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Acute Toxicity Test of Ethanol Leaves Extract *Ruellia tuberosa* L. in Wistar White Male Rats Using Fixed Dose Procedure Method

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ABSTRACT

Ruellia tuberosa L. has been widely used by the community as a traditional medicine, but its toxicity test data is still minimal. This study aims to acute toxic effects of biochemical parameters, macroscopic and histopathological liver, kidney and heart organs. The test animals consisted of 25 male white Wistar rats and were divided into five groups. The preliminary test: normal group (Na-CMC 0.5%) and the treatment group with varying doses of 5, 50, 300, and 2000 mg/KgBW and the test animals were observed for 24 hours. The results showed that there were no toxic symptoms or death in the normal group and the 2000 mg/kgBW dose group. The ethanol extract of *Ruellia tuberosa* L at a dose of 2000 mg/kgBW has no significantly affect the biochemical parameters ($p>0.05$) with the average levels of biochemical parameters in the normal group, namely SGOT 147.89 ± 16.41 U/L, SGPT 90±8.05 U/L, creatinine 0.65±0.04 mg/dL, ureum 37.22±8.32 mg/dL, while in the 2000 mg/kgBW dose group obtained SGOT 146±34.87U/L, SGPT 90±3.19 U/L, creatinine 0.63±0.05 mg/dL, and ureum 32.36±5.89 mg/dL. Ethanol extract of *Ruellia tuberosa* L at a dose of 2000 mg/KgBW had no effect on the macroscopic condition of the liver, kidney, and heart organs ($p>0.05$). While in the microscopic condition of the liver and kidney organs experienced a moderate degree of damage (multifocal) and in the heart organ experienced a mild degree of damage (focal).

Keywords:
Acute Toxicity; biochemical; fixed dose procedure; histopathology; *Ruellia tuberosa* L

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1. Introduction

Since ancient times, people's knowledge of herbal medicine based on tradition has been used to cure diseases. The use of herbal medicine has increased around the world due to its presumptive efficiency, availability, and general acceptance [1]. Although believed to be safe, herbal preparations may contain impurities, a combination of many toxic compounds, contaminants, microbes and heavy metals [2,3]. After the administration of certain chemicals into the biological system, various types of interactions may possibly cause adverse outcomes, sometimes even fatal. For these reasons, toxicology studies are mandatory to assess the safety of new drugs and herbal

compounds in the development stage. Toxicity test data assess the regulatory safety of natural herbal products [4]. The safety of new drugs that need to be carried out are acute, subacute, and chronic toxicity tests. Acute toxicity is the initial step that needs to be taken as the first step to screen for safety and evaluate the toxicity of compounds [5].

Ruellia tuberosa L is one of the basic ingredients of traditional medicine from the Acanthaceae family, which is widely found in tropical areas [6]. The 96% ethanol extract of *Ruellia tuberosa* L. contains secondary metabolite compounds such as alkaloids, flavonoids, saponins, tannins, steroids and terpenoids [7]. The ethanol extract of *Ruellia tuberosa* L. can provide antibacterial activity with a minimum inhibitory concentration against Escherichia coli of 500 mg/mL with an inhibition percentage of 99.1%. The minimum inhibitory concentration of the ethanol extract of *Ruellia tuberosa* L. against Bacillus subtilis is 1.000 mg/ml, with an inhibition percentage of 99%[8]. The results of a study conducted methanol extract of *Ruellia tuberosa* L. showed toxicity if used at more than a dose of 5000 mg/kg in rabbit test animals[9]. Ethanol extract of *Ruellia tuberosa* L. has potent antioxidant activity in 2-diphenyl-1 picryl-hydrazine-hydrate (DPPH) and azinobis-(3-ethylbenzothiazoline - 6 - sulfonate) (ABTS) with an IC_{50} value of 25.18 µg/mL in the DPPH test and an IC_{50} value of 18.22 µg/mL in the ABTS test [10]. To determine the safety of *Ruellia tuberosa* L., a toxicity test is needed.

This study evaluated the acute oral toxicity of *Ruellia tuberosa* ethanol extract using a limit dose of 2,000 mg/kg in Wistar rats, assessing mortality and clinical signs, body weight change, serum AST/ALT, urea, creatinine, and histopathology of liver, kidneys, and heart.

2. Methods

Materials

The sample in this study, *Ruellia tuberosa* L., was obtained from Ogan Ilir, South Sumatra and identified at the Andalas University Herbarium, with letter number 803/K-ID/ANDA/XI/2023. Male white rats of the Wistar strain, filter paper (Whatman®), 96% ethanol solvent (PT. Dira Sonita), 0.5% Na-CMC (Brataco®), distilled water (Brataco®), creatinine reagent and urea reagent (Dialab®), SGPT and SGOT reagents (Dialab®), 10% formalin, alcohol, xylol (Merck & Co®), liquid paraffin (Merck & Co®).

Preparation of Extract *Ruellia tuberosa* L.

Ruellia tuberosa L. was extracted by the maceration method. 1 kg of *Ruellia tuberosa* L. simplisia was macerated with 96% ethanol with a ratio of 1:10 for 3 x 24 hours and filtered. Remaceration was carried out for 2x24 hours and then filtered again. The filtrate obtained was then concentrated with a rotary evaporator at a maximum temperature of 60°C.

Acute Toxicity (LD_{50}) Test

The test animals were male Wistar rats. Based on Ferderer's dosage calculation, 25 male white rats were required, with each group consisting of five rats. The test animals were randomly selected and divided into five treatment groups. The test rats used were healthy, without disabilities, and behaved normally with a body weight of 150-250 g and an age of 2-3 months. The test animals were acclimatised for 7 days. Environmental conditions were controlled at 20°C–26°C and 40%- 70% relative humidity (RH). The light cycle was set at 12 hours of light and 12 hours of darkness. Animals were considered in normal condition if they showed active behaviour, a stable appetite for nutritious feed, and had a clean physique without any abnormal clinical signs. Testing with these test animals has received ethical permission from the Ahmad Dahlan

University Research Ethics Committee under number 022312133. This research was divided into two stages: preliminary testing and main testing.

Preliminary Test

Male white rats of the Wistar strain Rats were acclimatized for seven days and then divided into five groups. The preliminary test: normal group (Na-CMC 0.5%) and the treatment group with varying doses of 5, 50, 300, and 2000 mg/KgBW, with each group consisting of five test animal. The treatment was given orally. Observations were made within the first 4 hours after dosing for 24 hours. The observation interval was at least 24 hours for each dose. Symptoms of toxicity were observed through changes in gait, salivation, diarrhea, seizures, tremors, and death.

Main Test

In the primary test, the dose used is obtained from the preliminary test results. If death occurs at the initial dose in the preliminary test, the dose in the primary test is reduced; if there are no symptoms of death or toxicity, the primary test uses the highest dose in the preliminary test. Routine observations are carried out within the first 30 minutes after administration of the test preparation and periodically every 4 hours for the first 24 hours, then once a day for 14 days. Observations include the presence or absence of symptoms of death or toxicity shown by the test animals. Symptoms of toxicity were observed through changes in gait, salivation, diarrhea, seizures, tremors, and death. In the main test, biochemical parameters were also measured, including SGPT, SGOT, urea, creatinine, and macroscopic and histopathological liver, kidney, and heart organs.

Blood Biochemistry Analysis

Blood biochemical analysis of SGOT and SGPT levels was performed using a Clinical Chemistry Analyzer at a wavelength of 340 nm. Determination of creatinine levels was performed using a Clinical Chemistry Analyzer at a wavelength of 550 nm. Determination of urea levels was performed using a Clinical Chemistry Analyzer at a wavelength of 578 nm. Urea levels were measured using three working reagents.

Histopathology Preparation of Organs

Histopathological preparations of the liver kidney, and heart were performed with Hemooxylin-Eosin (HE) staining. Samples of the liver organs of the test animals were taken and immersed in formalin buffer solution (NBF). Organ samples were scaled with thin slices to be stored in a tissue cassette and fixed in NBF solution. The fixed sample was then dehydrated and cleared with a solution of 70% alcohol, 80% alcohol, 90% alcohol, absolute alcohol, toluene, and paraffin, gradually within one day. The organ samples were blocked by embedding cells with liquid paraffin poured, then cooled. Blocks that have cooled, sectioning using a microtome with a thickness of ± 4-5 microns. After that, it stained with Hematoxylin-Eosin and mounting media.

Data Analysis

The research data analysis was conducted using the SPSS software. The research results were analysed statistically using the Normality Test. To determine the difference in body weight of the test animals before and after treatment, the body weight data were analysed using a paired T-test. For organ weight, SGOT, SGPT, creatinine, and urea levels, an independent T-test was performed to compare the 2 treatment groups.

Ethical Approval

This study involving animal experimentation was conducted in compliance with ethical standards. The research was approved by the Ethics Committee of Universitas Ahmad Dahlan (approval number 022312133). All procedures followed the institution's animal guidelines, ensuring humane treatment of the animals.

3. Results and Discussion

The thick extract of *Ruellia tuberosa* L. obtained was 142.28 grams, yielding a rendemen sebesar 14.22%. The 96% ethanol extract of *Ruellia tuberosa* L. yielded 30.35 g, corresponding to a 10.12% yield [8]. The higher the extract yield, the higher the content of active substances attracted to the raw materials [11]. The yield percentage indicates the effectiveness of the extraction process. The effectiveness of extraction is influenced by stirring and temperature factors, which can increase the extraction rate by increasing contact between the sample and the solvent. Extraction efficiency also depends on the extraction time within a specific period [12]. Phytochemical tests were carried out on the ethanol extract of *Ruellia tuberosa* L., which includes flavonoids, tannins, phenolics, steroids, alkaloids, saponins, and triterpenoids. This study is similar to one that found that the 96% ethanol extract of *Ruellia tuberosa* L. contains secondary metabolites, including alkaloids, flavonoids, saponins, tannins, steroids, and triterpenoids [7].

Preliminary Test

Based on the preliminary test results, there were no toxic symptoms caused by test animals, such as walking on the stomach, walk backward, weakness, tremors, diarrhea, and salivation at all dose levels of the ethanol extract of *Ruellia tuberosa* L. leaves. If administering the test preparation at a dose of 2000 mg/kgBW in the preliminary test did not show toxic symptoms and death in the test animals, then the dose of 2000 mg/kgBW was determined as the test dose in the primary test.

Main Test

The main test was started using the highest dose in the preliminary test. Based on the results of the main test observations, there was no death response or toxic symptoms such as walk backward, walk with the stomach, tremors, diarrhea, salivation, and weakness in test animals in the normal group, and the 2000 mg/kg BW dose group during the 14day observation period. The results of the primary test can be seen in **Table 1**.

Table 1. Results of Main Test Observations

Group	Treatment	Number of rats	Number of dead rats	Clinical signs of toxicity					
				1	2	3	4	5	6
Normal	Na-CMC 0.5%	5	0	-	-	-	-	-	-
Test	Dose 2000 mg/kgBW	5	0	-	-	-	-	-	-

Note:
1: Walk backwards, 2: Walk with stomach, 3: Tremors, 4: Diarrhea, 5: Salivation,
6: Limp, −: There are no symptoms, +: There are had symptoms, BW: Body weight

Table 1 shows that after 14 days of observation, there was no death or toxic symptoms in the test animals. This indicates that the test preparation does not cause acute toxicity effects. Before detonation, the test animals were weighed first and then sacrificed. Macroscopic observations were made of the liver, kidneys, and heart of the test animals that had been detropated. The results of observations of the liver, kidneys, and heart showed no difference in the shape and color of the liver, kidneys, and heart in the normal group of rats and the 2000 mg/kg BW dose group. The observations on the liver showed that the macroscopic liver of the normal group and the 2000 mg/kg BW dose group was dark red, had a smooth surface, and had a chewy consistency. A normal liver has a flat, soft, and dark red surface, while an abnormal liver has a mottled surface,

cysts, and discoloration [13]. This shows that the liver of the normal group and the 2000 mg/kg BW dose group were macroscopically normal.

Measurement of Test Animal Body Weight

Data on the measurement of the weight of the test animals to determine the difference in the weight of the test animals before and after treatment in the normal group and the test dose of 2000 mg/Kg BW. Based on the statistical analysis results, it states that the value ($p>0.05$) means that there is no significant difference in the weight of the test animals either before or after treatment from the normal group and the test dose of 2000 mg/Kg BW. The results of the measurement of the weight of the test animals can be seen in Table 2.

Based on **Table 2**, the measurement of the body weight of the test animals during 14 days of observation experienced an increase and decrease of no more than 10%. Changes in the body weight of test animals decreased by no more than 10% and did not indicate any toxic effects after administration of the test preparation [14]. The increase and decrease that occurred in test animals can be caused by the erratic appetite of test animals, which less supportive environmental conditions can also influence.

Table 2. Results of measuring the weight of test animals

Group	Day 0	Day 14
Normal	189.38 ± 4.04	184.15 ± 3.31
Dose 2000 mg/kgBW	183.74 ± 7.54	190.78 ± 3.49

Biochemical Parameter Level Check

The results of the average measurement of SGPT, SGOT, creatinine, and urea levels in the normal group and the 2000 mg/kg BW dose group can be seen in Table 4. Normal SGOT levels in male white rats range from 45.7-80.8 U/L. Normal SGPT levels are 63-175 IU/L in white rats [15]. Normal urea levels in the blood of rats are 15-22 mg/dL [16]. Normal creatinine levels in the blood of rats are in the range of 0.4-0.8 mg/dL [16].

Table 3. Levels of SGOT, SGPT, creatinine, and urea of test animals

Group	Biochemical parameters (± SD)			
	SGOT (U/L)	SGPT (U/L)	Creatinine (mg/dL)	Urea (mg/dL)
Normal	147.89 ± 16.41	90 ± 8.50	0.65 ± 0.04	37.22 ± 8.32
Dose 2000 mg/kgBW	146.68 ± 34.87	90 ± 3.19	0.63 ± 0.05	32.36 ± 5.89

Based on the results of the examination of the biochemical parameter values in **Table 3**, it is known that the average values of SGOT and urea are not within the normal range. However, the values of SGPT and creatinine are within the normal range. Increased SGOT levels can be caused by enzyme production, increased muscle mass, red blood cells, and liver metabolic activity due to the development of test animals [17]. This is reinforced by the fact that the SGOT enzyme is found in the liver and blood cells, heart cells, and muscle cells. Therefore, increased SGOT levels do not always indicate liver cell abnormalities [15]. SGPT levels are in cells under normal conditions. When the lysis process occurs in liver cells, SGPT enters human blood, resulting in high levels of SGPT in the blood. Suppose there is an increase in SGPT. Then, it can be a sign of damage to liver cells because the SGPT enzyme in large quantities is only found in liver cells [18].

Liver damage due to poisoning or infection causes SGPT and SGOT to increase 20-100x from the highest normal levels.

The increase in SGOT levels in the 2000 mg/kg BW test dose group cannot be considered toxic regarding SGOT value parameters. Increased SGOT levels can be caused by inflammation, which causes changes in cell membrane permeability, and cells release high concentrations of SGOT enzymes into the bloodstream. The above factors most likely caused this study's increase in SGOT levels. This is evidenced by the fact that increased SGOT levels also occurred in the normal group.

Based on the testing results, the blood biochemical parameters levels show an increase in urea levels exceeding normal limits. High urea levels can be caused by the provision of high protein feed during testing, which affects the distribution of amino acids in the blood, thereby affecting urea levels. In addition, increased urea levels can also be caused by the saponin content in the ethanol extract of *Ruellia tuberosa* L., which can increase the permeability of the lipid bilayer of red blood cells. This study's increase in urea levels is likely due to hypovolemia or lack of fluid volume. Urea and creatinine levels are often used as indicators to determine kidney conditions. Prerenal uremia is suspected if urea levels increase, but creatinine levels remain normal. Prerenal uremia is caused by decreased blood flow to the kidneys. Increased biochemical parameter values cannot be directly interpreted as a result of liver or kidney function damage. This can be caused by biological factors that cause an increase in each parameter.

Histopathology of rat liver, kidney and heart

After 14 days of administration of the test preparation in *Ruellia tuberosa* L. ethanol extract, the hepatic, kidneys, and heart organs were histopathologically examined. The result of histopathological observations of each group can be seen in **Table 4,5,6** and **Figure 1,2,3**.

Tabel 4. Score level in histological images of hepatic cell

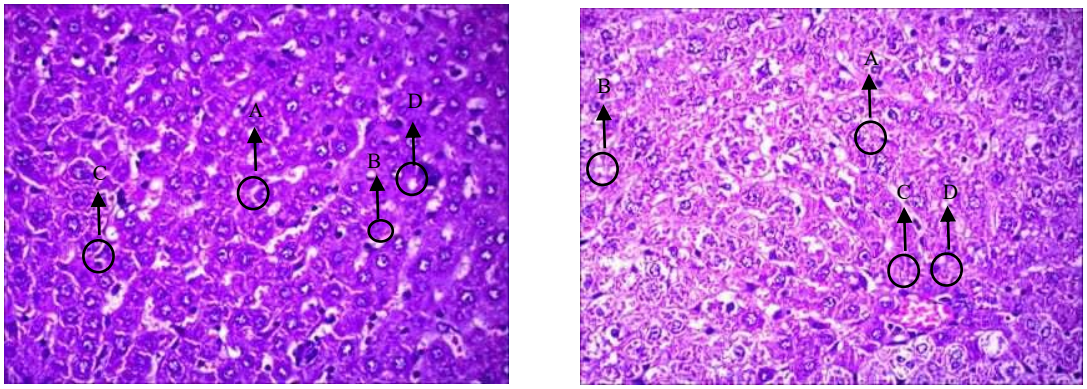
Group	Hydropic Degeneration	Steatosis	Necrosis
Normal	1	1	1
Dose 2000 mg/Kg BW	2	2	2

Table 5. Score level in histological images of kidneys cell

Group	Hydropic Degeneration	Steatosis	Necrosis
Normal	1	0	1
Dose 2000 mg/Kg BW	2	0	2

Table 6. Score level in histological images of heart cell

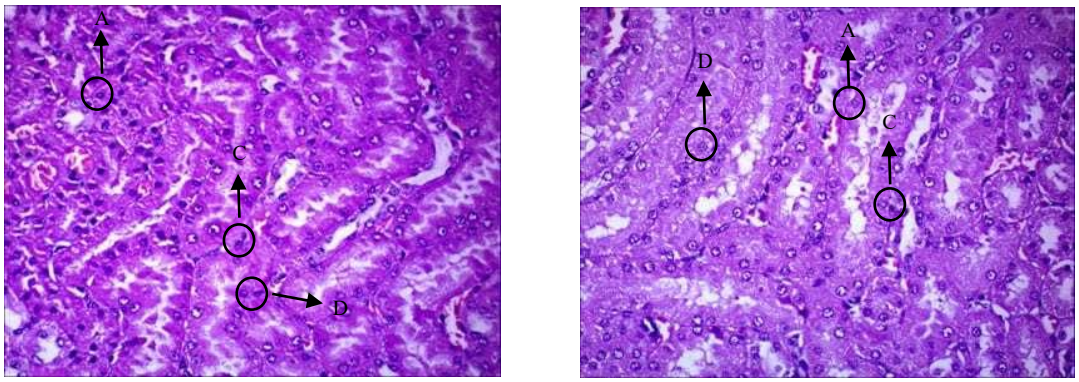
Group	Congestion	Oedema	Inflammation	Necrosis
Normal	1	1	1	1
Dose 2000 mg/Kg BW	1	1	1	1



(a) (b)

A: Normal cells, B: Fatty degeneration, C: Hydropic degeneration, D: Necrosis

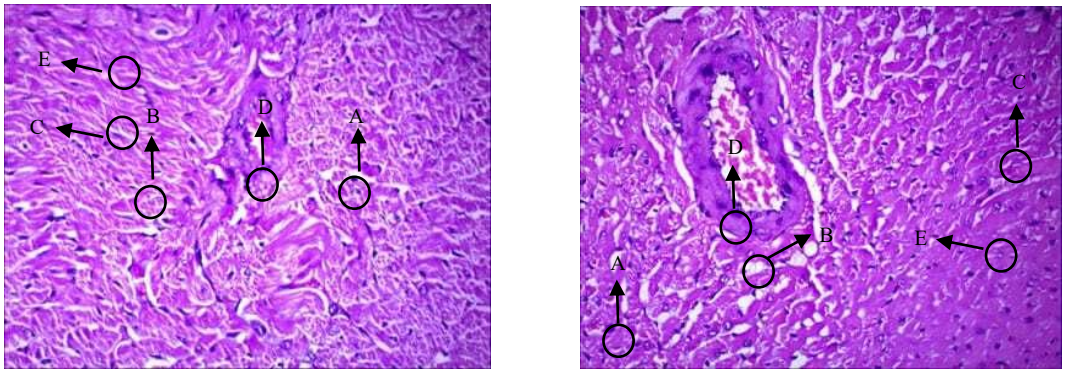
Figure 1. Histopathological picture hepatic cell (a) group normal, (b) group dosage 2000 mg/kgBW



(a) (b)

A: Normal cells, B: Fatty degeneration, C: Hydropic degeneration, D: Necrosis

Figure 2. Histopathological picture kidneys cell (a) group normal, (b) group dosage 2000 mg/kgBW



(a) (b)

A: Normal cells, B: Fatty degeneration, C: Hydrophic degeneration, D: Necrosis

Figure 3. Histopathological picture heart cell (a) group normal, (b) group dosage 2000 mg/kgBW

Based on **Figure** 1, there is a difference in the level of hepatocyte cell damage in the normal group with the test dose group. This hepatocyte cell damage is reversible and irreversible. The level of hepatocyte cell damage in the image is observed in the presence of hydropic degeneration, fatty degeneration, and necrosis. The histopathology results of the liver organ in the normal group given Na CMC showed a score of 1 with hydropic degeneration, fatty degeneration, and focal necrosis (mild). In the 2000 mg/kg BW dose group, a score of 2 was obtained, which observed fatty degeneration, inflammatory cell infiltration, and multifocal necrosis (moderate).

The histopathology results of the liver organ in the normal group that experienced mild damage can be caused by trauma (shock conditions), infection of infectious agents, and exposure to toxins [19]. In addition, it can occur due to the physiological process of apoptosis experienced by normal cells. Naturally, cells in the body will experience aging and cell death; in cell regeneration, they will be replaced with new cells.

Fatty degeneration in the liver indicates an imbalance in the metabolic process that causes decreased liver function. Morphological changes occur due to the accumulation of fat and cytoplasm. This steatosis is characterized by the formation of round vacuoles in hepatocyte tissue[20]. Degeneration in the low and moderate categories, such as liver function, is still normal because the nature of this degeneration is reversible.

Hydropic degeneration is characterized by swelling of cells containing fat and glycogen. This is caused by impaired active transport that prevents cells from pumping Na+ ions so that the Na+ concentration comes out and causes cell swelling [21]. Moderate liver damage in the 2000 mg/kgBW dose group.

The results of microscopic observations of the kidney organs showed no fatty degeneration (steatosis) in the normal group or the test dose group. Kidney histopathology in the normal group showed a score of 1 with the formation of hydropic degeneration and necrosis with mild damage. The 2000 mg/kgBW dose group showed a score of 2, which showed hydropic degeneration and necrosis with moderate damage. Changes in the histopathological structure can be caused by several factors, including entering toxic substances into the body, where the kidneys' primary function is the excretion of metabolic waste products.

Histopathological observation of the heart of the normal group and the 2000 mg/kg BW dose group showed that there was mild (focal) damage to the heart organ, which could occur due to an excessive immune response resulting in histopathological changes in the form of necrosis and inflammation, as well as changes in blood fluid metabolism which could cause blockage (congestion). The damage between the normal treatment group and the 2000 mg/KgBW test dose treatment group showed the same score. It can be concluded that there is no difference in the heart organ damage score from the 2000 mg/KgBW test dose group when compared to the normal group.

Based on the results of this study, further research, including subchronic toxicity testing, is recommended. This is necessary to disseminate the substance's safety profile for long-term use, identify target organs that may be damaged by accumulation of the substance, and establish a No Observed Adverse Effect Level (NOAEL). Furthermore, histopathological examination of vital organs and a more comprehensive blood biochemistry analysis are highly recommended to detect functional changes that may not be detected by acute testing.

4. Conclusion

Ethanol extract of *Ruellia tuberosa* L. leaves at a dose of 2000 mg/kgBW did not cause death, but caused signs of biochemical and histopathological toxicity in the liver, kidneys and heart, so it is necessary to carry out a subchronic toxicity test.

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Conflicts of Interest:
The authors declare no conflict of interest regarding the publication of this paper.

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