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ORIGINAL ARTICLE

Antibacterial Effects of Salvia Officinalis Extract on P.gingivalis & F.nucleatum: An In Vitro Study

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Abstract

Background: The side effects of chemical mouthwashes have led to the increasing use of herbal mouthwashes. *Salvia officinalis* (S. *officinalis*) is one of the most valuable medicinal plants from the Lamiaceae family with a broad-spectrum antimicrobial activity. This study aimed to assess the antibacterial effects of *S. officinalis* against P. gingivalis and F. nucleatum and to compare their susceptibility patterns to the extract using standard in vitro assays.

Materials & Methods: This in vitro Study evaluated the effects of *S. officinalis* hydroalcoholic extract against standard-strain *Porphyromonas gingivalis* (*P. gingivalis*; ATCC3327) and *Fusobacterium nucleatum* (*F. nucleatum*; ATCC2558). The antibacterial activity of different concentrations of the extract was evaluated by measuring the diameter of the growth inhibition zones on Mueller-Hinton agar culture medium, and by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract in 96-well plates against the bacteria. Data were analyzed by the Kruskal-Wallis and Mann-Whitney U tests (α=0.05).

Results: The mean diameter of the growth inhibition zones caused by 10, 12, and 15 mg/mL concentrations of the extract was significantly larger in the *P. gingivalis* culture compared with the *F. nucleatum* culture (P<0.001). Also, the extract at 10, 12, and 15 mg/mL inhibited bacterial growth by 50% (MIC50), 80% (MIC80), and 100% (MBC), respectively.

Conclusion: *S. officinalis* hydroalcoholic extract showed antibacterial effects against *P. gingivalis* and *F. nucleatum* periopathogenic microorganisms. Its MICs were 10 and 12 mg/mL, and its MBC was 15 mg/mL in vitro.

Keywords: Salvia officinalis; Antibacterial Agents; Periodontal Diseases; Porphyromonas gingivalis; Fusobacterium nucleatum.

Introduction

Infectious oral diseases caused by oral bacteria are among the most common human diseases and can be categorized into two main groups: dental caries and periodontal disease. Periodontal diseases can also be divided into two categories: gingivitis, which refers to inflammation of the gingiva, and periodontitis, which refers to inflammation of the tooth-supporting structures and the periodontium (cementum, periodontal ligament, and bone) (1). Periodontal diseases develop due to an imbalance between bacterial proliferation and the host immune response. The diagnosis of periodontitis is based on bleeding on probing, periodontal pocket formation, gingival hyperplasia, attachment loss, and bone resorption,

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which can lead to subsequent tooth mobility and eventual tooth loss if left untreated. During periodontal disease progression, metabolic bacterial products can induce leukodiapedesis and the subsequent release of pro-inflammatory cytokines (2-4). Although periodontal disease is more common in adults, some subtypes may also affect children. The prevalence of periodontal disease increases with age (5). According to the available data, periodontal disease is more common in males (56%) than in females (38%) (6).

Prevotella intermedia, Tannerella forsythia, Porphyromonas gingivalis (P. gingivalis), Campylobacter rectus, Eikenella corrodens, Fusobacterium nucleatum (F. nucleatum), Aggregatibacter actinomycetemcomitans, Peptostreptococcus micros, Treponema denticola, and Selenomonas species are among the most important periopathogenic microorganisms (7,8).

Various antimicrobial agents, such as triclosan, fluoride, iodide, and chlorhexidine, are used to prevent or control oral bacterial infections (9). Chlorhexidine is among the most commonly used antimicrobial mouthwashes; however, despite its strong antimicrobial activity, it can lead to tooth discoloration and alter taste perception (10). On the other hand, medicinal herbs and traditional medicine have gained increasing popularity in recent years as alternatives or adjuncts to synthetic drugs (11).

Salvia officinalis (S. officinalis), or common sage, belongs to the mint family, Lamiaceae. It is a perennial, evergreen subshrub with 30- to 60-cm woody stems. It has grayish leaves and blue to purplish flowers that appear around May or June, and rarely white flowers. It is the most valuable member of the mint family, Lamiaceae, regarding its therapeutic effects, as it has antibacterial, antifungal, and anti-inflammatory properties (12). Its leaves have energizing and boosting effects due to their essential oils and tannins (13,14). They are also used topically for disinfection of ulcers and enhancement of wound healing (14). In traditional medicine, S. officinalis is used to treat respiratory infections and gastrointestinal diseases. S. officinalis has greater therapeutic effects and fewer side effects (in certain concentrations) compared with antibiotics (15). Due to the long-term use of S. officinalis in traditional medicine and the absence of any report regarding any possible adverse effect (when used in certain concentrations), researchers have become interested in assessing its potential clinical applications (16). Considering the recent interest in medicinal herbs as a new source of antimicrobial agents and the lack of studies on the antimicrobial effects of S. officinalis against periopathogens, this study aimed to assess the antibacterial effects of S. officinalis against two main periopathogenic microorganisms, namely F. nucleatum and P. gingivalis.

Materials and Methods

In this in vitro Study, standard strains of P. gingivalis (ATCC 33277) and F. nucleatum (ATCC 2558) were used. In this in vitro Study, the statistical unit was defined as an independent experimental replicate (plate). For each bacterial strain, antibacterial assays were performed at three extract concentrations and a control condition, with each condition tested in triplicate independent experiments. Sample size selection was based on commonly accepted practices in in vitro antimicrobial studies and CLSI recommendations rather than on clinical power calculations. The study was approved by the ethics committee of Islamic Azad University, Isfahan Branch (Khorasgan) (IR.IAU. KHUISF.REC.1400.243).

Preparation of the extract

S. officinalis was purchased from a reliable botanical store, and its hydroalcoholic extract was obtained in the School of Pharmacy of Isfahan University of Medical Sciences. For this purpose, S. officinalis was diced into

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small pieces and ground in a ball mill; 700 g of the ground herb was weighed on a digital scale (A&D, Japan), placed in a glass desiccator, and 4 L of 70% ethanol (as the solvent) was added. After 24 hours of desiccation, S. officinalis extract was obtained by maceration. Maceration was repeated 5 times (maceration was performed for 24 hours, and the extract was filtered each time). The final extract was then filtered by a filter paper (Merck, Germany) and concentrated in a rotary evaporator (Heidolph, Germany). The mean of five weighing repetitions was recorded as the dry weight of the extract, and its concentration was calculated in milliliters.

Bacterial culture

Standard strains of P. gingivalis (ATCC 33277) and F. nucleatum (ATCC 2558) were obtained from Isfahan University of Medical Sciences. The Brucella agar basic medium (Merck, Germany) supplemented with 5% defibrinated sheep blood (Iran), 5 mg/L hemin, and 10 mg/L vitamin K1 (Sigma, Germany) was used for bacterial growth and proliferation. The culture plates were incubated under anaerobic conditions (80% N2, 10% H2, and 10% CO2) at 37°C for 48 hours.

For antimicrobial testing, an overnight culture of the bacteria was prepared in BHI broth; the bacteria were harvested by centrifugation, and a standard suspension of each bacterium was prepared in saline, containing 1.5 × 10^8 colony-forming units per milliliter (CFUs/mL). The turbidity of the microbial suspension was adjusted to 0.5 McFarland standard (OD at 600 nm of 0.08–0.1). Antimicrobial susceptibility testing was carried out using disk diffusion and broth microdilution methods in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (17).

Assessment of antibacterial activity by the disc-diffusion method

In this study, 2, 6, 9, 10, 12, and 15 mg/mL concentrations of the extract were prepared, and 50 µL of each concentration was inoculated on the discs; 3 to 5 minutes after complete absorption, the discs were incubated at 37°C to dry (18).

A total of 100 µL of a standard microbial suspension of each bacterial type was inoculated onto Mueller-Hinton agar containing 5% blood using the lawn culture method. Then, the discs were placed on the plate and incubated at 37°C under anaerobic conditions for 24 hours. The plates were then evaluated for the presence of growth-inhibition (halo) zones. The diameters of the growth inhibition zones around the discs were measured with a millimeter ruler.

Assessment of antimicrobial activity by the broth microdilution method

The sensitivity of each bacterial strain to S. officinalis extract was evaluated by the broth microdilution method using round-bottom 96-well microtiter plates (18). A total of 100 µL of different concentrations of the extract (2, 6, 9, 10, 12, and 15 mg/mL) prepared by dilution was added to each well, along with 100 µL of culture medium and 20 µL of a 0.5 McFarland standard-concentration bacterial suspension.

The positive control wells contained 100 µL of saline instead of the extract, while the negative control wells contained 20 µL of sterile saline instead of bacterial suspension.

The contents of each well were mixed in a Microplate Reader equipped with a shaker for 2 minutes, and the well contents were then analyzed by spectrophotometry at 620 nm to measure the optical density (OD) in triplicate.

The percentage of bacterial growth inhibition in the presence of different concentrations of the extract was calculated using the following formula: X=100-[OD×100)/ODc]

Where ODT is the mean optical density of the treatment wells, and ODc is the mean optical density of the positive control wells. Prior to this calculation, the OD

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of the negative control wells was subtracted from the OD of the other wells.

Each well was also evaluated to determine the minimum inhibitory concentration (MIC) of the extract. To determine the minimum bactericidal concentration (MBC) of the extract, the contents of the wells showing no bacterial growth were cultured on Mueller-Hinton agar. Inoculated culture plates were incubated at 37°C for 24 hours under anaerobic conditions. After incubation, the minimum concentration of the extract that completely inhibited bacterial growth was recorded as MBC. Finally, the percentage of bacterial growth inhibition at different concentrations of the extract was calculated, and MIC50, MIC80, and MBC were determined according to standard definitions.

The antibacterial activity was assessed using independent experimental replicates (plates). Each experimental condition (each concentration for each bacterial species) was tested in triplicate, meaning the sample size for each concentration-species group was $n = 3$independent replicates. Given the non-normal distribution of the data, the results are presented as the Median and Interquartile Range (IQR). The independence of the samples was maintained throughout the study, with *P. gingivalis* and *F. nucleatum* tested in separate units. Considering the non-normal distribution of data, comparisons were carried out using the Kruskal-

Wallis (for general comparisons) and Mann-Whitney (for pairwise comparisons) tests by SPSS version 24. The level of significance was set at 0.05.

Result

Table 1 compares the mean diameter of the growth inhibition zones for P. gingivalis and F. nucleatum caused by different concentrations of S. officinalis. The results showed that across all three concentrations of the extract, the mean diameter of the growth inhibition zones for P. gingivalis was significantly larger than that for F. nucleatum (P<0.001 for all three). Also, significant differences in the diameter of the growth inhibition zones were observed across different concentrations of the extract for each bacterial type (P<0.001 for both). Thus, pairwise comparisons of different concentrations of the extract are summarized in Table 2. For both bacterial species, the largest inhibition zones were observed at 15 mg/mL, with significant differences compared to lower concentrations (Table 2). The diameter of the growth inhibition zone for P. gingivalis was significantly larger than that for F. nucleatum (P<0.05). No growth inhibition zone was observed at the 2, 6, and 9 mg/mL concentrations of the extract. The 2, 6, and 9 mg/mL concentrations of the extract had no inhibitory effect on P. gingivalis, whereas the 10 and 12 mg/mL concentrations inhibited its growth by 47.25% and 82.5%, respectively. The difference between these two concentrations was statistically significant (P<0.05).

Table 1. Mean diameter of the growth inhibition zones (mm) for *P. gingivalis* and *F. nucleatum* caused by different concentrations of *S. officinalis* extract (Mann–Whitney U test)

Concentration (mg/mL)	*P. gingivalis* Mean ± SD	*F. nucleatum* Mean ± SD	P value
10	11.30 ± 1.63	6.98 ± 0.83	<0.001
12	13.00 ± 2.55	9.63 ± 0.60	<0.001
15	17.45 ± 2.35	15.13 ± 1.81	<0.001

Table 2. Pairwise comparisons of different concentrations of the extract regarding growth inhibition zones for each bacterial type using the Mann-Whitney test with Bonferroni adjustment

Extract concentration (mg/mL)	*P. gingivalis* Difference	*P. gingivalis* P value	*F. nucleatum* Difference	*F. nucleatum* P value
10-12	-8.500	0.112	-18.650	0.001
10-15	-30750	<0.001	-39.325	<0.001
12-15	-22.200	<0.001	-20.675	<0.001

The 2 and 6 mg/mL concentrations of the extract had no inhibitory effect on F. nucleatum, but the 9, 10, and 12

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mg/mL concentrations inhibited its growth by 31.45%, 62.4%, and 82.6%, respectively. The difference among the three concentrations was significant in this regard (P<0.05). Table 3 presents the mean OD of different concentrations of S. officinalis extract against P. gingivalis and F. nucleatum. The Kruskal-Wallis test showed a significant difference in OD between the different concentrations of the extract and the positive and negative control groups for P. gingivalis (P=0.003). Thus, pairwise comparisons were performed using the Mann-Whitney test. Table 4 presents pairwise comparisons of the ODs of the positive and negative control groups at different concentrations of the extract, separately for each bacterial type. As shown, the OD of the negative control group was significantly lower than that of the 2 mg/mL (P=0.001), 6 mg/mL (P=0.004), and 9 mg/mL (P=0.008) concentrations of the extract. However, the OD of the negative control group did not differ significantly from that of the 10 mg/mL (P=0.183), 12 mg/mL (P=0.418), and 15 mg/mL (P=0.954) concentrations of the extract, indicating low bacterial growth and optimal antibacterial activity at these concentrations. The OD of the positive control group did not differ significantly from that of the 2, 6, 9, and 10 mg/mL concentrations of the extract (P>0.05), indicating that these concentrations had no significant antibacterial activity.

Table 3. Mean optical density of different concentrations of *S. officinalis* extract against *P. gingivalis* and *F. nucleatum*

Concentration (mg/mL)	*P. gingivalis*	*F. nucleatum*
	Mean ± SD	Mean ± SD
2	0.805 ± 0.004	0.798 ± 0.012
6	0.802 ± 0.003	0.801 ± 0.008
9	0.802 ± 0.003	0.672 ± 0.012
10	0.615 ± 0.005	0.551 ± 0.002
12	0.475 ± 0.004	0.472 ± 0.006
15	0.404 ± 0.004	0.331 ± 0.002
Negative control	0.404 ± 0.005	0.404 ± 0.005
Positive control	0.975 ±0.003	0.800 ± 0.006
P value	0.003	0.003

Table 4. Pairwise comparisons of the optical density of the positive and negative control groups with different concentrations of the extract, separately for each bacterial type

Concentration (I)	Concentration (II)	*P. gingivalis*		*F. nucleatum*	
		Statistic	P value	Statistic	P value
Negative control	2 mg/mL	18.333	0.001	14.333	0.013
	6 mg/mL	16.500	0.004	15.333	0.008
	9 mg/mL	15.167	0.008	9	0.119
	10 mg/mL	7.667	0.183	6	0.298
	12 mg/mL	4.667	0.418	3	0.603
	15 mg/mL	0.333	0.954	-3	0.603
Positive control	2 mg/mL	7.667	0.183	-1	0.862
	6 mg/mL	5.833	0.311	0	1
	9 mg/mL	4.500	0.435	-6.333	0.272
	10 mg/mL	-3	0.603	-9.333	0.106
	12 mg/mL	-6	0.298	-12.333	0.032
	15 mg/mL	-10.333	0.073	-18.333	0.001

The Kruskal-Wallis test also revealed a significant difference in OD between the different concentrations of the extract and the positive and negative control groups for F. nucleatum (P=0.003, Table 3). Thus, pairwise comparisons were performed using the Mann-Whitney test (Table 4). As shown, the OD of the negative control group was significantly lower than that of 2 mg/mL (P=0.013) and 6 mg/mL (P=0.008) concentrations of the extract. Thus, these two concentrations had insignificant antibacterial activity. However, the 9 mg/mL (P=0.119), 10 mg/mL (P=0.298), 12 mg/mL (P=0.603), and 15 mg/mL (P=0.603) concentrations of the extract showed no significant difference compared with the negative control group, indicating optimal antibacterial activity. The OD of the positive control group was significantly higher than that of 15 mg/mL (P=0.001) and 12 mg/mL (P=0.032) concentrations of the extract but had no significant difference with the OD of 2 mg/mL (P=0.862), 6 mg/mL (P=1.00), 9 mg/mL (P=0.272), and 10 mg/mL (P=0.106) concentrations.

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The results showed that the 10 mg/mL concentration of the hydroalcoholic extract of S. officinalis inhibited 50% of the bacteria and thus had an MIC50 of 10 mg/mL. The 12 mg/mL concentration of the extract inhibited the growth of 80% of the bacteria. Thus, its MIC80 was 12 mg/mL. The 15 mg/mL concentration of the extract inhibited the growth of 100% of the bacteria. Thus, the MBC of the extract against the two bacteria was 15 mg/mL.

Discussion

Recent studies have highlighted the growing interest in plant-derived antimicrobial agents as potential adjuncts in periodontal therapy, particularly against key periodontal pathogens (19, 20, 21, 22, 23). Given the lack of information on the effects of S. officinalis on periopathogenic microorganisms, this study assessed the effects of different concentrations of the hydroalcoholic extract of S. officinalis on P. gingivalis and F. nucleatum in vitro. In this study, the antimicrobial activity of the extract was evaluated using two methods: disc diffusion and broth microdilution. Comparison of growth inhibition zone diameters is not a suitable method for assessing the antimicrobial effects of an extract on two bacterial types, as it is a qualitative technique that can only detect the presence or absence of antimicrobial activity (17). Thus, in the present study, the OD of extracts at different concentrations was also evaluated to compare their effects on the two bacterial types.

Conde-Hernández et al. (24) aimed to determine the best technique for obtaining S. officinalis extract and showed that different extraction methods from plant leaves affected the extract's antimicrobial properties. Delamare et al. (25) reported that the essential oil of S. officinalis at a concentration of 0.5 mg/mL exhibited bacteriostatic activity against Staphylococcus aureus. Haznedaroglu et al. (26) demonstrated that S. officinalis essential oil inhibited the growth of Gram-positive and Gram-negative bacteria except Pseudomonas aeruginosa.

In the present study, the 2, 6, and 9 mg/mL concentrations of the extract did not inhibit the growth of P. gingivalis, but the 9 mg/mL concentration inhibited the growth of F. nucleatum. Thus, the extract had a greater effect on F. nucleatum. The present results were consistent with previous findings on other pathogenic bacteria and confirmed the bactericidal and bacteriostatic effects of S. officinalis. The present study showed that S. officinalis extract at 10 and 12 mg/mL had MICs of 50 and 80, respectively, and an MBC of 15 mg/mL. The present results were consistent with the findings of previous studies. According to Holetz et al (27), MIC < 100 mg/mL indicates optimal antibacterial activity of the extract, values between 100 and 500 mg/mL indicate moderate antimicrobial activity, values between 500 and 1000 mg/mL indicate mild antimicrobial activity, and values higher than 1000 mg/mL indicate no antibacterial activity. The hydroalcoholic extract of S. officinalis was evaluated in the present study; however, Mandes et al. (28) evaluated the effects of the S. officinalis dichloromethane crude extract, its dichloromethane-soluble fractions, and its pure substances against periopathogens, including P. gingivalis, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, and F. nucleatum. Similar to the present study, they determined the MIC of the extract by the broth microdilution method using 96-well plates. They reported that the S. officinalis dichloromethane extract and its fractions had the lowest MIC values of 50-400 µg/mL, while the pure substance, manool, showed MIC values ranging from 3-50 µg/mL against different periopathogens. Overall, these values were lower than those obtained in the present study, indicating more satisfactory antibacterial activity of the pure substance manool (27). Doudi et al. (29) evaluated the antibacterial effects of the ethanolic extract of S. officinalis against Staphylococcus aureus, Escherichia

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coli, Pseudomonas aeruginosa, and Klebsiella pneumonia using the disc diffusion and broth microdilution methods. They reported that the MICs of the alcoholic extract of S. officinalis against Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Escherichia coli were 18.75, 33.75, 31.25, and 26.56 mg/L, respectively. Since they evaluated different types of bacteria, whereas the present study focused on S. officinalis, it may be concluded that S. officinalis has an inhibitory effect on a wide range of bacteria. Also, its alcoholic extract has a stronger effect on P. gingivalis and F. nucleatum (29). Kermanshah et al. (30) assessed the effects of the hydroalcoholic extract of S. officinalis on Streptococcus mutans, Lactobacillus rhamnosus, and Actinomyces viscus, cariogenic bacteria, using the broth microdilution method. They reported the MICs of the hydroalcoholic extract of S. officinalis to be 6.25, 1.56, and 12.5 µg/mL for the three bacteria mentioned above, respectively, which were close to the values obtained in the present study (30). Sookto et al. (31) reported the MIC and MBC of S. officinalis extract against Candida albicans to be 2.7 mg/mL, which was lower than the value obtained for two periopathogenic microorganisms in the present study.

In general, Gram-positive bacteria are more sensitive to S. officinalis essence than other bacteria. Izadi and Mirazi (32) found the maximum and minimum diameters of the growth inhibition zones for Staphylococcus aureus and Pseudomonas aeruginosa, respectively, in the presence of a 300 mg/mL concentration of the extract. The MIC of S. officinalis against the Gram-positive and Gram-negative bacteria ranges from 16 to 256 mg/mL. Alfahdawi et al. (33) evaluated the effect of S. officinalis extract against different pathogenic bacteria compared with a chemical mouthwash using the Mueller-Hinton agar method. They reported that pure S. officinalis essential oil and its alcoholic extract had greater inhibitory effects on the bacteria, whereas its aqueous extract showed no significant antibacterial activity. Although they evaluated different types of bacteria, their results on the overall antimicrobial efficacy of S. officinalis were consistent with the present findings. Eghbal et al. (34) assessed the efficacy of S. officinalis at 2, 6, 9, 12, and 15 mg/mL, compared with chlorhexidine, against common oral infections by measuring growth inhibition zones and determining MICs. Their results were in line with the present findings, despite evaluating different types of bacteria. Thus, different concentrations of S. officinalis extract may serve as an alternative to chemical antimicrobial mouthwashes (34). Beheshti-Rouy et al. (35) studied the effect of aqueous extract of S. officinalis in the form of a mouthwash on Streptococcus mutans colony count and indicated a reduction in bacterial colony count after using the mouthwash. Shahriari et al. (18) assessed the effect of pure S. officinalis powder at four different volume percentages on Streptococcus mutans and Lactobacillus by measuring the size of growth inhibition zones. They concluded that this powder had antimicrobial effects on both bacteria, but was more active against Streptococcus mutans. Differences in MIC values of S. officinalis reported in the literature may be due to differences in the timing of plant harvesting, the method of preparing the extract, storage conditions for the culture media, and the type of bacterial strains evaluated. The cytotoxicity or biocompatibility of sage was not evaluated in this study. Future studies are recommended to address this topic.

Although the present study was conducted in-vitro, the findings provide preliminary insights into the potential clinical applicability of *Salvia officinalis* extracts in periodontal therapy. The demonstrated antibacterial activity against key periopathogenic bacteria suggests that *S. officinalis* may serve as a candidate for use in adjunctive antimicrobial formulations such as mouthwashes, gels, or local delivery systems. To enable

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clinical translation, further investigations, including cytotoxicity assays, biofilm-based models, animal studies, and well-designed clinical trials, are required to determine optimal concentrations, safety, and therapeutic efficacy under in vivo conditions.

Conclusion

The hydroalcoholic extract of S. officinalis showed optimal antimicrobial activity against P. gingivalis and F. nucleatum periopathogens. Its MICs were 10 and 12 mg/mL, and its MBC was 15 mg/mL in vitro.

Conflict of Interests: The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial, in this article

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