called karyorrhexis. The pyknosis core is the initial stage of necrosis. Fibrosis causes excessive extracellular matrix deposition, directly affecting liver function and shape. Fibrosis can progress to cirrhosis [18]. Toxic effects of drugs can be seen in the kidneys. The kidney is a body organ that is susceptible to the

influence of chemical substances because the kidney receives 25-30% of blood circulation for cleaning, so as a filtration organ, the possibility of pathological changes occurs [19]. Exposure to toxic doses of drugs will certainly affect the histology of the kidneys. Histological changes in the kidney include pyknosis, karyoexis, karyolysis, congestion, necrosis, and intratubular hemorrhage. Based on this, an acute toxicity test was carried out, which was measured by determining the LD50 of nanosilver inulin, observing symptoms of toxicity, and observing the profile semiquantitative histology of the kidney and liver organs from the dose administered.

Materials and Methods

Materials

AgNO3 (Merck KgaA, Germany), gembili tubers harvested in Cepogo, Boyolali, Indonesia, 96% ethanol (Merck, Germany), distilled water (Purelizer, Indonesia), Balb-C male mice (CV Dunia Kaca, Indonesia), KBr pellets (Fanny Pakan Ternak, Indonesia), 10% neutral formalin buffer (wako pure chemical corporation, Japan), xylol (Merck KgaA, Germany), paraffin (Vale Gardens, Worcestershire, United Kingdom), Canada balsam (oxford lab fine chem, UK), Hematoxylin -Eosin (Vector laboratories, United States. Instrument: Analytical balance (US Solid Precision, US), Eppendorf micropipette (Multipette, Germany), UV-Vis spectrophotometer (Genesys 150, USA), Particle Size Analyzer (HORIBA, USA), 1 mL syringe (One Med, Indonesia), FT- IR Spetroschopy (FindLight, USA), incubator (Sakura EM200T, Japan), deck glass (OneLab, Indonesia), Humidity chamber (Presto Stantest Pvt. Ltd, India), microscope (Olympus CX23, Japan), Optilab viewer (Olympus, Japan).

Sample Preparation

Gembili tubers harvested in the Cepogo, Central Java, Indonesia, were determined in the biology laboratory, Faculty of Mathematics and Natural Sciences, UNS, Surakarta, Indonesia. The tubers are crushed and dissolved in hot water, and the filtrate is added with 30% ethanol in a ratio of 1:3, then cooled in the refrigerator until a precipitate forms. Separate the precipitate and solvent; the precipitate is dried in a low-temperature oven [10].

Biosynthesis Process

Inulin weighed 10.0 grams and dissolved in 250.0 mL of warm water at 40°C, stirred until dissolved and homogeneous. Dissolve 85.0 mg silver nitrate (AgNO3) powder in 500.0 mL of warm water at 40°C and stir until dissolved and homogeneous. Take a volume of 7.0 mL of inulin solution and add 36.0 mL of silver nitrate solution; the dissolved mixture is heated at 60°C and stirred until homogeneous for 15 minutes. The solution was kept for 24 hours to maximize the biosynthesis process, namely changing the color of the solution to brownish. The solution that has changed color is ready for characterization [10][20] Glibowski et al. (2011).

Nanosilver Characterization

The nanosilver inulin solution was scanned using a UV-Vis spectrophotometer in the wavelength range 190-500 nm, aqua dest as a blank. The biosynthesis solution was analyzed for the particle distribution and shape using a Transmission Electron Microscope (TEM) method so that the results obtained were the optimum conditions that produced the expected particle size. Nanosilver size was characterized using a PSA (Particle Size Analyzer)

instrument. Nanosilver characterization was done by taking 1.0 mL of biosynthesized colloid and placing it in a cuvette to analyze particle size, potential zeta, and polydispersity index [10].

Animals Treatment

Submission of Ethical Clearance before the research process to obtain Ethical Clearance from the Ethics Commission for using test animals [13]. Ethical clearance for this research was submitted to the Research Ethics Committee of Dr. Moewardi Hospital, Surakarta, Central Java, Indonesia. This study used male mice with the Balb-C strain aged 16-20 weeks and weighing 20-30 grams. Acclimatize the test animals for three days before treatment. The test animals were placed at room temperature 22°±3°C, and the lighting was adjusted to 12 hours of light and 12 hours of darkness, and they were not exposed to direct sunlight.

The test animals were divided into seven treatment groups: three control groups and four dose groups, each with five test animals. Determination of test animal groups is based on the provisions of Indonesian Food and Drugs Regulatory Agency (BPOM RI) Regulation Number 10/2022 concerning Guidelines for In Vivo Preclinical Toxicity Tests [13]. The test animals were given samples orally according to the treatment dose group in Table 1.

Table 1. Treatment Group of Anmimal Test in Acute Toxicity Test

Treatment group	Number of Animal Test	Treatment	Amount of died animal
Group 1	5	Water	0
Group 2	5	Silver Nitrate solution	0
Group 3	5	Inulin solution	0
Group 4	5	Nanosilver inulin at dose 4 mg/kg BW	0
Group 5	5	Nanosilver Inulin st dose 16 mg/kg BW	0
Group 6	5	Nanosilver Inulin at dose 64 mg/kg BW	2
Group 7	5	Nanosilver inulin at dose 256 mg/kg BW	1

The behavior of the test animals was observed after 4 hours of treatment and 6 hours of treatment, and observations were continued after 24 hours of treatment to observe the test animals die. Observations were carried out for 14 days to evaluate the reversibility of toxic effects. All behavioral changes in the test animals are recorded [21] (Malole and Pramono, 1989). Toxic symptoms observed include changes in behavior, licking movements, scratching, "twitching," tremors, writhing, reactivity to stimuli (irritability, passivity), cerebral reflexes and spinal cord, pupil size, secretions, breathing, cardiac palpitations, skin, hair, death [22] (Wahyono et al., 2007). Dissection of test animals to remove organs, kidneys, and liver after 14 days of observation, then examining the histological profile of these organs.

Profile Histology Organs

Animal organs were soaked in 50% alcohol for 24 hours, then transferred to 70% alcohol for 1 hour, then soaked in 80% alcohol for 1 hour, and finally soaked in 95% alcohol for 1 hour

and 2 hours, respectively. The soaking process in alcohol was completed, and then the mixture was soaked in xylol solution for 1 hour each. The organs are drained and embedded using liquid paraffin at a temperature of 58°C for 24 hours to form a paraffin block. Kidney and liver organs were cut using a rotary microtome with a thickness of 3-5 μm in cross-section. The deparaffinization process is carried out by cutting paraffin blocks 4-5 microns thick, incubating at 58°C for 20 minutes. The paraffin blocks are soaked successively in xylol 1-4, 95% alcohol, 70% alcohol, and water for 5 minutes each, finally soaked in hematoxylin paint for 7-10 minutes, then washed with water.

Observation of the profile histology organs using a microscope at 400x magnification with ten fields of view with the Optilab Viewer program. Damage to the kidneys takes the form of intra-tubular bleeding, pyknosis, congestion, karyolysis, karyorrhexis, and necrosis [23](Hidayat et al., 2013). The results of the histological change based on microscope photos as semiquantitative data on the condition of the liver were observed for damage to the liver organ, namely sinusoidal dilatation, necrosis (which consists of the pyknotic, karyorrhexis, and karyolysis stages), fibrosis and cirrhosis. The histology scoring parameters of the kidney organ were assessed as follows: (0) indicates no change, and (1) (2) and (3) indicate mild, moderate, and severe changes, respectively. Meanwhile, the assessment was determined by the following percentage: changes of less than 30% (<30%) indicate mild changes, changes of less than 30% – 50% (<30% – 50%) indicate moderate changes, and changes of more than 50% (> 50%) showed weight changes (Arsad et al., 2014).

Analysis Data

Data were processed using SPSS 25.0 with the One-way ANOVA method. Data on the number of test animals that died were analyzed using the Thompson-Weil method to determine nanosilver inulin's potential acute toxicity (LD50). Histological profile data of kidney and liver organs were expressed as X ± SE. Semiquantitative histology organs were considered non-parametric, so data were analyzed using the Kruskal-Wallis method, and Mann-Whitney U was used to compare each parameter between groups. The mean difference was considered significant when the significance value was <0.05.

Results and Discussion

Results of plant determination with document number No. 130/UN27.9.6.4/Lab/2022 states that the tubers used in this research are the species Dioscorea esculenta L.

The nanosilver biosynthesis process is carried out at a temperature of less than 60°C for 15 minutes. Based on research by Kaviya et al. [24] (2011), the reaction in the biosynthesis process at 60°C was completed quickly and produces smaller nanoparticles than at room temperature. This occurs because increased hydroxyl groups (R-CHO) in the inulin chain cause active reduction of Ag+ to Ag° upon heating to form nanosilvers [25](Xu et al., 2018).

Nanosilver Characterization

The biosynthesis process with the gembili tuber inulin bioreductor showed a change in the color of the solution to brownish. The color change indicates the initial success of the nanosilver biosynthesis process. This was proven by the characterization using the UV-VIS spectrophotometer, FT-IR, and PSA methods. The maximum wavelength of the nanosilver inulin solution was 440 nm. These results indicate that nanosilver has been formed because it

produces an absorbance peak in the SPR (Surface Plasmon Resonance) range of nanosilver, namely a wavelength of 400-500 nm [24](Kaviya et al., 2011).
TEM results show a heterogeneous size distribution with round particle morphology; this is similar to the research conducted by Charoenwongpaibon et al. [26](2019), which states that inulin synthesis Nanoparticles (INNP) show that INNP morphology has a round shape. Biosynthesis of silver nanoparticles using Dioscorea bulbifera with the results are generally round, triangular, and hexagonal structures. The starch of the extract Dioscorea bulbifera acts as a capping agent responsible for reducing Ag+ to Ag°. The research of Pugazhendi et al. [27] (2016) also stated that TEM results from silver nanoparticle biosynthesis using Dioscorea alata show the morphological structure in a round shape.

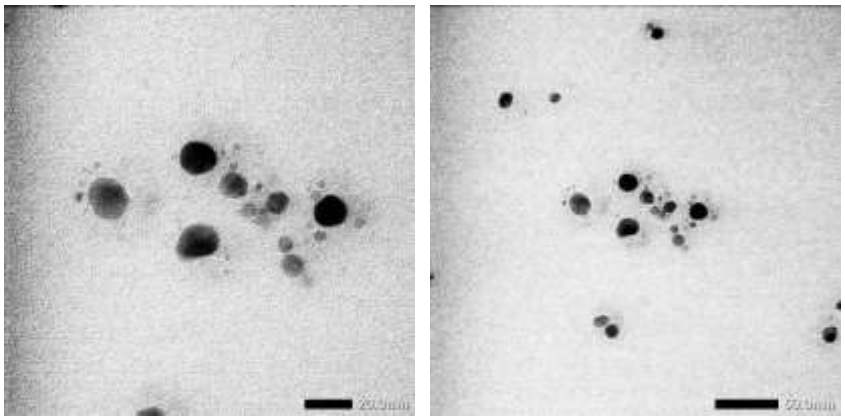

Figure 1. TEM analysis results of nanosilver inulin in 20-50 times magnification, with round shape and heterogeny of distribution particle

Particle size analysis using dynamic light scattering technique. The detected analog signal is converted into a digital signal, becoming an arithmetical series. Data obtained from measurements include Z-average, PI (Polydisperse Index), and zeta potential. Z-average is the average particle diameter; the narrower the curve formed, the better the results are. PI (Polydisperse Index) is the magnitude of the distribution distance particle size. Polydisperse shows the distribution distance of the sample. It is comprehensive, has multiple peaks, and has heterogeneous particle sizes [28] (Fadillah, 2019). Zeta potential is a charge parameter of electricity between colloidal particles.
The analysis results obtained a Z-average value for nanosilver inulin of 109.3-128.9 nm. PI (Polydisperse Index) value 0.378-0.395. A Polydisperse Index value close to zero indicates a homogeneous or uniform distribution of particles, while a PI value that exceeds 0.5 indicates the particle has a high heterogeneity [29](Taurina et al., 2017). Furthermore, the zeta potential value is -29.1 to -28.4 mV. Zeta potential shows the strength of the particles that repel each other. The resistance becomes stronger to produce a stable dispersion of the preparation; if the force of the particles repels the weaker, the particle experiences the tendency to aggregate and causes less stable dispersion of the preparation. According to Atun and Handayani [30] (2017), the potential value of the preparation for zeta is stable and is more than +25 mV or less than -25 mV.

Animals Treatment

Research Ethics Commission Dr. Hospital Health Moewardi, Surakarta, Indonesia, issued a Certificate of Ethical Eligibility Number 1.107 / VIII / HREC / 2022 and declared it meets the ethical clearance rules on 25 August 2022. Determination of the LD_{50} value was obtained using the Thompson-Weil because it does not require several animal experiments. This method also uses an accurate calculation list of LD_{50}. LD_{50} data taken from the amount Balb-C mice that died in each group were then tabulated and calculated using the Thompson-Weil formula (table 2) [31] (Nonci et al., 2014).

Table 2. Classification of compound toxicity levels based on the Indonesian Food and Drug Supervisory Agency

Toxicity Level	Oral LD50 dose	Clasification
1	≤5 mg/kg BW	Super toxic
2	5-50 mg/kg BW	Very toxic
3	>50-500 mg/kg BW	Toxic
4	500-2000 mg/kg BW	Moderate toxic
5	2000-5000 mg/kg BW	Mild toxic
6	>5000 mg/kg BW	No toxic

Dyspnea is caused by a decrease in the heart's cardiac output, which occurs during activities that result in respiratory muscle ischemia and ultimately cause respiratory muscle fatigue [32] (Kasron et al., 2022). The cause of decreased appetite is caused by the hormone ghrelin produced by the stomach and Pancreatic Peptide (PYY) 3-36, produced by the large intestine. Ghrelin works when hungry by stimulating NPY, while PYY3-36 is active during meals and then inhibits NPY. In its entire state, the nucleus tractus solitaries (NTS) gets signals from the hypothalamus and digestive organs to increase cholecystokinin by the duodenal mucosa. As a result, feed intake decreases (table 3) [33].

Based on Table 4 and Table 5, it is clear that the administration of nanosilver inulin affects the profile of semiquantitative histology organs observed in mice as the treatment dose increases. Liver and kidney damage due to toxic substances can be influenced by the type of active substance and the number of doses given. The higher the concentration of a substance, the greater the toxic response caused. Thus, it can be concluded that supplementation of nanosilver inulin in mice can cause damage to the microanatomic structure of the liver and kidney, which is characterized by changes in cell nuclei (pyknotic nuclei, karyolysis, karyorrhexis) sinusoidal dilation, and fibrosis. The higher the dose changes, the more severe the histopathology of the mice's liver, as shown by the comparison between the control group mice. That showed more significant liver damage ($p<0.05$) than dose 4 mg/kg BW compared to the control group. Dose 4 mg/kg BW and 64 mg/kg BW, there is a significant difference in damage to karyorrhexis and karyolysis. Dose 4 mg/kg BW and 256 mg/kg BW, there is a difference in significant damage to pyknotic damage. This means doses 64 mg/kg BW and 256 mg/kg BW cause more damage and are significant compared to doses 4 mg/kg BW.

Table 3. The rusults of toxicity symptoms evaluation of animal tests based on the group treatment

Groups Treatment	**4 hours**	**6 hours**	**24 hours**	**14th days**
Netral, water	Test animals showed no symptoms of toxicity			
Silver Nitrate solution	Test animals showed no symptoms of toxicity			
Inulin solution	Test animals showed no symptoms of toxicity			
Nanosilver inulin at dose 4 mg/kg BW	Test animals showed no symptoms of toxicity			
Nanosilver Inulin st dose 16 mg/kg BW	Test animals showed no symptoms of toxicity			
Nanosilver Inulin at dose 64 mg/kg BW	The test animals were limp and had reduced activity; one mouse died	The test animals were limp and had reduced activity; one mouse died	Test animals showed no symptoms of toxicity	Test animals showed no symptoms of toxicity
Nanosilver inulin at dose 256 mg/kg BW	Test animals look weak, have difficulty breathing, have a decrease in activity, have glazed eyes, lose appetite, have hiccups, and have one mouse die	Test animals look weak, have difficulty breathing, have a decrease in activity, have glazed eyes, lose appetite, have hiccups, and have one mouse die	Test animals showed no symptoms of toxicity	Test animals showed no symptoms of toxicity

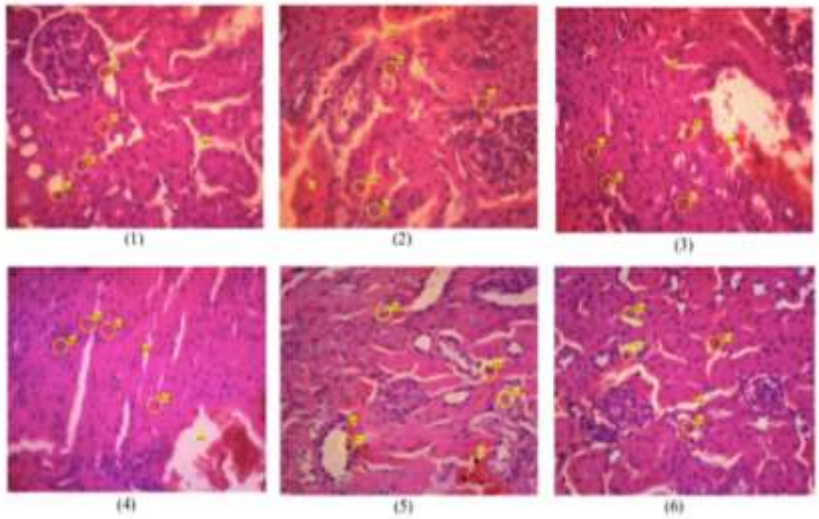

Figure 2. The analysis results of kidney histology organ after administration based on treatment group (1) Aquades group; (2) AgNO3 group; (3) Inulin group; (4) Dose 4 mg/kg BW; (5) Dose 64 mg/kg BW; (6) Dose 256 mg/kg BW

Table 4. The results of semiquantitative histological observations of kidney organ

Damage Categories	Water group	Silver nitrate group	Inulin group	Nanosilver 4 mg/kg BW	Nanosilver 64 mg/kg BW	Nanosilver 256 mg/kg BW	Sig.
Intratubular hemorrhage	1.33±0.33	1.33±0.33	1.33±0.67	1.67±0.33	3.0±0.00	3.0±0.00	0.025
Congestion	0.67±0.67	1.33±0.33	0.67±0.33	1.33±0.33	2.33±0.33	2.33±0.33	0.073
Pycnosis	1.0±0.00	1.0±0.00	1.33±0.33	2.0±0.00	1.67±0.33	2.0±0.00	0.035
Karyoexis	1.0±0.00	1.0±0.00	1.0±0.00	1.33±0.33	2.0±0.58	2.67±0.33	0.037
Karyolysis	1.0±0.00	1.0±0.00	1.33±0.33	1.0±0.67	2.33±0.67	2.67±0.33	0.040

*0.0 (no damage), 1 (mild damage <30%), 2 (moderate damage 30-50%), 4 (damage >50%)

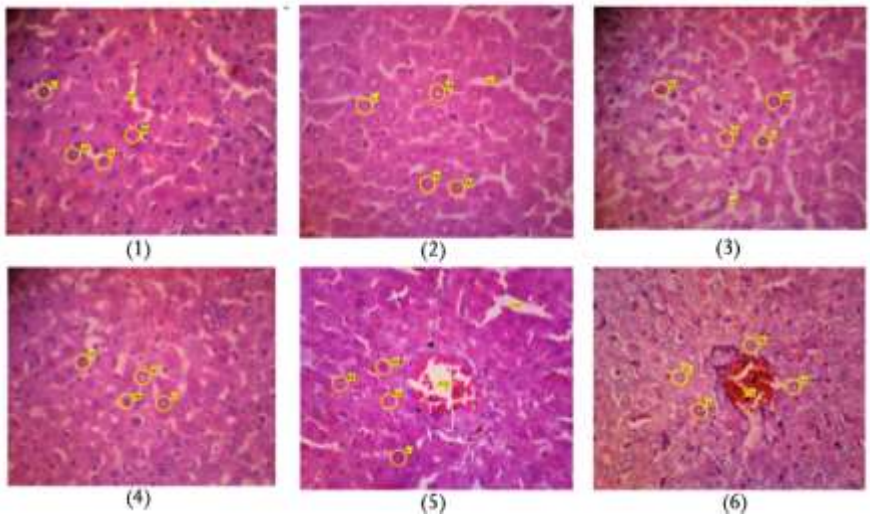

Figure 3. The analysis results of hepar histology organ after administration based on treatment group (1) Aquades group; (2) AgNO3 group; (3) Inulin group; (4) Dose 4 mg/kg BW; (5) Dose 64 mg/kg BW; (6) Dose 256 mg/kg BW

Table 5. The results of semiquantitative histological observations of hepar organ

Damage Categories	Water group	Silver nitrate group	Inulin group	Nanosilver 4 mg/kg BW	Nanosilver 64 mg/kg BW	Nanosilver 256 mg/kg BW	Sig.
Sinus dilatation	1.33±1.16	1.00±0.33	2.00±1.00	1.00±0.00	1.33±0.58	2.33±1.12	0.05

Pycnosis	1.00±0.00	1.00±0.00	1.67±0.58	1.67±0.58	2.33±0.58	3.08±0.00	0.05
Fibrosis	0.00±0.00	0.00±0.00	0.00±0.00	0.33±0.58	2.00±1.00	1.00±0.00	0.05
Karyoexis	1.00±0.00	1.33±0.58	1.33±0.58	2.00±0.00	3.00±0.00	2.67±0.58	0.05
Karyolysis	1.00±0.00	2.00±1.00	1.33±0.58	1.67±0.58	3.00±0.00	2.67±0.58	0.05
Syrosis	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.05

*0.0 (no damage), 1 (mild damage <30%), 2 (moderate damage 30-50%), 4 (damage >50%)

Conclusion

The highest dose of nanosilver inulin did not cause death in 50% of the test animals. However, according to the Indonesian Food and Drug Monitoring Agency, 64 mg/kgBW and 256 mg/kgBW were categorized as toxic. Symptoms of toxicity and death of test animals appeared in the 64 mg/kgBW and 256 mg/kgBW dose groups, namely weakness, difficulty breathing, decreased activity, droopy eyes, reduced appetite, and hiccups. Microscopically results, administration of nanosilver inulin had a significant effect on the histological profile of the kidney organ in the form of intratubular hemorrhage, pyknosis, karyoexis, and karyolysis as well as insignificant damage (sig. >0.05) in the form of congestion. A dose of 4 mg/kgBW caused the least amount of histological damage in liver.

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Ethics statement: Research Ethics Commission Dr. Hospital Health Moewardi, Surakarta, Indonesia, issued a Certificate of Ethical Eligibility Number 1.107 / VIII / HREC / 2022 and declared it meets the ethical clearance rules on 25 August 2022.

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