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Effectiveness of Administering Red Belt Leaf Extract (*Piper Crocatum*) Against *Aeromonas Hydrophila* Bacteria in Vitro

Muhammad Jusril[1*], Arief Prajitno[2], Mohamad Fadjar[2]

[1] Master's Degree Program in Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, Indonesia.
[2] Department of Aquaculture, Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, Indonesia.

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Corresponding Author:
Muhammad Jusril
ariiefpratama99@gmail.com

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Abstract: Diseases caused by pathogenic bacteria such as *Aeromonas hydrophila* are a major health concern. However, the use of red betel leaves (*P. crocatum*) can serve as a potential solution to this problem due to their antibacterial properties. This research aimed to study the antibacterial activity of red betel leaf extract (*P. crocatum*), the structure and character of the bacteria inhibited by the extract, and the bioactive components present in the leaves. The results of the disc test showed that the average zone of inhibition was 14.28 mm, while the MIC test results revealed that a dose of 100 mg/L could inhibit bacterial growth (bacteriostatic). Moreover, the crude extract of red betel leaf (*P. crocatum*) damaged the cell walls and cell membranes of *A. hydrophila* bacteria, thereby affecting their character and structure. The UV-VIS analysis of red betel leaf extract (*P. crocatum*) and FTIR analysis with 96% ethanol absorption indicated the presence of flavonoids, saponins, tannins, and alkaloids. These findings suggest that red betel leaves can be used as a natural remedy to fight bacterial infections caused by pathogenic bacteria such as *Aeromonas hydrophila*.

Keywords: *Aeromonas hydrophila*; Antibacterial activity; Phytochemical test; *Piper crocatum*

Introduction

The bacteria that commonly infect freshwater fish farming is known as *A. hydropila* bacteria. According to Amanu et al. (2014), *A. hydropila* bacteria attack freshwater fish and factors that contribute to the occurrence of the *A. hydropila* infection are fish stress, particularly due to high stocking densities, poor water quality, and high level s of organic matter. These bacteria attack the host's defense mechanisms, allowing the bacteria to multiply quickly in the host and eventually causing death.

Aeromonas hydrophila is a type of bacteria that produces hemolysin and aerolysin enzymes. These enzymes can cause damage to the skin tissue, leading to hemorrhaging. The hemolysin toxin breaks down the red blood cells, causing them to come out of the blood vessels, resulting in a reddish appearance of the skin surface. This bacterial infection is likely to occur in environments with low oxygen levels, high temperatures, and high accumulation of organic waste or fish metabolism byproducts, as well as high stocking densities in aquaculture waters (Kinetasari et al., 2023).

In an effort to reduce deaths from *A. hydropila* attacks, the use of antibiotics is often employed. However, continuous use of antibiotics can lead to environmental pollution and accumulation of antibiotic residues in the environment. According to Rahmah et al. (2023), red betel leaf extract can be used as a treatment alternative. They explain that red betel leaves contain active ingredients such as alkaloids, saponins, polyphenols, and flavonoids. Alkaloids act as antibacterials by disrupting the peptidoglycan components of bacterial cells. This prevents the cell wall layer from forming completely, leading to cell death.

This research aims to determine the effectiveness of red betel leaf extract (*Piper crocatum*) in vitro against *A. hydropila* bacteria.

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Method

Location and Time of Research

The research was conducted at the Disease and Fish Health Laboratory, Faculty of Fisheries and Marine Sciences, Brawijaya University, Malang, in Juni 2023.

Extraction

To extract from red belt leaves by taking 1,000 grams of wet leaves. These leaves are dried in the sun, but not directly exposed to sunlight. Once the leaves are dried and refined, 200 grams of leaf powder is soaked in 2 liters of 96% ethanol solvent using 1:10 ratio (Navirius et al., 2023). It is left to soak for three days before filtering through filter paper to get the filtrate. The filtrate is evaporated using a vacuum rotary evaporator, to obtain a thick paste from the ethanol extract of red belt leaves (Navirius et al., 2023).

MIC Test (*Minimum Inhibitory Concentration*)

Irwandi et al. (2020) explanation of the MIC test process, the initial step is to prepare a sterile test tube. This test tube should be filled with 4.5 ml of sterile TSB media. After that, 0.5 mL of red betel leaf (*Piper crocatum*) extract should be added to each test tube containing TSB at a predetermined dose.

Seven test tubes were used in the experiment. Five of them were filled with a predetermined dose concentration of red betel leaf extract, while the remaining two tubes (numbers 6 and 7) were filled with negative and positive controls, respectively. The negative controls were given synthetic antibiotics (*Chloramphenicol*) at a concentration of 50 ppm with a volume of 0.5 mL, whereas the positive controls were given no extract. After that, 0.1 mL of bacterial isolate (10^7 CFU/mL) was added into each test tube, which was then incubated at 32°C for 24 hours. At the end of the incubation period, the turbidity level of the test medium was checked, and the absorbance was measured using a spectrophotometer at a wavelength of 600 nm. The Minimum Inhibitory Concentration (MIC) test is also based on the turbidity indicator in Tryptic Soy Broth (TSB) media that has been inoculated with bacteria and extracts at different doses and incubated for 24 hours, compared to the control tube.

Disc Test

The disc test is a process used to determine the inhibitory power of red betel leaf extract by measuring the clear zone created around a disc paper. The dose of the extract used in the test is predetermined based on the results of the previous MIC test, which are 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, and 250 mg/L. Positive control treatment uses antibiotics, while negative control does not involve any treatment. To start the process, 0.1 mL of bacterial density 10^7 CFU/mL is poured onto the media. Then, paper discs are soaked in red betel leaf extract for 10-15 minutes. The media treated with bacteria and paper discs are incubated for 18-24 hours at 32 °C. Finally, the diameter of the clear zone around the paper disc is measured using a caliper to observe the media.

FT-IR Test (Fouries Transfrom-Infrared)

The FT-IR spectrophotometer is a tool used to identify the different functional groups present in a compound by analyzing the wavelengths of light they absorb. This analysis relies on the unique molecular vibrations of each compound, which produce characteristic absorption spectra in the mid-infrared range (4000-400 cm^{-1}). Using FTIR spectroscopy, scientists can quickly analyze compounds in a non-destructive way, with minimal sample preparation required (Kumayas et al., 2015).

Analyze the characteristics of a compound, the FTIR spectrophotometric method can be used on isolates using KBr pellets. This method determines the functional groups present in the isolate. First, 1 mg of the sample is crushed with 100 mg of KBr (Potassium Bromide) and mixed thoroughly. Next, the sample is measured for infrared absorption at wave numbers (400-450 cm^{-1}), and further analyzed (Triyasmono et al., 2020).

SEM Test (Scanning Electron Microscope)

The purpose of SEM observations is to identify any damage to the bacterial cell morphology caused by extracts that contain active ingredients (Hariati et al., 2018). To prepare for the observations, two treatments were administered to *A. hydropila* preparations. The first treatment was a normal one for panda bacteria, while the second treatment involved giving *A. hydrophila* bacteria red betel leaf extract at the optimal dose. Once the preparations were ready, they were observed using SEM (Scanning Electron Microscope). To analyze the damage to *A. hydropila* bacteria, SEM images were compared between normal conditions and bacteria treated with extracts. Images of damage to the bacterial cell walls were also examined. Based on the results of the damage analysis, the next stage of the study will be conducted.

Data Analysis

Data were analyzed using SPSS 26.0 for Windows. ANOVA test (0.05) will determine the efficacy of red betel leaves (*P. crocatum*) against A. hydrophila

Result and Discussion

Phytochemical Screening

The study conducted on the extraction of red betel leaves (*P. crocatum*) using 96% ethanol as a maceration solvent reveals that there are active compounds present

in red betel leaves. The phytochemical test results of the extract from red betel leaves are listed in the table 1.

Table 1. Phytochemical Test Results of Red Betel Leaves Crude Extract

Identification of Compound	Characteristics	Results
Flavonoid	Orange, pink, brick red, dark red	(+) Positive
Alkaloid		
Meyer	White precipitate	(+) Positive
Dragendrof	Orange precipitate	(+) Positive
bouchardat	Brown deposits	(+) Positive
Tanin/Fenol	Blackish brown, blackish blue	(+) Positive
Terpenoid		
Steroid	Bluish green	(-) Negative
Triterpenoid	Orange, orange-brown	(+) Positive
Saponin	Permanent foam	(+) Positive

According to the phytochemical test results, red betel leaves contain active compounds such as flavonoids, alkaloids, tannins, triterpenoids, and saponins. Identified alkaloids, flavonoids, tannins, and triterpenoids in red betel leaf extracts. In addition, research by Egra et al. (2019) revealed that red betel leaf extract contains tannins, flavonoids, alkaloids, and saponins.

Extract quality can be affected by biological and chemical factors. According to Athaillah et al. (2020), biological factors are related to the natural materials used, such as the type of plant, plant age, parts utilized, and environmental conditions like water, temperature, light, and soil. These factors can impact secondary metabolite compounds present in plant parts used for extracts, such as leaves. Young leaves have various antibacterial substances, but the substances in old leaves start to degrade. This deterioration can affect the quality of the extract created if old leaves are still used.

Flavonoids in red betel extract can form complex compounds with proteins and cell walls, inhibiting bacterial growth (Kinetasari et al., 2023). Alkaloids react with the amino acids present in bacterial cell walls, leading to structural changes. This alteration affects the formation of the peptidoglycan layer on bacterial cells, resulting in incomplete formation of the cell wall layer. As a consequence, bacterial cells become lysed, and in some cases, they may even die (Marwulan et al., 2023). Tannins function by interacting with bacterial cell membranes, deactivating enzymes involved in bacterial regeneration, and disrupting or deactivating the function of bacterial genetic material (Anarkhis et al., 2023). Saponin has been found to work as an antibacterial agent by causing the bacterial cell wall to lyse, leading to the leakage of alkaline phosphate. When the concentration of saponin is increased, it dissolves the protein, allowing intercellular compounds to diffuse through the bacterial cell wall and outer membrane (Gerrine et al., 2023).

MIC Test (*Minimum Inhibitory Concentration*)

The MIC test results are determined based on the absorbance value, which indicates the effectiveness of red betel leaf extract (*P. crocatum*) in hindering the growth of *A. hydropila* bacteria. The absorbance values obtained from the test are presented in Table 2.

Table 2. MIC Test Result

Concentration (mg/L)	Absorbance Value
Control (-)	0.95
1 mg/L	0.88
10 mg/L	0.81
100 mg/L	0.58
500 mg/L	0.66
1000 mg/L	0.65
Control (+)	0.04

Based on the results of the MIC test, as presented in Table 2, it is evident that red betel leaf extract with ethanol solvent, at a concentration of 100 mg/L, has the lowest absorbance value of 0,57792, which is similar to that of the Negative Control. Furthermore, the data collected at all concentrations indicate a decrease in the absorbance level with an increase in the dosage of the extract given. Therefore, it can be inferred that the growth of *A. hydropila* bacteria can be suppressed due to the presence of antibacterial compounds in the red betel leaf extract.

After analyzing the table, it was decided that the minimum dose reference for determining the disc test dose would be 100 mg/L of red betel leaf extract. According to Putri et al. (2008), the spectrophotometer results cannot differentiate between the level of turbidity of the pigment from the extract and the turbidity of the bacterial cells. Therefore, the Optical Density (OD) value obtained is a combination of both. Seniati et al. (2019) added that a spectrophotometer can measure the density of cells in a suspension with OD (amount of light absorbed and scattered) as a unit of count. This is because OD is directly proportional to the density of cells in a bacterial suspension. After obtaining the MIC results, the disc test is performed to obtain more accurate results.

Disc Test

The disc test is a method that measures the clear zone formed around a disc of paper. This zone indicates the ability of red betel leaf extract (*P. crocatum*) to hinder the growth of *A. hydropilla* bacteria. To determine whether the extract can inhibit the growth of *A. hydrophila*, the inhibitory response is observed after 24 hours, while to check if the extract can kill *A. hydrophila* bacteria, the response is observed after 48 hours. The dose used in the disk test is based on the results of the

MIC test, starting from 50 mg/l and gradually increasing to 100 mg/l, 150 mg/l, 200 mg/l, and 250 mg/l, using a bacterial density of 107 CFU/mL. The results of the inhibition zone measurements are presented in Table 3.

Table 3. Disc Test Result

Concentration (mg/L)	Average Inhibition Zone Diameter (mm)		Inhibitory Response
	24 Hours	48 Hours	
Control (-)	0±0.00	0±0.00	Weak
50	8.66±0.08	8.40±0.07	Medium
100	10.85±0.11	10.42±0.11	Strong
150	13.06±0.08	12.67±0.08	Strong
200	14.41±0.26	13.98±0.25	Strong
250	14.82±0.33	14.28±0.32	Strong
Control (+)	19.69±0.51	20.09±0.52	Strong

The study measured the diameter of the inhibition zone at various doses and at different time intervals. The results showed that an increase in the number of extract doses given could result in a larger diameter of the inhibition zone. When using an extract concentration of 50 mg/L, the inhibition zone was categorized as moderate, with a diameter of 8.66 mm. On the other hand, concentrations of 100 mg/L, 150 mg/L, 200 mg/L, and 250 mg/L resulted in a strong inhibition zone category, with an average diameter of 10.85 mm, 13.06 mm, 14.41 mm, and 14.82 mm, respectively. The research proved that the larger the concentration used, the larger the inhibition zone formed. Various factors affect the size of the inhibition zone in bacteria, such as growth sensitivity, media components, incubation time, and metabolic activity of microorganisms. The amount of active substance present in the solution also affects the size of the clear zone (Marfuah et al., 2018).

After measuring the diameter of the inhibition zone at all doses with a time interval of 48 hours, it was observed that the disc inhibition zone had decreased, except for the Negative Control (+) which showed an increase up to 20.09 mm. The results of the disc inhibition test presented in the table above demonstrate that every concentration of red betel leaf extract decreased after 24 hours, specifically at 48 hours. This is due to the fact that red betel leaf extract is bacteriostatic. Sanurat et al. (2019) indicate that the mechanism of action of antibacterial compounds can be divided into two groups: bacteriostatic and bactericidal. In case an antibacterial compound inhibits the growth of bacteria, it is included in the bacteriostatic group. If it kills bacteria, it is included in the bactericidal group. The decrease in the size of the inhibition zone during the 48th incubation period may also be caused by various factors such as the nature of bacteria, the ability of the compound to suppress bacterial growth, or the state of the active ingredients of the antibacterial compound used (Sofidiana et al., 2022).

FTIR Test

Red betel leaf extract was subjected to FTIR test analysis to confirm the presence of active ingredients at the functional group level. The UV-Vis and FTIR results showed similarities in the analysis of the content of active compounds present in the extract. In other words, the infrared spectrum provides information about the vibrations of the atoms in a compound. FT-IR identifies molecules from a group of compounds found in the infrared peak. Submolecular groups produce peaks in certain spectral regions, which form the basis for interpreting the vibrational spectrum. The FTIR spectrophotometric analysis results show 7 absorption band frequency regions with different functional groups. The analysis results are presented in Figure 1.

In Figure 1, the spectrum data reveals several key findings. Firstly, there is an absorption at a frequency of 813,666 cm^{-1}, indicating the presence of an aromatic C-H group. This frequency aligns with the absorption wave number of 1.000-650 cm^{-1}, as noted by Mabruroh et al. (2019). Secondly, other spectrum data shows absorption at frequencies of 1.030,713 cm^{-1} and 1.071,069 cm^{-1}, indicating the presence of a C-O group. This aligns with the statement of Alchaddad et al. (2015), which notes that the C-O group occurs at wave numbers 1.300-1.000 cm^{-1}.

The analysis results reveal two important observations. Firstly, there is an absorption at a frequency of 1.399,069 cm^{-1}, which identifies the presence of the aromatic C=C group. This is in agreement with Tiwow et al. (2021) and the wave number range of 1.500-1.400 cm^{-1}. Secondly, there is an absorption at a frequency of 1.646,882 cm^{-1}, which is indicative of the C=C stretching group. This is consistent with Alchaddad et al. (2015) who noted this frequency at a range of 1.680-1.600 cm^{-1} the frequency absorption at 2.109,941 cm^{-1} indicates the presence of the C=O carbonyl group, according to Tiwow et al. (2021), and a wave number frequency of 2.300-1.800 cm^{-1}. Finally, the spectrum data shows absorption at a frequency of 2.926,624 cm^{-1}, indicating the presence of the C-H alkane group, and the absorption wave number of 3.850-2.960 cm^{-1}.

The FTIR analysis conducted on the red betel leaf extract (*P. crocatum*) revealed that it contains flavonoids and tannins that possess antibacterial properties. Flavonoids, as explained by Fatmawati et al. (2021), have the ability to prevent bacterial growth by damaging cell walls, inactivating enzymes, binding cell adhesions, and disrupting cell membranes. This leads to instability in the bacterial cell wall and cytoplasm, causing disruption in selective permeability, active transport function, and control of bacterial cell protein structure. Consequently, the bacterial cell loses its shape, and lysis occurs due to the release of macromolecules and ions from the cell.

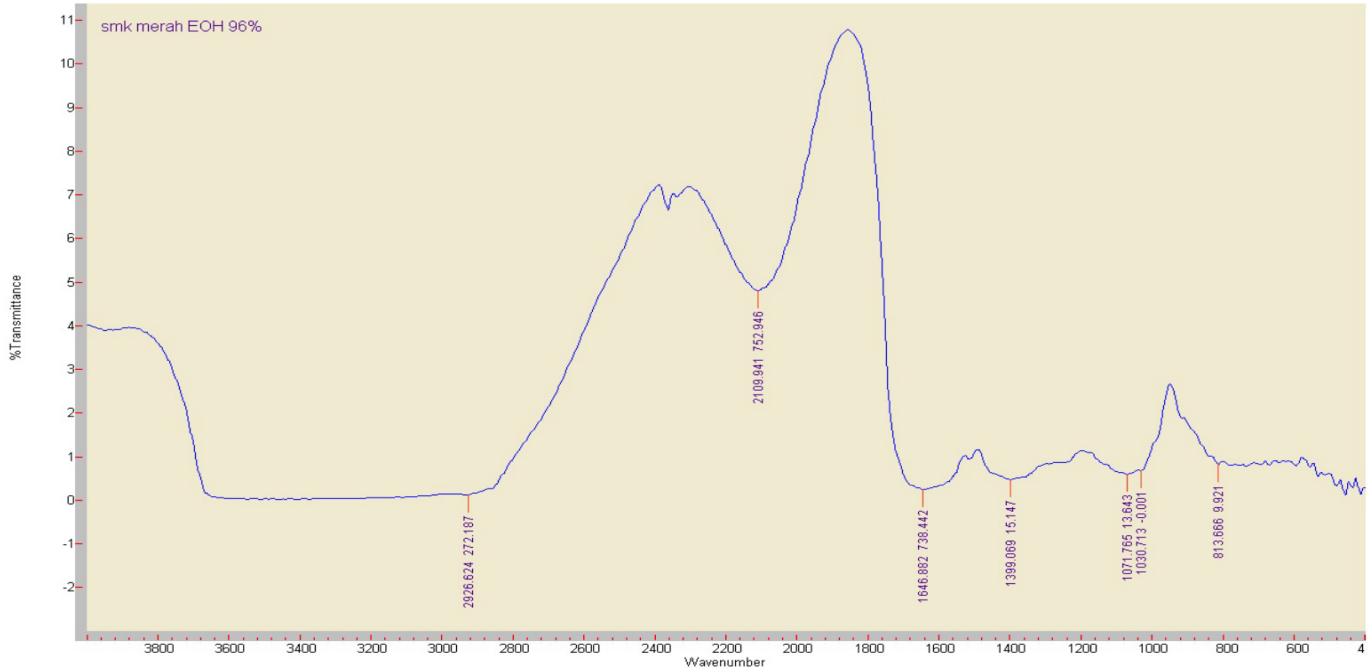

Figure 1. FTIR test results of red betel leaf extract (*P. crocatum*)

SEM Test (Scanning Electron Microscope)

Before treatment, structural changes in *A. hydrophila* bacteria were determined via SEM testing. Post-treatment, SEM testing was conducted to observe changes after the bacteria were given red betel leaf extract (*P. crocatum*). SEM test results are shown in Figure 2.

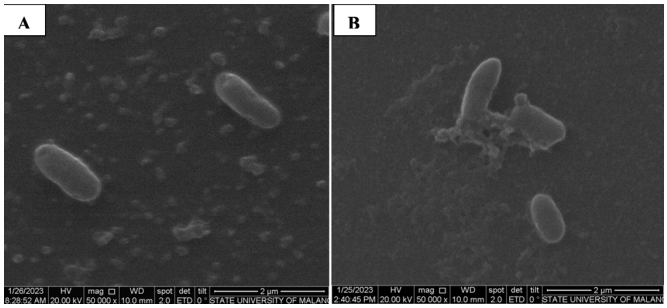

Figure 2. Morphology of *A. hydrophila* bacteria before and after treatment with red betel leaf extract (*P. crocatum*).

In Figure 2A, we can see the structure of the *A. hydropila* bacteria without any damage to its cell wall. In contrast, Figure 2B depicts the morphology of the *A. hydropila* bacteria that experienced lysis due to cell wall instability. This instability led to a disruption in the bacteria's metabolism. The cause of this damage was the red betel leaf extract, which contains active antibacterial compounds, including flavonoids, alkaloids, tannins, terpenoids, and saponins.

Antimicrobial alkaloids can inhibit bacterial growth by preventing the enzymes responsible for DNA replication from functioning properly. A lack of DNA replication can hinder bacterial division, effectively preventing bacterial growth. Additionally, the alkaloids in the extract can disrupt the formation of cross bridges in the peptidoglycan components of bacterial cells, preventing the cell wall layer from forming completely and leading to the death of specific cells (Ernawati, 2015). Flavonoid compounds, which are phenolic compounds, can cause protein denaturation and damage cell walls. The inhibition of bacterial colony growth is thought to be caused by damage to the structural components of the bacterial cell membrane. Damage to the cell membrane disrupts the transport of nutrients (compounds or ions) through the membrane, which causes bacterial cells to lack the necessary nutrients for growth (Sulistiyono et al., 2018).

Tannins possess antibacterial properties, which are attributed to their ability to deactivate microbial cell adhesins, enzymes and interfere with protein transport in the inner layers of cells. Tannins also target cell wall polypeptides, leading to an imperfect cell wall formation, causing bacterial cells to lyse due to osmotic and physical pressure, ultimately leading to the death of bacterial cells. On the other hand, terpenoid compounds act as antibacterial substances, thought to damage the cell membrane by lipophilic compounds. Terpenoids react with porins (transmembrane proteins) on the outer membrane of bacterial cell walls, forming strong polymer bonds and damaging porins, ultimately reducing the permeability of bacterial cell walls. Due to this, the bacterial cells experience a lack of nutrition, which leads to the inhibition of their growth or their death (Pambudi et al., 2021).

Conclusion

The Red Betel leaves (*P. crocatum*) contain active compounds such as flavonoids, alkaloids, tannins, and triterpenoids based on the results of phytochemical tests. According to the MIC and inhibition tests, the crude extract of Red Betel leaves (*P. Crocatum*) can kill *A. hydrophila* bacteria at a dose of 250 mg/l, as it has bacteriostatic properties. The SEM test results indicate that the crude extract can damage the cell walls of *A. hydrophila*, causing lysis of bacterial cells.

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Author Contributions
Conceptualization, Jusril M., Prajitno, A., and Fadjar, M.; methodology, Jusril, M. Gerrine, G. and Kenitasari, R. E.; validation, Prajitno, A. and Fadjar, M.; formal analysis, Jusril, M.; investigation, Jusril, M. and Kenitasari, R.; resources, Jusril, M., Prajitno, A., and Kenitasari, R. E.; data curation, Jusril, M.; writing—original draft preparation, Jusril M.; writing—review and editing, Jusril, M.; visualization, Jusril, M.; supervision, Prajitno, A. and Fadjar, M.; project administration, Jusril, M., Prajitno, A., Fadjar, M. and Kenitasari, R. E.; funding acquisition, Jusril, M. and Prajitno, A.

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Conflicts of Interest
The authors declare no conflict of interest.

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