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(RESEARCH ARTICLE)

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Antimicrobial activity test of ethanol extracts of bangle rhizomes (*Zingiber montanum*) against fish-decomposing bacteria *Pseudomonas* sp. and *Vibrio* sp.

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Abstract

Fish spoilage often occurs if not handled properly. Fish are prone to spoilage due to their high water content. Several natural antimicrobial alternatives have begun to be used to slow down spoilage in fish. Bangle rhizome (*Zingiber montanum*) is a traditional medicinal plant utilized by the community due to its secondary metabolites, such as flavonoids, alkaloids, saponins, and essential oils, which have potential antibacterial properties. This study aims to determine the antimicrobial activity of bangle rhizome ethanol extract against *Pseudomonas* sp. and *Vibrio* sp., which are pathogenic bacteria in fishery products.

Objective: The purposes of this study is to determine the antimicrobial activity of bangle rhizome *Zingiber montanum* ethanol extract against *Pseudomonas* sp. and *Vibrio* sp., to identify the optimal concentration of the bangle rhizome ethanol extract as antimicrobial, and to identify the types of secondary metabolites contained in the bangle rhizome that exhibit antimicrobial activity.

Results: The test results showed that in the well diffusion antimicrobial test, the best results were obtained at the highest concentration, namely 100%, with an average inhibition zone diameter of 6.83 mm for *Pseudomonas* sp. and 5.8 mm for *Vibrio* sp. Meanwhile, in the antimicrobial microdilution test, the highest inhibition percentage was observed at a concentration of 80%, with 2.07% for *Pseudomonas* sp. and 1.27% for *Vibrio* sp.

Keywords: Bangle Rhizome; Antimicrobial; *Pseudomonas* sp.; *Vibrio* sp.; Well Diffusion; MIC

1. Introduction

Indonesia's aquatic biodiversity is a key sector in the global food industry and a vital source of livelihood for coastal communities. However, most pathogenic diseases originate from aquatic organisms infected with bacteria, viruses, fungi, and parasites [1]. This situation has driven the use of biological agents as natural biocontrol agents, including probiotics, prebiotics, and phytogenics derived from plant compounds [2]. Generally, biological agents are frequently used for food animals, including fish. Fish is an important food source for the community, rich in nutrients such as protein, healthy fats, vitamins, and minerals. Fish are generally handled and monitored regularly because they are a natural food source. However, handling fish also presents significant challenges, as fish are highly perishable, with rapid spoilage occurring shortly after capture, especially in tropical temperatures. If not handled properly, spoilage will occur more rapidly [3]. The decay in question involves a decline in fish quality, affecting its appearance, aroma, taste, and meat texture. Increased pH and nitrogen compounds promote bacterial growth, which is a key process causing fish spoilage and has the greatest impact on the quality of fresh fish [4].

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Pathogenic bacteria that commonly infect fishery products and are also dangerous to humans include *Vibrio* sp., which belongs to the Gram-negative group [5], and typically inhabits water, air, and soil, and can cause disease in humans. The species that frequently infect humans are *Vibrio parahaemolyticus* and *Vibrio cholerae*, which cause the *Vibrio*sis disease [6]. In addition to *Vibrio* sp., *Pseudomonas sp.* which belong to the Gram-negative group, can also cause infectious diseases in animals, such as otitis, folliculitis, urinary tract infections, hemorrhagic pneumonia, and reproductive diseases like mastitis, abortion, and endometritis [7].

To address fish spoilage, several methods are typically employed, one of which involves the use of chemical antimicrobials. However, excessive or indiscriminate use can lead to microbial resistance. Many researchers have turned to alternative antimicrobial agents derived from plant extracts, one of which is the bangle rhizome. Bangle (*Zingiber montanum*) is a tropical or subtropical herb native to Southeast Asia. The part of the bangle plant commonly used in traditional medicine is the rhizome [8], which is effective as a remedy for fever, abdominal pain, constipation, colds, intestinal worms, and gout [9].

The chemical constituents of the bangle rhizome consist primarily of essential oils, which contain terpinen-4-ol, sabinene, arylbutanoids, phenylbutanoids, curcuminoids, and sesquiterpenes [10]. The phytochemical groups in bangle rhizomes dissolved in ethanol are known to contain tannins, alkaloids, steroids, terpenoids, and flavonoids [11]. According to several studies, bangle rhizomes are capable of inhibiting bacterial growth, such as *Escherichia coli* and *Streptococcus*, as evidenced by clear zones at four concentrations, with an average inhibition zone for *Escherichia coli* of 10–20 mm, while for *Streptococcus* it reached 5–10 mm [12]. In antimicrobial studies against *Shigella dysenteriae, activity was* quite high at a 25% concentration (8.80 ± 1.36 mm), with the diameter increasing continuously up to a 100% concentration (14.30 ± 0.39 mm) [13].

This study was conducted to determine the inhibitory activity of bangle rhizome extract, to identify the optimal concentration of the extract, and to identify the bioactive compounds in the bangle extract using ethanol as a solvent to analyze the secondary metabolites present in the bangle rhizome.

2. Materials and method

2.1. Study Design

This study employed an experimental design to test the antimicrobial activity of ethanol extracts of bangle rhizomes (*Zingiber* montanum) against *Pseudomonas* sp. and *Vibrio* sp. The study was conducted at the Microbiology Laboratory of the Department of Biology, University of Lampung, from November 2025 to February 2026. This study used a completely randomized design (CRD) with three concentrations in the well diffusion test, namely 50%, 75%, and 100%, as well as six concentrations in the microdilution test, namely 50%, 60%, 70%, 80%, 90%, and 100%, with five replicates.

2.2. Extraction of Bangle Rhizome

The bangle rhizomes were first prepared to make crude drug. Next, 450 g of the bangle rhizome crude drug was soaked in 2,250 ml of 96% ethanol. Maceration was carried out for 3 × 24 hours at room temperature, with occasional stirring. Subsequently, the macerate was strained and filtered using a rotary evaporator at a temperature below 50 °C.

2.3. Phytochemical Test

After a slightly viscous concentrated extract was produced, phytochemical testing was performed on the extract using five tests: alkaloids using three reagens, which is Mayer, Dragendroff, and Bouchardart; flavonoids; saponins; tannins; and triterpenoids. The method followed the research conducted by Konay et al., [14].

2.4. Bacterial Inoculation

Tryptone Soy Agar (TSA) medium, prepared at a concentration of 0.8 grams in 20 ml of distilled water, was sterilized beforehand. Next, it was divided into 5-ml portions in each test tube, which were then tilted and compacted. Afterward, the sterile TSA medium was inoculated with *Pseudomonas* sp. and *Vibrio* sp., then incubated for 24 hours at 37 °C.

2.5. Qualitative Test of Protease Enzyme

Qualitative test for protease enzyme activity was conducted to assess protease activity in *Pseudomonas* sp. and *Vibrio* sp. using a specialized test medium, namely Skim Milk Agar (SMA), with a composition following Amanina et al. [15], dissolved in 100 mL of sterile distilled water. Next, the poured and solidified SMA medium was tested by placing a single

loop of *Pseudomonas* sp. and *Vibrio* sp. bacterial isolates onto the surface of the SMA medium, then tightly wrapped and incubated at 37 °C for 24 hours.

2.6. Well Diffusion Test

Well diffusion tests were performed after both bacteria had been homogenized and diluted in physiological saline, and their turbidity was assessed using a McFarland 0.5 scale. Next, 0.1 mL of the bacterial suspension was applied to the surface of Mueller-Hinton Agar (MHA) and spread evenly using a cotton swab. Next, holes were made in the medium using a sterile straw for the number of replicates used, and then each hole was treated with 0.5 ml of bangle rhizome extract at adjusted concentrations (50%, 75%, and 100%). This test used chloramphenicol as a positive control and sterile distilled water as a negative control.

2.7. Microdilution Test

The microdilution test used a 96-well microplate, in which both bacteria were homogenized and diluted beforehand in physiological NaCl, then their turbidity was compared using a McFarland 0.5 scale. Mueller-Hinton Broth (MHB) was prepared to be mixed with bangle rhizome extract at the prepared concentrations (50%, 60%, 70%, 80%, 90%, and 100%) and to be mixed with both bacterial suspensions. After that, 200 µL of the mixture of the extract medium and the suspension medium was added to each microplate well. Two columns were also provided for positive and negative controls, as well as one row for a blank in each test to serve as a comparison for the measurement results. After that, the microplates were measured using a microplate reader before and after incubation to obtain the optical density (OD) at a wavelength of 630 nm. They were then incubated for 24 hours at 37˚C.

2.8. Data Analysis

Data analysis of the research results from the well diffusion test to determine the inhibition zone and the dilution test to determine the minimum inhibitory concentration (MIC) used the Kruskal-Wallis statistical test followed by the Dunn post hoc test [16].

3. Result and Discussion

3.1. Phytochemical Test

Based on the results obtained, the phytochemical content in the bangle rhizome (*Zingiber montanum*) extract contains bioactive compounds belonging to the alkaloid, flavonoid, saponin, and terpenoid groups. These results can be seen in Table 1, where nearly all tests yielded positive indicators.

Table 1 Results of Phytochemical Testing

No	Compound Class	Result	Description
1	Alkaloid (Mayer)	Positive	Yellow precipitate
2	Alkaloid (Dragendrof)	Positive	Brick-red precipitate
3	Alkaloids (Bouchardart)	Positive	Brown precipitate
4	Flavonoids	Positive	Color turns orange
5	Saponins	Positive	Foam appears
6	Tannin	Negative	Color unchanged (remains orange)
7	Terpenoids	Positive	Brownish-red precipitate

However, in the tannin test, no color change to blackish green occurred. This is due to several factors, one of which is the high content of terpenoids in the bangle rhizome. According to research conducted by Riaz et al., [17] in the Zingiberaceae family, some phytochemical test results are not visible or are faint in the tannin test using ethanol as a solvent. This is because in some Zingiberaceae species, terpenoid content is inversely proportional to tannin content, meaning the amount produced is not very high. The bangle rhizome functions as a food reserve and a storage site for secondary metabolites. The bioactive compounds abundant within it are essential oils. The extraction of essential oils is due to the polar nature of the solvent used. According to Pramushinta et al., [18] 96% ethanol is a semi-polar solvent with low water content, so the absorption of polar bioactive compounds such as tannins is not maximized.

3.2. Protease Enzyme Activity Test

The results of the protease enzyme activity test for both bacteria showed that they were qualitatively capable of degrading the protein content in the SMA medium. This can be seen in Figure 1, with the formation of a clear zone around the colonies.

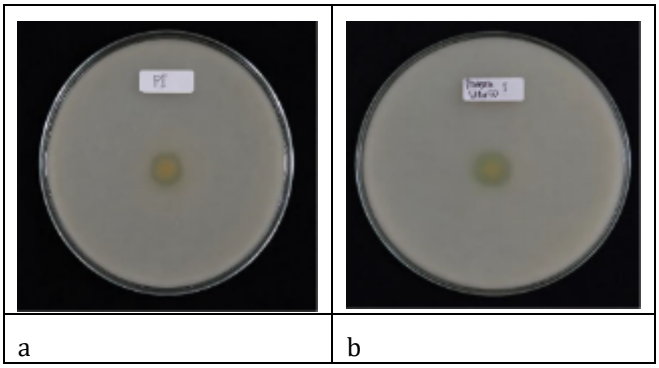

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Figure 1 Results of the Protease Enzyme Activity Test on (a) *Pseudomonas* sp. and (b) *Vibrio* sp., indicated by white arrows

Protease enzymes are classified as hydrolases because their reactions involve water at specific substrate bonds. Generally, the process of fish spoilage by protease enzymes begins when macromolecular protein compounds are broken down into peptide compounds and amino acids, causing the fish meat to soften.

These compounds are then further processed into volatile compounds that cause foul odors, such as putrescine, CO_2, H_2S, and ammonia [19]. *Pseudomonas* sp. produces alkaline proteases that degrade myofibrillar proteins [20], and *Vibrio* sp. produces metalloproteases containing zinc ions as catalytic cofactors to break peptide bonds [21].

3.3. Well Diffusion Test

In the well diffusion test, the ability of Zingiber montanum rhizome extract against *Pseudomonas* sp. and *Vibrio* sp. was assessed using the well diffusion method, which is expected to produce clear zones. The calculation of the inhibition zone's was performed using a gravimetric formula by transferring the zone pattern to a plastic sheet to measure the inhibition zone area and well diameter [22].

$$\mathbf{LZ/S} = \frac{\boldsymbol{Weight\ of\ the\ Zone\ or\ Well\ Replica}(\mathbf{g})}{\mathbf{Weight\ of\ 1x1\ Replica\ (g)}} \boldsymbol{x\ 1\ cm^2}$$

After calculating the area, the process continues using the equivalent diameter formula, which calculates an area considered equivalent to the zone area, so that the results of the zone area and the borehole area yield a representative diameter in millimeters. To calculate the zone area from which data will be collected, the total zone area (Zone Area) is subtracted from the borehole area [23].

$$\boldsymbol{Diameter} = \sqrt{\frac{\boldsymbol{\pi\ x\ Area}*}{\mathbf{4}}}$$

*Where Area = Zone Area, then:

Zone Area (AZ) = Total Area - Well Area

The results of the well diffusion test for *Pseudomonas* sp. showed average diameter values as presented in Table 2, where the inhibition zone produced by the highest extract concentration was at 100%, with an average of 6.83 mm. This value still falls within the moderate inhibition category. Meanwhile, the average diameter in the positive control also falls within the moderate category.

Table 2 Average Inhibitory Zone Diameter (mm) against the Growth of *Pseudomonas* sp. and *Vibrio* sp.

Treatment	**Average Inhibitory Zone Diameter for *Pseudomonas* sp. (mm)**	**Average Inhibitory Zone Diameter for *Vibrio* sp. (mm)**
K(+) (Chloramphenicol)	9,19	12,7
K(-) (Aquadest)	-	-
UP1 (50 %)	5,19	2,97
UP2 (75 %)	6,35	5,41
UP3 (100 %)	6,83	5,8

The results obtained from this test that *Pseudomonas* sp. is a Gram-negative bacterium with a four-layered cell wall consisting of lipoproteins, an outer membrane, phospholipids, and lipopolysaccharides. The cell wall of *Pseudomonas* sp. is also covered by a thick capsule, making it difficult for compounds to penetrate into the cell [24]. Furthermore, the high lipid content in this cell wall also reduces its permeability, making it difficult for antibacterial agents to penetrate and enter the bacterial cell [25]. These criteria explain why the positive control for *Pseudomonas* sp. produced a strong effect, while the bangle rhizome extract yielded a diameter in the moderate category.

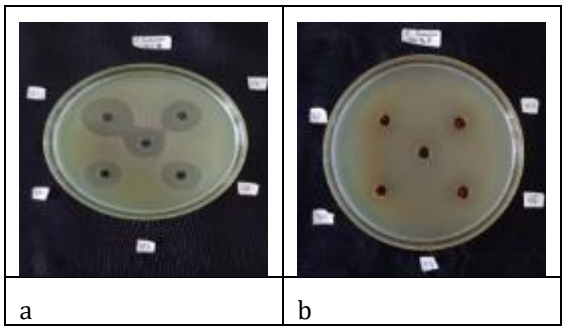

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Figure 2 Results of the Well Diffusion Test for *Pseudomonas* sp. in (a) Positive Control and (b) 100% Concentration

Meanwhile, for *Vibrio* sp., the inhibition zone results showed an increase in the average diameter, with the largest value obtained at the 100% concentration. The positive control, however, was already classified as strong. This also indicates that the bacterial resistance of *Vibrio* sp. to chemical antimicrobials is not as strong, yet it yields the same effect as the bangle rhizome extract, where the highest concentration falls into the moderate category. This differs from the conditions observed with *Pseudomonas* sp., which may indicate the presence of some technical errors. Non-circular or suboptimal diameter results can be caused by environmental contamination—which is particularly susceptible during the process of punching the test medium—or by improper administration of the bangle rhizome extract (e.g., insufficient or excessive extract during pipetting). Additionally, the viscosity of the extract affects the absorption process in the test medium, causing the absorbed extract to not spread evenly, so that the test bacteria don't receive the antimicrobial extract uniformly [26].

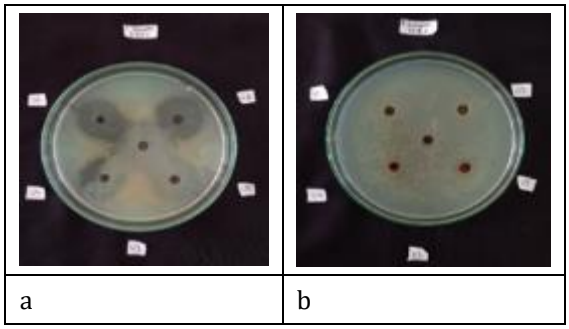

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Figure 3 Results of the Well Diffusion Test for *Vibrio* sp. in (a) Positive Control and (b) 100% Concentration

3.4. Microdilution Assay

In the microdilution test results for *Pseudomonas* sp. and *Vibrio* sp., the qualitative observations were not significantly different to the naked eye due to the intense color of the ethanol extract of bangle rhizome. However, when measured by *optical density* (OD), a difference was observed between the results before and after incubation. The average absorbance value was calculated by subtracting the pre-incubation OD value from the post-incubation value, and the inhibition percentage can be determined using the following formula and comparison table [27].

$$\textbf{\textit{Inhibition Rate}}\ (\%) = \left(\frac{\boldsymbol{K\,(-)Absorbance - Total\ Absorbance\ Sample}}{\boldsymbol{K\,(-)\ Absorbance}}\right) \times \mathbf{100}$$

In the results of the microdilution test of bangle rhizome extract, the highest average percentage was found at a concentration of 80% (2.074%), while the lowest average absorbance was observed at a concentration of 80% (-0.962). These results indicate that the highest concentration was not as effective at inhibiting bacterial growth as the 80% concentration, but it was still capable of inhibiting bacterial growth within a 24-hour period, as evidenced by the OP measurements taken before and after incubation. The 100%, 90%, and 80% concentrations yielded fairly good average inhibition results, as shown in Table 3. When compared to the average results, there is a direct correlation: the lower the absorbance value, the higher the antimicrobial activity is considered to be, while the highest percentage of inhibition is observed. However, the highest concentration was at 80%, so this result warrants further attention in the subsequent discussion, as there is a deviation from the expected concentration sequence.

Table 3 Results of the Microdilution Test of the Ethanol Extract of *Zingiber montanum* Rhizome against *Pseudomonas* sp**.**

Treatment	**Before Incubation (mm)**	**After Incubation (mm)**	**Average of Absorbance (nm)**	***Inhibition Rate* (%)**
K(+) (Chloramphenicol)	0,05	0,112	0,062	-
K(-) (Media)	0,05	1,034	0,984	-
UP1 (50 %)	1,934	1,397	-0,537	1,466
UP2 (60 %)	2,468	1,728	-0,74	0,804
UP3 (70 %)	2,199	1,267	-0,932	1,381
UP4 (80 %)	2,500	1,538	-0,962	2,074
UP5 (90 %)	2,187	1,243	-0,944	1,986
UP6 (100 %)	2,617	2,063	-0,554	1,853

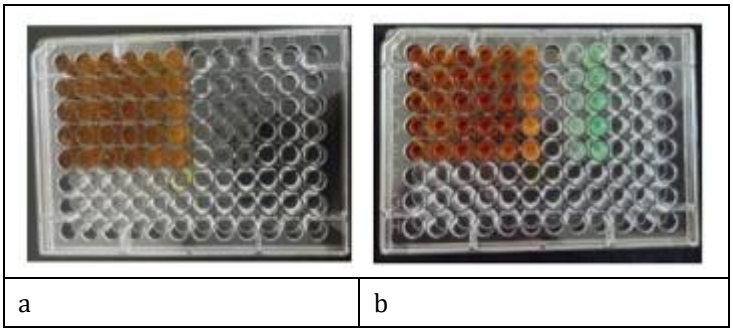

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Figure 4 Results of the Microdilution KHM Test of *Zingiber montanum* Rhizome Extract against *Pseudomonas* sp. at (a) Before Incubation and (b) After Incubation.

Meanwhile, for *Vibrio* sp., the lowest average absorbance value was obtained at a concentration of 70% (-1.061), while the highest inhibition percentage was obtained at a concentration of 80% (1.27). The following results did not decrease

linearly from the highest concentration to the lowest concentration to determine the minimum inhibitory concentration (MIC) value; therefore, some errors should be noted

Table 4 Results of the Microdilution Test of the Ethanol Extract of *Zingiber montanum* Rhizome against *Vibrio* sp.

Treatment	**Before Incubation (mm)**	**After Incubation (mm)**	**Average of Absorbance (nm)**	***Inhibition Rate* (%)**
K(+) (Chloramphenicol)	0,050	0,051	0,001	-
K(-) (Media)	0,059	0,805	0,746	-
UP1 (50 %)	1,267	1,541	0,274	0,179
UP2 (60 %)	2,356	1,617	-0,739	0,884
UP3 (70 %)	2,637	1,576	-1.061	1,095
UP4 (80 %)	3,198	2,442	-0,756	1,27
UP5 (90 %)	2,899	2,183	-0.716	1,10
UP6 (100 %)	3,62	3,103	-0.517	0,788

The lack of a significant increase in results from 50% to 100% may be due to several reasons, such as the blank OD value obtained not being very representative due to the color intensity of the ethanol extract of the bangle rhizome, causing the absorbance reading or OD value to fluctuate. Additionally, this phenomenon may also result from the complex nature of the compounds, causing the solution's absorption capacity at specific wavelengths to be suboptimal [28]. It is advisable to pay attention to the concentration of the bacterial inoculum that has been diluted with physiological NaCl, the lipophilicity of the oil, which must be well emulsified so that it can come into good contact with the bacteria, and sonication is required to reduce the oil content and agitation during incubation to prevent two-phase separation [29].

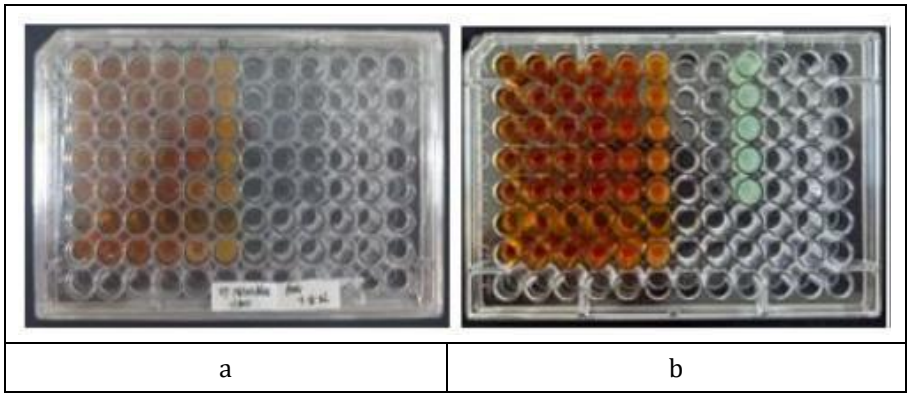

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Figure 5 Results of the microdilution test of *Zingiber montanum* rhizome extract against *Vibrio* sp. at (a) before incubation and (b) after incubation.

4. Conclusion

The conclusion drawn from this study is that the ethanol extract of *Zingiber montanum* rhizome exhibits antimicrobial activity against *Pseudomonas* sp. and *Vibrio* sp., with the best well diffusion test results for both bacteria at a 100% concentration (6.83 mm for *Pseudomonas* sp. and 5,8 mm for *Vibrio* sp.). Meanwhile, in the microdilution test, the MIC values based on the percentage inhibition of *Pseudomonas* sp. and *Vibrio* sp. were at a concentration of 80% (2,07% for *Pseudomonas* sp. and 1,27% for *Vibrio* sp.). Compounds identified through phytochemical analysis of the ethanol extract of *Zingiber montanum* rhizome using the method yielded positive results for the alkaloid, flavonoid, saponin, and terpenoid groups.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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