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Original Article

EFFECTIVENESS OF BATAK LEEKS EXTRACT AGAINST *S. MUTANS* AND *E. FAECALIS* AS ANTIBACTERIAL AND ANTIBIOFILM AGENT

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ABSTRACT

Objective: *S. mutans* biofilms have become the main cause of dental caries. Meanwhile, *E. faecalis* biofilms cause endodontic infection. Batak leeks are native North Sumatra herb that contains antibacterial properties. This study aims to determine Batak leeks extract's antibacterial and antibiofilm effectiveness towards *S. mutans* and *E. faecalis.*

Methods: This study was a laboratory experimental research with a posttest-only control group design. The sample size was 27, consisting of 3 samples each with concentrations of 0.78%; 1.56%; 3.25%; 6.25%; 12.5%; 25% and 50% Batak leek extract and 3 negative control samples and 3 positive. The Kirby-Bauer disc diffusion method was used to determine MIC, the streaking method to determine MBC and the Static Microtiter Plate Assays method for antibiofilm determination. Data analysis used Oneway ANOVA and Kruskall Wallis tests followed by post-hoc LSD and Mann-Whitney tests.

Results: The results showed that Batak leeks extract contained MIC values at 0,78% concentration levels with an average inhibitory zone of 6,80±0,10 mm in S. *mutans* and E. *faecalis*. MBC values were undetected because there were no group capable of reducing bacteria at 98-98% and the highest antibiofilm reduction was found in 50% concentration extract with values of 85.58% for S. *mutans* and 90.29% for *E. faecalis*.

Conclusion: It concluded that 0.78% concentration of Batak leeks extract has an antibacterial effect, but at 50% concentration was the most influential in inhibiting *S. mutans* and *E. faecalis*, and antibiofilm ability starts at the concentration level of 1.56%.

Keywords: *S. mutans*, *E. faecalis*, Batak leeks extract, Antibacterial, Antibiofilm

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INTRODUCTION

The oral cavity is the second body part to contain various microbiotas after the intestines, with 700 species of microorganisms consisting of bacteria, fungi, and protozoa [1]. Several bacterias that can cause various infections in the oral cavity tissues are *Streptococcus mutans (S. mutans)* and *Enterococcus faecalis (E. faecalis)* [2-4]. Both bacteria have several virulence factors that play a role in forming colonies and significantly contribute towards forming neither the cariogenic nor the cariogenicity biofilm [5, 6]. *S. mutans* biofilm adheres to the enamel surface and is the main cause of dental caries; meanwhile, *E. faecalis* biofilms adhere to the dentine wall and root canals, which leads to endodontic infection [5, 7].

Biofilm on both bacteria has five stages of formation: attachment, colonization, proliferation, cell maturation, and dispersion [8, 9]. Extracellular matrix formed during the biofilm formation stage gives a defense mechanism from external factors, such as preventing dissociation and raising mechanic stability. Thus, the biofilm survives in harsh environments, host immunity, and antimicrobial treatment [5].

A strong antiseptic that is commonly used is chlorhexidine. Chlorhexidine contains bactericidal and bacteriostatic properties against various bacteria, including bacteria found in plaque, such as *S. mutans* and *E. faecalis* [10, 11]. However, Chlorhexidine causes side effects that include changes in tongue color, teeth, and reversible silicate or composite restorations, thus making it necessary to find potential natural antibacterial and anti-biofilm derived from medicinal plants [12].

Batak leeks *(Allium chinense G. Don)* is a native herb that grows in the higher plateau of North Sumatra, for example, Sidikalang, Deli Serdang, Berastagi, and Tapanuli. They are commonly used as vegetables, cooking spices, and medicine [13, 14]. *Allium chinense* contains nutritional substances capable of suppressing cholesterol levels, preventing hypertension, antioxidants, antibacterial, antibiotic, and cancer [13]. Phytochemical test results using n-hexane extract reveal that Batak leeks *(Allium chinense G. Don)* contain alkaloids, flavonoids, phenols, and are high in saponins [15].

Research conducted by Rhetso *et al.* reveals antibacterial activity between Batak leeks n-hexane extract against *S. aureus, P. aeruginosa*, and *A. niger* [15]. Another research by Fahmi also discovered methanol extract, total ethyl acetate and ethyl acetate residue of Batak leeks *(Allium chinense)* were effective against gram-positive bacteria *B. cereus* and gram-negative bacteria *S. thypii* by finding the diameter of the inhibition in each treatment [16].

Based on the above background, research regarding antibacterial activity and antibiofilm of Batak leeks *(Allium chinense G. Don.)* extract against *S. mutans* and *E. faecalis* is still very limited. Thus, researchers are interested in investigating Batak leeks' antibacterial and anti-biofilm effectiveness *(Allium chinense)* toward *S. mutans* dan *E. faecalis*.

MATERIALS AND METHODS

This research is a laboratory experimental research with the post-test-only group design approach. The composing of the Batak leeks extract and phytochemical screening tests were conducted at the Medicinal Plant Research and Development Laboratory (ASPETRI) and the Phytochemical Laboratory, Faculty of Pharmacy Universitas Sumatera Utara. Identification and sample testing were conducted at the Microbiology Laboratory Faculty of Pharmacy Universitas Sumatra Utara, and the Integrated Laboratory of Faculty of Medicine Universitas Sumatera Utara. The sample used was the *S. mutans* ATCC®25175 and *E. faecalis* ATCC®29212 bacteria. This research sample size consists of 27 items with 3 samples each from 7 groups concentration of 70% ethanol extract of Batak leeks *(A. chinense),* namely 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, and 0.78%, as well as 3 samples for the 0.2% chlorhexidine group as a positive control and 3 samples for the DMSO group as a negative control. The research consent form was approved by the Health Research Ethics

Committee of the Universitas Sumatera Utara No. 20/KEPK/USU/2023/.

Extract assembly

Starting with the assembly of simplicia consisting of 1 kg of Batak leeks obtained from Berastagi (North Sumatera), sterilized and cut into small portions, then dried in the dehumidifier and crushed into powder using a pestle and a blender. The Batak leeks powder was then incorporated with 70% ethanol and proceeded with the maceration method using a rotavapor until it became a thick extract.

The thick extract was first subjected to a phytochemical test prior to conducting the antibacterial and anti-biofilm test to determine the phytochemical compound contained in the Batak leeks extract. Subsequently, the thick extract from the Batak leeks was then diluted with Dimethyl Sulfoxide (DMSO) with concentration levels of 50%; 25%; 12.5%; 6.25%; 3.125%; 1.56%; and 0.78%.

Antibacterial test

The Kirby-Bauer disc diffusion is a method used for diffusion. Paper discs were dripped with various extract concentrations, and the control groups were placed on a Muller Hinton Agar (MHA) (HiMedia®, India) media homogenized with *S. mutans* and *E. faecalis*. The Petri cups were then incubated for 24 h at a temperature of 37 °C. After 24 h, observation and evaluation were performed to determine the most effective concentration. Minimum Inhibitory Concentration (MIC) values were obtained by measuring the diameter of the clear zone surrounding the paper discs using vernier callipers [17].

Minimum Bactericidal Concentration (MBC) values were determined by calculating the number of colonies. The clear zone was formed from each concentration in the MIC test from streaking using a sterile cotton swab, then dipping it for 10 min in each test tube containing Muller Hinton Broth (MHB) (HiMedia®, India) media. Take 1 ml of MHB from each test tube and drop it into sterile petri dishes, add Plate Count Agar (PGA) (HiMedia®, India) then homogenized and incubate for 24 h at 37 °C. The colonies formed in the Petri dishes are then calculated using the colony counter (Scan®300Interlab, France) machine. A concentration is considered MBC when said concentration can reduce 98-99% from the initial number of bacteria [18, 19].

Antibiofilm test

The Static Microtiter Plate Assays method uses suspensions of *S. mutans* dan *E. faecalis* bacteria cultured in Trypticase Soy Broth (TSB)+1% Glucose. The bacterial suspension is par with the McFarland 0.5 (1.5x108 CFU/ml) standard. 100 µl of 70% ethanol extract of Batak leeks with various concentrations and 100 µl of each bacterial suspension was put into a 96-well microplate and incubated at 37°C for 48 h. After incubation, rinsed with Phosphate Buffer Saline Solution (PBS) and fixated with a Bunsen burner, the next step is to administer 200 µl of the violet crystal solution (1 % w/v), then incubate for 15 min at room temperature, after that rinsed with PBS. After the microplate dries, put 96% ethanol onto the microplate and incubate for 15 min at room temperature, then, the microplate is measured using the microplate reader with a wavelength of 595 nm [20].

Data analysis

Testing of the inhibition activity of the *S. mutans* bacteria used the Kruskal Wallis followed by the Mann-Whitney test because the data was not distributed normally. Meanwhile, the *E. faecalis* bacteria used the one-way ANOVA test preceded by the Post Hoc LSD test. Testing for the bacteria-killing rate and the *S. mutans* and *E. faecalis* anti-biofilm used the Kruskal Wallis followed by the Mann-Whitney test. Significant values in this study were $p<0.05$.

RESULTS

Phytochemicals screening test result of 70% ethanol extract of Batak leeks *(Allium chinense G. Don)* in this research reveals that alkaloids, flavonoids, glycosides, saponins, tannins, and triterpenoid/steroids compound substances were found (table 1).

MIC based on the diameter of the lowest inhibition zone of 70% ethanol extract of Batak leeks towards the *S. mutans* dan *E. faecalis* bacteria was found at concentration levels of 0.78% with the average inhibition zone of 6.80±0.10 mm each (table 2). Post hoc testing of the *S. mutans* bacteria inhibition activity from multiple concentrations of 70% ethanol extract of Batak leeks *(Allium chinense G. Don)* reveals several significant differences, except at concentrations of 3.125% with 1.56%, 6.25% with 1.56%, 12.5% with 1.56%, 12.5% with 3.125%, 12.5% with 6.25%, and 25% with 12.5%. (table 5). Post hoc testing of the *E. faecalis* bacteria inhibition activity towards various 70% ethanol extracts of *Batak leeks* reveals significant differences, except at a concentration of 12.5% with 6.25%, which doesn't show a significant difference (table 6).

Table 1: Phytochemical screening test results of 70% ethanol extract of Batak leeks *(Allium chinense G. Don)*

S. No.	Secondary metabolites	Reactor	Results
1.	Alkaloid	Dragendroff	+
		Bouchardat	+
		Meyer	+
2.	Flavonoid	Mg Powder+Amil	+
		Alcohol+HCl	+
3.	Glycosides	Molish+H_2SO_4	+
4.	Saponin	Hot water/shaken	+
5.	Tannin	$FeCl_3$	+
6.	Triterpenoid/Steroid	Lierberman-Bourchat	+

Table 2: Inhibitory activity test of 70% ethanol extract of batak leeks (*Allium chinense* G. Don) against *E. faecalis* and *S. mutans* bacteria

S. No.	Extract concentration	*E. faecalis (A)*		*S. mutans (B)*	
		Inhibitory zone in mm (mean±SD)	*p*	Inhibitory zone in mm (maen±SD)	*p*
1.	0.78%	6.80±0.10	0.001*	6.80±0.10	0.002*
2.	1.56%	7.40±0.10		8.13±0.92	
3.	3.125%	8.13±0.06		8.00±0.10	
4.	6.25%	8.67±0.06		8.40±0.20	
5.	12.50%	9.23±0.15		8.73±0.90	
6.	25%	9.83±0.15		9.93±0.31	
7.	50%	10.47±0.15		10.93±0.40	
8.	Chlorhexidine 0.2%	13.20±0.61		20.27±0.47	
9.	DMSO	0.00±0.00		0.00±0.00	

*p≤0.05=significant with the *one-way* ANOVA test. N=3

MBC were undetected because the entire concentration test could not reduce 99%-100% of the bacterial colonies. The highest reduction percentage of the *S. mutans* bacteria is at concentration extract of 50%, 89.86%, and 89.41% for *E. faecalis* (table 3). The post hoc testing of the *S. mutans* and *E. faecalis* bacteria inhibition activity from various concentration levels of 70% ethanol extract of Batak leeks *(Allium chinense G. Don)* reveals that all concentration pairs have a significant difference (p≤0.05), the higher the concentration extract of Batak leeks, the higher the chance of reducing bacteria (table 5 and table 6).

Table 3: Killing rate test of 70% ethanol extract of *batak leeks* (*Allium chinense G. Don*) against *E. faecalis* and *S. mutans bacteria*

S. No.	Extract concentration	*E. faecalis (A)*			*S. mutans (B)*		
		Number of bacterial colonies (CFU/ml)	% Reduction	*p*	Number of bacterial colonies (CFU/ml)	% Reduction	*p*
1.	0.78%	0.30±0,03	55.76	0.000*	698±25.58	63.15	0.001*
2.	1.56%	0.24±0,01	58.65		605±1.00	69.05	
3.	3.125%	0.20±0,00	64.41		531±9.16	71.96	
4.	6.25%	0.14±0,01	71.74		418±2.52	77.93	
5.	12.50%	0.13±0,12	78.70		310±12.66	83.63	
6.	25%	0.11±0,00	84.90		272±6.56	86.64	
7.	50%	0.11±0.00	89.41		192±10.21	89.86	
8.	Chlorhexi-dine 0.2%	0±0.00	100		0±0.00	100	
9.	DMSO	1.13±0.07	0		1894±128.22	0	

*p≤0.05= significant with the *Kruskall-Wallis* test. N=3

Results of the *S. mutans* and *E. faecalis* anti-biofilm from various concentration extract of 70% ethanol extract of Batak leeks show that the lowest concentration of 0.78% is capable of reducing *S. mutans* by 34.69% and *E. faecalis* by 73.17% (table 4). The post hoc anti-biofilm activity test of the *S. mutans* bacteria from various concentration extract of 70% ethanol extract of Batak leeks *(Allium chinense G. Don)* shows that there are significant differences, except at concentrations of 3.125% with 56%, 6.25% with 3.125%, 12.5% with 6.25%, 25% with 6.25%, 25% with 12.5% and 50% with 6.25% that display there is no significant difference in anti-biofilm activity (table 5). The post hoc anti-biofilm activity test of the *E. faecalis* bacteria towards various concentrations of 70% ethanol extract of Batak leeks shows significant differences, except at concentrations of 12.5% with 6.25%, 25% with 12.5% and 50% with 25% that doesn't show a significant difference (table 6).

Table 4: Antibiofilm test of 70% ethanol extract of batak leeks (*Allium chinense G. Don*) against *E. faecalis* and *S. mutans* bacteria

S. No.	Extract concentration	*E. faecalis*			*S. mutans*		
		Optical density (OD)	% Reduction	*p*	Optical density (OD)	% Reduction	*p*
1.	0.78%	0.30±0.03	73.17	0.001*	0.36±0.04	34.69	0.002*
2.	1.56%	0.24±0.01	78.55		0.20±0.04	63.16	
3.	3.125%	0.20±0.01	82.35		0.16±0.02	70.99	
4.	6.25%	0.14±0.01	87.11		0.11±0.03	80.24	
5.	12.50%	0.13±0.12	82.35		0.10±0.02	81.49	
6.	25%	0.11±0.01	89.59		0.09±0.00	83.27	
7.	50%	0.11±0.01	90.29		0.08±0.01	85.58	
8.	Chlorhexidine 0.2%	0	100		0±0.00	100	
9.	DMSO	1.13±0.07	0		0.56±0.05	0	

**p*≤0.05= significant with the *Kruskall-Wallis* test. N=3

Table 5: Post hoc test results for differences in inhibitory activity, killing rate, and antibiofilm of 70% ethanol extract of batak leeks against *S. mutans*

p-value		0.78%	1.56%	3.125%	6.25%	12.5%	25%	50%	K(+)	K(-)
0.78%	I								0.050*	0.037*
	II								0.050*	0.037*
	III								0.037*	0.050*
1.56%	I	0.050*							0.050*	0.037*
	II	0.050*							0.050*	0.037*
	III	0.050*							0.037*	0.050*
3.125%	I	0.050*	0.513						0.050*	0.037*
	II	0.050*	0.050*						0.050*	0.037*
	III	0.050*	0.127						0.037*	0.050*
6.25%	I	0.050*	0.513	0.050*					0.050*	0.037*
	II	0.050*	0.050*	0.050*					0.050*	0.037*
	III	0.050*	0.050*	0.127					0.037*	0.050*
12.5%	I	0.050*	0.275	0.513	0.513				0.050*	0.037*
	II	0.050*	0.050*	0.050*	0.050*				0.050*	0.037*
	III	0.050*	0.050*	0.050*	0.827				0.034*	0.046*
25%	I	0.050*	0.050*	0.050*	0.050*	0.077			0.050*	0,037*
	II	0.050*	0.050*	0.050*	0.050*	0.050*			0.050*	0.037*
	III	0.046*	0.046*	0.046*	0.507	0.507			0.037*	0.050*
50%	I	0.050*	0.050*	0.050*	0.050*	0.050*	0.050*		0.050*	0.037*
	II	0.050*	0.050*	0.050*	0.050*	0.050*	0.050*		0.050*	0.037*
	III	0.050*	0.050*	0.050*	0.513	0.050*	0.046*		0.037*	0.050*
K(+)	I									0.037*
	II									0.037*
	III									0.037*

**p*≤0.05=significant, I: MIC (Mann-Whitney), II: MBC (Mann-Whitney), III: Antibiofilm (Mann-Whitney)

Table 6: Post hoc test results for differences in inhibitory activity, killing rate, and antibiofilm of 70% ethanol extract of batak leeks against *E. faecalis*

p-value	No	0.78%	1.56%	3.125%	6.25%	12.5%	25%	50%	K(+)	K(-)
0.78%	I								0.000*	0.000*
	II								0.000*	0.000*
	III								0.037*	0.050*
1.56%	I	0.027*							0.000*	0.000*
	II	0.005*							0.000*	0.000*
	III	0.050*							0.037*	0.050*
3.125%	I	0.000*	0.000*						0.000*	0.000*
	II	0.000*	0.001*						0.000*	0.000*
	III	0.050*	0.050*						0.037*	0.050*
6.25%	I	0.000*	0.000*	0.000*					0.000*	0.000*
	II	0.000*	0.000*	0.010*					0.000*	0.000*
	III	0.050*	0.050*	0.050*					0.037*	0.050*
12.5%	I	0.000*	0.000*	0.000*	0.000*				0.000*	0.000*
	II	0.000*	0.000*	0.000*	0.007*				0.000*	0.000*
	III	0.050*	0.050*	0.050*	0.275				0.037*	0.050*
25%	I	0.000*	0.000*	0.000*	0.000*	0.000*			0.000*	0.000*
	II	0.000*	0.000*	0.000*	0.000*	0.000*			0.000*	0.000*
	III	0.050*	0.050*	0.050*	0.050*	0.184			0.037*	0.050*
50%	I	0.050*	0.050*	0.046*	0.046*	0.050*	0.050*		0.000*	0.037*
	II	0.000*	0.000*	0.000*	0.000*	0.000*	0.003*		0.000*	0.000*
	III	0.050*	0.050*	0.050*	0.050*	0.050*	0.127		0.037*	0.050*
K(+)	I									0.000*
	II									0.000*
	III									0.037*

*$p \leq 0.05$=significant, I: MIC (Mann-Whitney), II: MBC (LSD), III: Antibiofilm (Mann-Whitney)

DISCUSSION

The results of testing the antibacterial activity on the 70% ethanol extract of Batak leeks ($p \leq 0.05$) showed that there is inhibitory activity towards the *S. mutans* and *E. faecalis* bacteria. This is proven by the clear zone that forms surrounding the disc paper. MIC values of this research lie at concentration levels of 0.78% of the Batak leeks extract with an average inhibition zone of 6.80±0.10 mm towards *S. mutans* and *E. faecalis* (table 2).

This research is in line with Fahmi's research (2020), which reported that Batak leeks extract at concentration levels starting from 0.0166 % is capable of inhibiting *S. mutans* bacteria with an inhibition zone of 10.5 mm and at concentration levels starting from 0.0083% is also capable of inhibiting the *Bacillus cereus* bacteria with an inhibition zone of 10 mm [16]. Another research also reports that Batak leeks extract demonstrated the ability to inhibit the *Salmonella thypii* bacteria at a concentration of 40% with an inhibition zone of 13.2 mm [21].

The strength of bioactive compounds in inhibiting bacteria is correlated with the diameter of the formed clear zone [22]. The bigger the antimicrobial concentration, the faster the diffusion occurs, thus the antibacterial strength increases, and the diameter of the resulting inhibition zone is larger [23]. Based on that, a concentration of 50% with the largest diameter of the inhibition zone is established as the most effective concentration of Batak leeks extract in inhibiting the bacterial growth of *S. mutans* and *E. faecalis*.

According to Davis and Stout (1971) in Sirri *et al.* (2022), it was pronounced that the diameter of the weak inhibition zone is ≤5 mm, the moderate inhibition zone is 5-10 mm, the strong inhibition zone is 10-20 mm and the strongest inhibition zone is ≥20 mm [24]. Therefore, extract with a strong inhibition zone was found at concentration levels of 50% with inhibiting strength on *S. mutans* and *E. faecalis* bacteria at 10.93 mm and 10.47 mm. Extract with a moderate inhibition zone was found at minimum concentration levels of 0.78% with an average inhibition diameter for *S. mutans* and *E. faecalis* bacteria at 6.8 mm and 6.8 mm (table 2).

In this research, MBC values were undiscovered in the test concentration group except for the positive control (chlorhexidine 0.2%). MBC values are the lowest concentration extract capable of reducing the initial number of colonies in the negative control for 98%-99% [19]. The highest concentration in the study was found at 50%, which reduced the initial amount of bacterial colonies by 89.86% for *S. mutans* bacteria and 89.41% for *E. faecalis* bacteria (table 3).

The results of the anti-biofilm activity on the 70% ethanol extract of Batak leeks ($p \leq 0.05$) reveal the presence of inhibition activity on the *S. mutans* and *E. faecalis* biofilm. In this research, it is discovered that the higher the concentration of the 70% ethanol extract of Batak leeks, the lower the Optical Density (OD) value and the greater the value of the biofilm inhibition (table 4). Biofilm forming capability from strain is categorized by comparing the Optical Density (OD) value in each strain with negative control (ODNC) i.e. there is no formation (ODNC>OD), weak formation (ODNC<OD ≤ 2 × ODNC), moderate formation (2 × ODNC<OD ≤ 4 × ODNC), nor strong biofilm (OD>4 × ODNC). Based on that, the concentration extracts capable of reducing *S. mutans* and *E. faecalis* biofilms is found starting from 1.56% extract (table 5 and table 6) *S. mutans* and *E. faecalis* are gram-positive bacteria with cell walls with thick peptidoglycan arrangement close to the cell surface [25]. That can cause bioactive compounds with antibacterial properties to become easily associated with the bacteria's surface, potentially damaging and hindering cell wall synthesis. Antimicrobials diffuse through the biofilm matrix and inhibit biofilm production in several ways, such as disruption of cell signalling system, suppression of alarm systems to avoid bacterial overreaction, and the downregulation of gene expression of transport binding proteins responsible for biofilm production. The potential membrane that is disrupted to inhibit production may reduce EPS so that the ability of the microorganism to withstand harsh environmental conditions and antimicrobial therapy can be reduced [26, 27].

Several compounds contained in 70% ethanol extracts of Batak leeks have antibacterial and antibiofilm characteristics, such as alkaloids, flavonoids, glycosides, saponins, tannins, and triterpenoids/steroids. Flavonoids on the Batak leeks extract inhibit nucleic acid synthesis, cytoplasmic membrane, and energy metabolism of bacteria [28]. Flavonoids also cause changes in protein structure and nucleic acid capable of denaturing EPS [29]. Tannin found in the extract also possess bacteria cell inhibiting properties by denaturing the cell protein. Other substances, such as alkaloids, can disrupt peptidoglycan components in bacterial cells and cause cell death [30, 31]. As an antibacterial, Saponin can cause protein and enzyme leakage in cells. At the same time, triterpenoid reacts with porins (transmembrane protein) on the outer wall of the bacteria cell's membrane, forming strong polymer bonds that can cause damage to the porins [30].

CONCLUSION

Based on this research results, 70% ethanol extracts of batak leeks *(Allium chinense G. Don)* are capable of inhibiting *S. mutans* and *E. faecalis* bacteria at concentration levels of 0.78% and concentration of 50% possess the highest inhibition and antibiofilm capability from the Batak leeks extract starting from concentration levels of 1.56%.

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AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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