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Acute Toxicity Evaluation of *Phyllanthus Niruri* NEDDS Formulation According to OECD 425

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Abstract: A self-nanoemulsifying drug delivery system **(**SNEDDS) is a preparation that can increase the solubility of poorly soluble compounds, including *Phyllanthus niruri*. However, increasing solubility and bioavailability carries the risk of increased toxicity. Previous research showed that SNEDDS of *Phyllanthus niruri* (SNPN) at a dose of 100 mg/kgBW has hepatoprotective activity through decreasing ALT and AST enzyme levels after administering paracetamol at a dose of 3 g/kgBW in rats. However, at a dose of 200 mg/kg, ALT and AST levels increased, indicating potential toxicity. This study aims to determine the LD_{50} and to conduct histopathological observations of the liver and kidneys following oral administration of SNPN, in accordance with OECD 425 guidelines. Dose determination follows the recommendations of the AOT 425 StatPgm software, which includes the limit test (2000 mg/kg BW) and the main test (175, 550, and 2000 mg/kg BW), with observations made over 14 days. Next, the animals were sacrificed and necropsied to collect the liver and kidney organs for histopathological observation. LD_{50} determination was performed using AOT 425 StatPgm, and the level of organ damage was assessed using histopathological scoring. The result showed that the LD_{50} value of SNPN exceeded 2000 mg/kgBW, placing it in category 5 (mild toxicity). Histopathological tests showed varying severity of changes between doses, indicating a toxic effect of SNPN administration on the microscopic structure of the target organs. In conclusion, SNPN administration has the potential to cause mild acute toxicity, as indicated by the LD_{50} results and histological changes in the liver and kidneys.

Keywords: Meniran, acute toxicity, nanoemulsion, female Wistar, histopathology

Introduction

Phyllanthus niruri (*P. niruri*) or *meniran* is a herbaceous plant proven preclinically and clinically to have activity as a hepatoprotector [1,2]. However, *P. niruri's* bioactive compounds have low water solubility. One study of the bioactive compound from *P. niruri*, quercetin, found a water solubility of 10 µg/mL [3]. In addition, another biomarker compound in *P. niruri*, phyllanthin, also has low solubility and bioavailability [4]. Therefore, developing a formulation that can increase the solubility of *P. niruri* extract is necessary.

A self-nanoemulsifying drug delivery system (SNEDDS) is a nano-based drug delivery system that can increase the solubility and bioavailability of a compound by combining oil, surfactant, cosurfactant, and drug [5,6]. One study on SNEDDS, a combination of *P. niruri* and garlic extracts, demonstrated increased immunomodulatory

activity, as evidenced by higher index values and phagocytosis ratios than those of extracts not formulated with nanoemulsion or positive controls [7].

In addition, a previous study reported that SNEDDS of *P. niruri* (SNPN) significantly suppresses elevated levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) compared to *P. niruri* extract alone. Other results show that SNPN at a dose of 100 mg/kg can suppress the increase in ALT and AST enzyme levels, and liver histopathology results do not show any necrosis after administration of high doses of paracetamol 3 g/kg body weight) in mice. However, higher doses of SNPN (200 mg/kg) may increase ALT and AST enzyme levels [8]. It is possible because the increase in bioavailability and effectiveness of the SNEDDS formula is also accompanied by greater toxic effects [9]. Therefore, further studies are needed to characterize the toxicity profile of SNPN and establish the formulation's safety profile.

Previous studies on the acute toxicity of SNPN were conducted on male Wistar rats with an LD_{50} value above 2000 mg/kgBW. To date, no data are available on the acute oral toxicity of SNPN formulations in the context of hepatoprotective activity in female Wistar rats, which are known to be more sensitive in toxicity testing. Therefore, this study aims to examine the acute oral toxicity profile of SNPN in female Wistar rats using the OECD 425 method.

Materials and Methods

Materials

P. niruri extract was obtained from PT. Solonat, Indonesia, distilled water (Pharmaceutical Technology Laboratory, Islamic University of Indonesia), propylene glycol (PT. Brataco, Indonesia), tween 80 (PT. Brataco, Indonesia), labrasol (PT. Brataco, Indonesia), ketamine, xylazine, female Wistar rats weighing 120-250 grams aged 8-12 weeks obtained from LPPT Gadjah Mada University, standard feed, mineral water, and wood shavings.

Methods

Experimental animals

This research has obtained approval from the Medical and Health Research Ethics Committee of the Faculty of Medicine, Universitas Islam Indonesia, No. 5/Ka.Kom.Et/70/KE/VII/2024. Eleven female Wistar rats from Integrated Research and Testing Laboratory (LPPT), Gadjah Mada University, aged 8-12 weeks, weighing between 120-250 grams, were acclimatized for 7-14 days, fed and watered ad libitum, and placed in clean, noise-free cages maintained at 27°C, with a relative humidity of 30-70%, adequate ventilation, and a daily lighting regime of 12 hours of light and 12 hours of darkness. Before treatment, the animals were fasted for 14-18 hours, but were still provided with water [10].

Synthesis of SNPN

SNPN was made by mixing *P. niruri* extract with Labrasol, tween 80, and propylene glycol until homogeneous using an ultrasonicator. Next, particle size, polydispersity index, and zeta potential were examined using a particle size analyzer (PSA).

Acute oral toxicity test by OECD method 425

The Limit Test

The limit test begins with the oral administration of SNPN preparations to one test animal at a dose of 2000 mg/kgBW, after the animal is fasted for 14–18 hours with continued access to drinking water. Thereafter, intensive observation for toxic symptoms is conducted for 30 minutes to 4 hours, followed by periodic observations for 24–48 hours after the relevant dose is administered. If the test animal survives, four more test animals are added. The LD_{50} value is greater than 2000 mg/kgBW if three or more of the total five test animals survive, based on the recommendations listed in the software [10,11].

The Main Test

The main test begins with the oral administration of SNPN to one test animal at a dose of 175 mg/kg BW according to the guidelines, after the animal is fasted for 14–18 hours with continued access to drinking water. Subsequently, observations are made for the first 30 minutes to 4 hours, followed by periodic observations for 24–48 hours after dosing. If the test animal survives, it is given additional feed at a higher dose of 550 mg/kgBW. Furthermore, if the animal remains alive, the software will recommend increasing the dose to 2000 mg/kgBW. Discontinuation of dosing and determination of the LD_{50} are carried out according to the recommendations in the AOT425 software [10].

Observation of toxic symptoms

Toxic symptoms observed include the condition of the skin, fur, eyes, and mucous membranes, as well as the function of the respiratory, autonomic, and central nervous systems, somatomotor activity, and behavior. Additionally, symptoms such as tremors, seizures, excessive salivation, diarrhea, weakness, sleep, and coma are monitored [10].

Histopathology tests of the liver and kidney

On the last day of testing, all animals in the limit test and the main test were sacrificed using a combination of ketamine and xylazine, with a dose of 300-360 mg/kg ketamine and 30-40 mg/kg xylazine administered intraperitoneally [12]. Then, a necropsy process was performed to collect the liver and kidney organs for histopathological testing. The liver and kidneys were then rinsed with 0.9% NaCl and immersed in 10% formalin. The liver and kidney tissues were cut at 5-7 µm, then placed on a glass slide and stained with hematoxylin and eosin (HE) for histopathological examination. Scoring of liver and kidney damage was performed using a modified scoring method that included the following categories: score 0 = normal or no damage, score 1 = focal damage, score 2 = multifocal, and score 3 = diffuse [13].

Data Analysis

The mortality data for the test animals were then analyzed using the "AOT425" software to obtain the LD_{50} value.

Result and Discussion

The test began with the preparation of SNPN using a combination of Labrasol, Tween 20, and PG (4:4:2). The results of the particle size, polydispersity index, and zeta potential tests are shown in Table 1. The particle size of SNPN in the table falls within the nano-size range, namely 10-200 nm [14,15]. In addition, the resulting polydispersity index value is below 0.7 or in the range of 0.05-0.7, indicating that SNPN has a uniform, well-distributed particle size[16,17]. Also, SNPN has a zeta potential of -4.17 ± 0.12, which, according to the literature, is considered a good value, as it falls between -30 mV and +30 mV [18]. However, a negative zeta potential indicates that the SNPN contains free fatty acids and is negatively charged, leading to repulsive forces that prevent aggregation of the preparation.

Table 1. Results of testing particle size, polydispersity index (PI), and zeta potential of SNPN

Particle size (nm)	PI	Zeta Potential (mV)
20.0	0.573	-4.3
19.6	0.596	-4.1
19.6	0.593	-4.1
19.73 ± 0.23*	0.59 ± 0.01*	-4.17 ± 0.12*

*Note: (*) Data are presented in the form of mean ± standard deviation (SD)

Acute toxicity testing was conducted by exposing test animals to the SNPN preparation. The results showed no toxic symptoms, including hair loss, skin redness, mucosal inflammation, panting, tremors, seizures, lethargy, coma, salivation, lacrimation, restlessness, diarrhea, or death, in the limit test or the main test after SNPN exposure. In addition, in the 14-day limit test with 5 test animals, there were no deaths, indicating that the LD_{50} is greater than 2000 mg/kg body weight in mice. These results were also confirmed by the main test, which showed no death at doses of 175, 550, and 2000 mg/kg body weight. Therefore, based on the observation results and in accordance with the AOT 425 StatPgm software guidelines, the LD_{50} value is set at more than 2000 mg/kg body weight. According to the 2022 BPOM acute toxicity category criteria, SNEDDS *P. niruri* preparations with an LD_{50} value exceeding 2000 mg/kgBW are assigned to toxicity level 5, which is mild toxicity [10]. The results indicate that the initial assumption of an LD_{50} value of less than 2000 mg/kgBW was not confirmed in this test. Several previous studies reported similar findings with *P. niruri* extract and herbal formulations containing the plant. Research on aqueous extracts of *P. niruri* leaves reported an LD_{50} value of 2590.984 mg/kgBW in Swiss albino mice [19]. This finding is reinforced by a study related to herbal combinations containing *P. niruri*, which had an LD_{50} value of more than 5000 mg/kgBW in Sprague-Dawley rats, without showing any acute toxic effects [20] These findings reinforce the understanding that although nano-delivery systems such as SNEDDS can increase the solubility and bioavailability of active substances, they do not always

significantly increase systemic toxicity [9,21,22]. Selecting a non-toxic base for SNPN is a crucial factor in obtaining a formula with a good safety profile. The use of non-ionic surfactants and co-surfactants, such as propylene glycol and Tween 80, has been reported to offer better safety compared to ionic surfactants. These selections yield an SNEDDS formulation with good bioavailability without increased toxicity [23,24].

Histological observations were also conducted to confirm the toxic state after SNPN exposure. Data from microscopic observations of liver histopathology are presented in Figure 1.

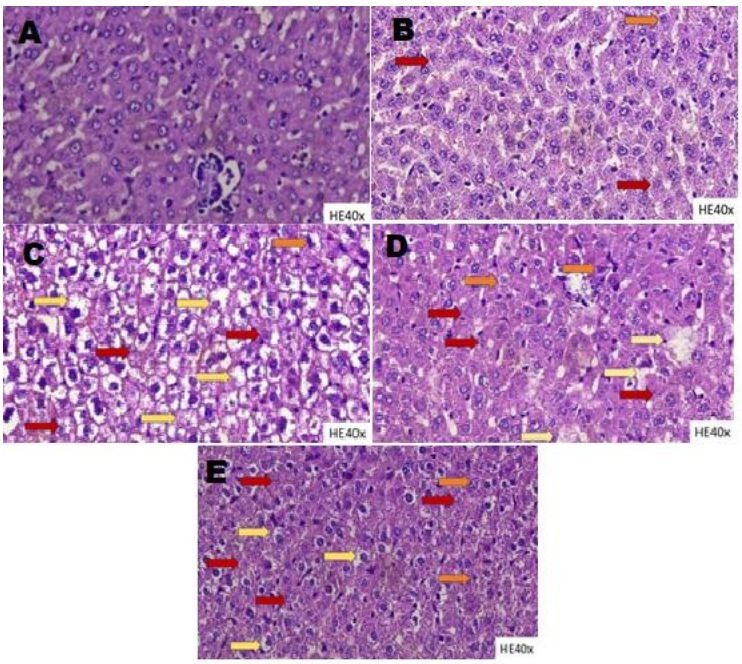

Figure 1. Histopathology of rat liver (40x magnification).

Caption: Orange arrows indicate hydropic degeneration, yellow arrows indicate fatty liver (hepatocyte steatosis), and red arrows indicate areas of necrosis. (A) Normal control group; (B) 175 mg/kgBW dose test group; (C) 550 mg/kgBW dose test group; (D) 2000 mg/kgBW dose main test group; and (E) 2000 mg/kgBW dose limit test group.

Figure 1 showed hydropic degeneration in all groups, characterized by small cytoplasmic vacuoles, a manifestation of impaired cell osmotic balance. This condition occurs due to disruption of the sodium pump, leading to fluid accumulation and retention and cell swelling [25]. In addition, fatty liver disease was also found in the treatment group with a dose of 550 mg/kgBW, the main test of 2000 mg/kgBW, and the limit test of 2000 mg/kgBW, which was marked by the presence of large, brightly colored vacuoles in the cytoplasm of hepatocytes, which was the impact of lipid accumulation [26]. These findings indicate that administering oil-based SNPN can cause toxic effects on the liver in the form of fatty liver.
Another observation was that necrosis occurred not only in the liver but also in the kidneys. Microscopic observations of kidney histopathology are presented in Figure 2.

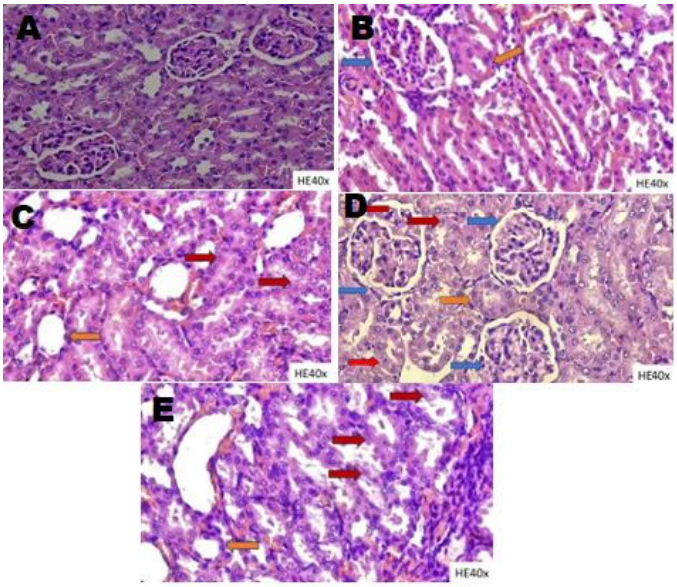

Figure 2. Histopathology of rat kidneys (40x magnification).

Caption: Orange arrows indicate normal tubules, blue arrows indicate normal glomeruli, and red arrows indicate necrotic tubules. (A) Normal control group; (B) 175 mg/kgBW dose test group; (C) 550 mg/kgBW dose test group; (D) 2000 mg/kgBW dose main test group; and (E) 2000 mg/kgBW dose limit test group.

Necrosis was observed in the liver in all groups, both the normal and treatment groups. In the kidneys, necrosis occurred in the 550 mg/kgBW group, the main test group at 2000 mg/kgBW, and the limit test group at 2000 mg/kgBW, characterized by karyolysis, in which the cell nucleus appeared faded or even disappeared [26]. This necrosis reflects cell death caused by SNPN administration. The presence of necrosis in all treatment groups indicates that exposure to SNPN can cause toxic effects on the kidney and liver tissue. Histopathology results were then assessed using a score (Table 2) to determine the distribution of signs of liver and kidney damage.

Table 2. Scoring of histopathological liver and kidney damage

Group	Hydropic Degeneration		Fatty Degeneration		Necrosis	
	H	K	H	K	H	K
Normal	0	0	0	0	0	0
175 mg/kgBB	1	0	0	0	1	0
550 mg/kgBB	1	0	3	0	3	2
2000 mg/kgBB (1)	2	0	3	0	3	3
2000 mg/kgBB (2)	2	0	3	0	3	3

Caption: (1) = Limit Test, (2) = Main Test
0 = No damage, 1 = Focal damage, 2 = Multifocal damage, and 3 = Diffuse.
H: Liver; K: Kidney

The scoring results showed that the distribution of liver and kidney damage correlated with the administered dose. Although SNPN administration had toxic effects on these organs, the pathologist's assessment stated that these effects were relatively mild.

Although distributed across several foci, they appeared to only occur at a few points in each foci. This result is consistent with the LD_{50} value, which is above 2000 mg/kgBW, according to the 2022 BPOM, which falls within the slight toxicity criteria.

Conclusion

The administration of SNPN preparations observed for 14 days did not cause death, and the LD_{50} value was above 2000 mg/kg BW, which is included in the slightly toxic category

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