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Antibacterial Activity of Nanoemulsions Based on Essential Oils Compounds Against Species of *Xanthomonas* that Cause Citrus Canker

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Abstract: Citrus canker is a bacterial disease that affects citrus crops, caused by microorganisms of the genus *Xanthomonas* responsible for great damage to citrus production. In this work, the antimicrobial effect of nanoemulsions based on the major compounds of essential oils (EOs) eugenol, thymol, geraniol and menthone were investigated against two strains of *Xanthomonas* that cause citrus canker (Xac and XauB). Nanoemulsions were produced for each compound using two different stirring speeds (7,000 and 12,000 rpm). All nanoemulsions underwent analysis to characterize particle sizes and stability. Thymol nanoemulsions had the smallest particle sizes (59.8 to 73.9 nm) and the highest stability and also showed the ability to inhibit the development of both strains of bacteria, with minimum inhibitory concentrations (MIC) of 0.03% (v/v), along with eugenol (0.03% (v/v) for Xac and 0.02% (v/v) for XauB) and geraniol nanoemulsions (0.06% (v/v) for both bacteria). Thymol was the compound with the highest minimum bactericidal concentrations (MBC), with values of 0.03% (v/v) for both bacterial strains. Eugenol showed MBC only against XauB, at a concentration of 0.03% (v/v). Geraniol did not show bactericidal activity, suggesting a bacteriostatic action of this compound with the tested microorganisms. In general, the antimicrobial activity of the nanoemulsions was increased with increased agitation speed, particle reduction and greater stability. Therefore, nanoemulsification can be an alternative to applying the antimicrobial activity of natural compounds in the control of citrus canker.

Keywords: citrus canker; *Xanthomonas citri* subsp. *citri*; *Xanthomonas fuscans* subsp. *aurantifolii* type B; eugenol; thymol.

1. Introduction

Citriculture is one of the most important agribusiness sectors worldwide, being a decisive branch of the economy of several countries and an important machine for generating jobs [1]. This phenomenon happens because, in addition to their consumption in nature, citrus fruits are also consumed through their processed products, such as juices and sweets [2]. In the

last decade, several diseases have been responsible for causing numerous losses to the citrus industry, such as citrus variegated chlorosis (CVC), huanglongbing or greening (HLB), sudden citrus death (CSD) and citrus canker [3-5].

Citrus canker, in particular, is a disease with a high impact on the global citrus industry, which can cause defoliation and premature fall of infected fruits [6]. It is caused by Gram-negative bacteria species of genus *Xanthomonas* [7], which can cause three main types of the disease: Canker A, caused by *X. citri* subsp. *citri* (Xac); Canker B, caused by *X. fuscans* subsp. *aurantifolii* type B (XauB); and Canker C, caused by *X. fuscans* subsp. *aurantifolii* type C (XauC). The host species may differ for each type, but the symptoms presented by the infected fruits are, in general, similar [8, 6].

There are still no curative methods for the treatment of citrus canker [9-10]. Prevention for places with no record of the disease is carried out by eradicating infected plants [11]. Where citrus canker is endemic, copper-based antimicrobial compounds are sprayed to control the disease [12]. However, in addition to not being a totally efficient measure, the use of copper agrochemicals can cause damage to human health and the environment in the long term [13]. There are also records of *Xanthomonas* species resistant to copper-based antibacterials [14-15].

In order to prevent major damage to agriculture crops caused by phytopathogenic microorganisms, the use of techniques that take advantage of natural and biodegradable alternatives has been extensively studied in recent years [16-17]. An example of this is the use of essential oils (EOs) and other plant extracts, which can provide antimicrobial activity due to their vast bioactive compounds [18, 16]. It has been reported that the use of these compounds isolated instead of the entire essential oils increases their action as potential antimicrobial agents [19]. Thymol (the major compound of thyme oil), eugenol (abundant in clove oil), geraniol (found in palmarosa oil) and menthone (a component of the essential oil of peppermint) are only a few of the natural compounds from EOs known to possess antimicrobial properties [20-21].

To be applied to fruits, however, it is necessary to create emulsions of EO's components in order to guarantee their function [22]. In this sense, nanotechnology is a possible ally to enhance the antimicrobial effects of natural compounds [22-23]. When being nanoemulsified, their physical-chemical characteristics are better preserved and their dispersion across biological membranes is greater [24].

Thus, to satisfy the increasing demands of consumers for sustainability and help agriculture have a less negative impact on the environment, alternatives to the overuse of agrochemicals must be found. Therefore, the present study aimed at the association of thymol, eugenol, geraniol and menthone with nanotechnology to investigate their antibacterial potential in the control of citrus canker.

2. Materials and Methods

2.1. Materials.

Thymol (lot T0501), eugenol (lot E51791), geraniol (lot 163333) and menthone (lot 95401) were obtained from Sigma-Aldrich Brasil Ltda. The bacterial strains used in the study were Xac strain 306 [25] and XauB strain ICPB11122 [8]. Both bacteria were gently given by the Laboratory of Applied Biochemistry and Molecular Biology (LBBMM) from the Department of Genetics and Evolution of Federal University of São Carlos (UFSCar), São

Paulo, Brazil. Tween 80 (ethoxylated sorbitan monooleate) was obtained from Labsynth Brasil Ltda.

2.2. Inoculum preparation.

The inoculum of both bacteria was prepared using Petri dishes with solid Luria-Bertani (LB) medium incubated for 3 days at 28ºC. Approximately two colonies isolated from each microorganism were collected and transferred to 50 mL of sterile LB liquid culture medium, kept under agitation at 180 rpm and 28ºC for 24 hours. After this period, an aliquot of 1 mL of each culture was collected and read in a spectrophotometer at a wavelength of 625 nm. The absorbance reading was compared with the reading of a solution corresponding to the 0.5 points on the McFarland Scale (approximately 1.5 x 10^8 CFU/mL). Then, dilutions were made in liquid LB to obtain a standardized inoculum for each bacterium with a 2 x 105 CFU/mL concentration. The concentration was confirmed by plating 100 µL of the inoculum in a solid LB medium with a sterile Drigalski loop [26-27].

2.3. Nanoemulsions preparation and characterization.

Each compound was diluted in sterile distilled water in order to obtain the final concentrations of 2% (v/v) for geraniol, 2% (w/v) for thymol and 1% (v/v) for eugenol and menthone. Tween 80 (0.05% v/v) were added as an emulsifying agent [28].

Each sample of thymol (NET), eugenol (NEE), geraniol (NEG) and menthone (NEM) was stirred for 4 min in Ultra-Turrax at two different speeds: 7,000 and 12,000 rpm, at room temperature, totaling 8 treatments.

The nanoemulsions were characterized with Zetasizer Nano ZS, from Malvern Panalytical. Particle size (in nm) and the polydispersity index (PDI) were measured. Three aliquots were collected from each sample and read at 0 and 24h to verify the stability of the nanoemulsions. Between readings, all samples were kept refrigerated at 4 ºC in a container with a barrier to the entry of light [29].

2.4. Minimum inhibitory concentration (MIC) assay.

In a sterile 96-well plate, 50 µL of liquid LB medium was deposited in each well, followed by the addition of 50 µL of bacterial inoculum at 2 x 10^5 CFU/mL, in order to obtain a final concentration of 10^5 CFU/mL in all wells, with a final volume of 100 µL. The two bacteria were tested separately. Then, in the wells of the first row, each nanoemulsion was added in the volume of 100 µL, reducing its initial concentration by half. Then, a serial dilution was performed, transferring 100 µL from the initial well to the subsequent wells in the vertical direction of the plate. Thus, all NEG and NET treatments were tested at the final concentrations of 1, 0.5, 0.25, 0.13, 0.06, 0.03, 0.02 and 0.01% (v/v), and the NEE and NEM treatments received the final concentrations of 0.5, 0.25, 0.13, 0.06, 0.03, 0.02, 0.01 and 0.004% (v/v). All nanoemulsions were tested in triplicate. Controls were also performed with the addition of distilled water at 1% (v/v) and Tween 80 at 0.05% (v/v). The plates were incubated, without shaking, at 28ºC for 24h [30].

After the incubation period, 30 µL of a 0.1% (w/v) solution of the 2,3,5-triphenyltetrazolic chloride reagent was added to each well of the microplates, returning them to the incubator for 30 min. This reagent is a dye that interacts with enzymes involved in cellular respiration to detect the wells in which there is still metabolic activity (reddish color).

Thus, the MIC was defined as the lowest concentration in which no color was visible in the corresponding well [31].

2.5. Minimum bactericidal concentration (MBC) assay.

The minimum bactericidal concentration was investigated by plating in solid LB medium 100 µL for each repetition of the wells in which MIC was defined and subsequent. The spreading of the aliquot was done using a sterile Drigalski loop. Negative controls were also plated. All plates were incubated at 28ºC for 24h. MBC was defined as the lowest sample concentration in which there was no visible microbial growth in the Petri dish [30].

2.6. Statistical analysis.

Data from nanoemulsions characterization were first analyzed using two-way ANOVA analysis of variance in triplicate. When necessary, the differences between treatments were determined using the Tukey Test ($p < 0.05$) for the data referring to the agitation speeds within each treatment and the Student T-Test ($p < 0.05$) for comparison between the readings at 0 and 24h.

3. Results and Discussion

3.1. Characterization of nanoemulsions.

The average values of the characterizations of the nanoemulsions are described in Table 1. Most of them were able to decrease the size of the particles (z-average) from the first stirring speed (7,000 rpm) to the second (12,000 rpm). This result coincides with the data already described in the literature. Otani *et al*. [29] used these same speeds to produce cinnamaldehyde nanoemulsions and obtained values more than twice as low in the rotation of 12,000 rpm compared to 7,000 rpm. This happens due to the fact that processes that demand high energy create deforming forces that overcome the pressure of Laplace and break the particles into smaller sizes [31].

Table 1. Characterization results of eugenol (NEE), geraniol (NEG), thymol (NET) and menthone (NEM) nanoemulsions. The sizes (z-average, in nm) and the polydispersion indices (PDI) of the particles are shown for each stirring speed used. Readings were done 0 and 24 hours after sample preparation.

Sample	Stirring speed (rpm)							
	7.000				12.000			
	0h		24h		0h		24h	
	z-average (nm)	PDI	z-average (nm)	PDI	z-average (nm)	PDI	z-average (nm)	PDI
NEE	190.2^{aB}	0.49^{aA}	270.6^{aA}	0.90^{aB}	131.2^{bA}	0.02^{bA}	137.5^{bA}	0.28^{bB}
NEG	98.7^{aB}	0.17^{aA}	150.0^{aA}	0.98^{aB}	99.4^{aB}	0.10^{bA}	156.2^{aA}	0.13^{bB}
NET	91.1^{bA}	0.06^{aA}	91.7^{aA}	0.58^{aA}	59.8^{cA}	0.26^{bA}	73.9^{bA}	0.14^{bB}
NEM	299.6^{bA}	0.86^{bA}	612.7^{bB}	0.55^{bA}	169.2^{cA}	0.94^{aA}	325.4^{bB}	0.74^{aB}

$^{a-c}$ Different letters indicate statistically relevant differences between stirring speeds within each treatment using the Tukey test ($p < 0.05$)

A-B Different letters indicate statistically relevant differences between the times 0 and 24h by the Student T-Test ($p < 0.05$)

The treatments with geraniol were the only ones that did not show a relevant reduction in the size of the particles, without any statistically significant difference between the averages

of the values presented for each of the two agitation speeds, which varied from 98.7 to 156.2 nm. This may be due to multiple factors, such as the need for a longer stirring time or the addition of different surfactants. Previous studies have shown that a longer stirring time can produce geraniol nanoemulsions with particle sizes of 68 nm in diameter [32].

In addition, the particle size and stability of an emulsion are related to the choice of the emulsifier, both because of its composition in relation to the active component and because of its hydrophilic-lipophilic balance (HLB) value, which represents the size and strength of the hydrophilic and lipophilic portions of the emulsifier molecule [33]. Therefore, the use of emulsifiers with HLB values different than that presented by Tween 80 (HLB 15) may be an alternative for the production of geraniol nanoemulsions with reduced particle sizes.

The smallest particle sizes were obtained at the second speed (12,000 rpm), ranging from 59.8 to 73.9 nm for NET treatments. These were also the treatments with the most stable z-average values, with no significant differences after 24h. This greater stability is directly related to the size of the particles since the lower the z-average values in a nanoemulsion, the more stable it shows over time [34]. A study by Su & Zhong [35] performed a test in which nanoemulsions containing 2% thymol (w/v) were produced by stirring in Ultra-Turrax for 2 minutes at 10,000 rpm, obtaining z-average values similar to that observed in the present work (from 38.67 to 58.08 nm).

The largest particle diameters were those of NEE (270.6 nm) and NEM (621.7 nm) treatments, at 7,000 rpm. These were also the most unstable samples after 24h, with statistically significant differences in values. This happens because there is a correlation between the particle size and the stability of the nanoemulsion. When the particles are larger, there are chances that sedimentation will occur during storage, along with coalescence [34].

Multiple studies with characterizations of nanoemulsions containing eugenol demonstrated results similar to the z-average values found for NEE. Hu *et al*. [36] obtained particles of 103.6 nm when producing nanoemulsions containing 1.25% eugenol (v/v) using different techniques and natural emulsifiers. In another study, Wang & Zhang [37] produced eugenol nanoemulsions (1% v/v) using different stirring methods, reaching z-average values of up to 158.4 nm in diameter. There are few reports in the literature about menthone-based nanoemulsions. However, a study by Liang *et al*. [38] used peppermint essential oil (rich in menthone) to produce nanoemulsions by stirring in Ultra-Turrax for 1 minute at 24,000 rpm, obtaining nanoparticles in the 200 nm diameter range, which corresponds to the values found in this study for the treatment of NEM. Yet, all results demonstrate that treatments with eugenol (NEE) and menthone (NEM) are particularly unstable, making its long-term use impracticable. For this to be corrected, further tests are needed to include other emulsifying agents and validate different methods of producing nanoemulsions.

The polydispersion index (PDI) showed a great distinction between the studied nanoemulsions. The statistical analysis pointed out relevant differences between the data obtained in the reading of 0 and 24h. This is related to the fact that the particles tend to agglomerate and form precipitates, which modifies the initial homogeneity of the sample [34]. In general, the PDI has a behavior dependent on the stability of the sample and the size of the particles: the highest values of PDI obtained were from the NEG (0.98) and NEM (0.94) treatments, which also showed high values of z-average. On the other hand, the one who presented low values of z-average also had smaller and less variable PDIs: NET (0.06). These results coincided with what was observed in a study by Pongsumpun *et al*. [39], in which the comparison of two methods for obtaining cinnamon essential oil nanoemulsions revealed that

the methodology responsible for obtaining the smallest particle diameters also produced the lowest PDI values. The only treatment that did not follow this pattern was NEE, which also demonstrated one of the lowest PDI values (0.10), but had relatively high z-average values. This may indicate that the surfactant used had a more stable interaction with eugenol than with the other treatments in which the z-average values were also high.

3.2. Minimum inhibitory concentration (MIC) assay.

The results for the tests of minimum inhibitory concentration (MIC) with Xac and XauB are described in Table 2. The thymol nanoemulsions (NET) were the ones that obtained the best result, with MIC of 0.03% for Xac in the sample with rotation of 12,000 rpm, and MIC of 0.02% for XauB also at 12,000 rpm.

Table 2. Results for the minimum inhibitory concentration (MIC) assay with *Xanthomonas citri* subsp. *citri* (Xac) and *Xanthomonas fuscans* subsp. *aurantifolii* (XauB). The concentrations of eugenol (NEE), geraniol (NEG), thymol (NET), menthone (NEM) present in their respective nanoemulsion are described in percentage (v/v), in the two stirring speeds used (7,000 and 12,000 rpm).

Sample	Stirring speed (rpm)			
	Xac		XauB	
	7.000	12.000	7.000	12.000
NEE	0.06 %	0.03 %	0.13 %	0.03 %
NEG	0.06 %	0.06 %	0.06 %	0.06 %
NET	0.06 %	0.03 %	0.13 %	0.02 %
NEM	> 0.5 %	> 0.5 %	> 0.5 %	> 0.5 %
Negative control	*	*	*	*
Tween 80	*	*	*	*

* There was no MIC in the concentrations tested.

These values coincide with a study by Nostro *et al*. [40] where the antimicrobial activity of thymol, carvacrol and oregano essential oil was tested against several strains of *Staphylococcus aureus*, obtaining MIC values between 0.03% and 0.06% (v/v). Kotan *et al*. [41], when evaluating the activity of multiple natural compounds against *Xanthomonas axonopodis* pv. *vesicatoria*, also obtained the lowest MIC in the treatment using thymol. In addition, some studies involving the use of essential oils having thymol as a major compound have already shown an antagonistic behavior of this compound against species of the genus *Xanthomonas* [42-43].

The high antibacterial activity of thymol is well described in the literature [44]. Banti *et al*. [45] tested the activity of essential oils containing thymol as the main constituent, finding antibacterial action even against strains resistant to antibiotics. Although the action of thymol against Xac and XauB has not yet been explored in the literature, the results of this study are consistent with what has been reported for other species of the genus *Xanthomonas* [42]. Da Silva *et al*. [43], when evaluating the cell viability of *Xanthomonas campestris* pv. *campestris* when exposed to thymol, reported damage to the cytoplasmic membrane that is probably related to the antibacterial mechanism of this compound.

The second best treatment was NEE, with MIC of 0.03% (v/v) at the rotation of 12,000 rpm for both bacteria tested. Marchese *et al*. [46] compared the activity of eugenol against several bacterial strains, finding MICs from 0.1 to 0.01% (v/v) against Gram-negative bacteria, which coincides with the results of the present study. Sauer *et al*. [47], when testing the anti-Xac activity of *Ocimum gratissimum* L. essential oil (containing 42.7% eugenol), obtained MIC

of 0.43 µL/mL. Considering the percentage of eugenol present in the oil, it is expected that the isolated compound has a lower MIC against Xac, as occurred in this work.

Geraniol treatments (NEG) showed the least activity, with MIC of 0.06% (v/v) in all rotations for Xac and XauB. Reichling *et al*. (2006) has already reported the antibacterial activity of geraniol in similar concentrations, with MIC of 0.05% (v/v) for *Escherichia coli* and 0.06% (v/v) for *Staphylococcus aureus*. Mirzaei-Najafgholi *et al*. [48] demonstrated that geraniol is able to inhibit the development of Xac at a concentration of 0.09% (v/v). As with eugenol, there have been no reports of anti-XauB tests using geraniol.

With the exception of geraniol treatments, all other nanoemulsions had an increase in the antimicrobial activity of the rotation from 7,000 rpm to 12,000 rpm. This phenomenon is consistent with what is described in the literature. Mutlu-Ingok *et al*. [49], when performing tests of antimicrobial activity with nanoemulsions of essential oils with different values of z-average, obtained an inverse relationship between the average size of the suspended particles and the antibacterial action. That is, the lower the z-average value, the greater the activity of the nanoemulsion. The same was reported by Otani *et al*. [29] in trials involving cinnamaldehyde nanoemulsions against *E. coli*, *S. aureus*, *L. monocytogenes* and *S. enterica*. In this work, treatments with smaller particle sizes showed the greatest inhibition halos.

This happens because the nanoparticles increase the sample's contact surface with the microorganism, improving the accessibility of bioactive compounds and their ability to enter cell membranes [34]. There are studies, however, that report a low influence of the size of the particles on the antimicrobial action, which is more susceptible to the components of the nanoemulsion and its compositions. For this reason, nanoemulsions with different z-average can have similar antimicrobial activities [50-51]. There are already some studies proving the effectiveness of nanoemulsions against species of the genus *Xanthomonas*, including using thymol as a bioactive component [52].

There was no MIC for NEM treatments. Menthone was tested at a reduced concentration, which may explain the lack of activity since its antimicrobial action has already been reported in concentrations above 2% (v/v) [53].

Among the three compounds that presented MIC, the treatments with thymol (NET) were more efficient against XauB than Xac, with concentrations of 0.03% (v/v) for Xac and 0.02% (v/v) for XauB at the same speed (12,000 rpm). Although not very significant, this difference may indicate that this compound acts differently for each species.

Studies have shown that Xac and XauB have differences in the protein composition of the periplasm region that give them different spectra of hosts and levels of pathogenicity, with Xac being a more virulent species than XauB [54]. Therefore, such distinctions in bacterial morphology may be related to the membrane permeability mechanisms through which thymol acts, allowing it to interact closer with XauB than Xac.

3.3. Minimum bactericidal concentration (MBC) assay.

The results for the tests of minimum bactericidal concentration (MBC) with Xac and XauB are described in Table 3. As with the MIC values, the treatments that presented the most MBCs were the nanoemulsions containing thymol (NET), being 0.03 % for both bacteria at 12,000 rpm and there was no MBC for the rotation of 7,000 rpm.

Table 3. Results for the test to assess the minimum bactericidal concentration (MBC) with *Xanthomonas citri* subsp. *citri* (Xac) and *Xanthomonas fuscans* subsp. *aurantifolii* (XauB). The concentrations of eugenol (NEE), geraniol (NEG) and thymol (NET) present in their respective nanoemulsion are described in percentage (v/v), in the two stirring speeds used (7,000 and 12,000 rpm).

Sample	Stirring speed (rpm)			
	Xac		XauB	
	7.000	12.000	7.000	12.000
NEE	> 0.5 %	> 0.5 %	> 0.5 %	0.03 %
NEG	> 1 %	> 1 %	> 1 %	> 1 %
NET	> 1 %	0.03 %	> 1 %	0.03 %
Negative control	*	*	*	*

* There was no MBC in the concentrations tested.

These results reflect that thymol inhibitory activity is related to bactericidal activity, since both MIC and MBC values had equal or very close concentrations. Nostro *et al*. [40], when evaluating the bactericidal activity of thymol against species of the *Staphylococcus* genus, obtained similar MBC values, ranging from 0.031 to 0.125% (v/v). However, the bactericidal action only occurred in treatments where the z-average was lower in the present study. This coincides with what was reported in a study by Sarwar *et al*. [55], where the evaluation of the bactericidal activity of chitosan nanoparticles against different Gram-positive and Gram-negative bacteria demonstrated lower MBC results for treatments with smaller particle sizes.

In the treatments with eugenol (NEE), only the bacterium XauB was susceptible, presenting the MBC of 0.03% in the rotation of 12,000 rpm. This difference in behavior between the two species is an expected phenomenon since there are considerable distinctions in each bacterium's morphology that can influence how the bioactive compound interacts with the cell membrane. Zandonadi *et al*. [54], when evaluating the periplasmic proteome of different strains of *Xanthomonas* that cause citrus canker, observed that there are distinctions in the proteins present in this region. Thus, even though the MIC values were the same, the bactericidal activity of eugenol appears to have mechanisms that are more effective against XauB than Xac, although Xac is, in general, more virulent.

There was no bactericidal action in the other rotations, nor for the treatments with geraniol (NEG). This absence of activity may indicate that the interaction of the compounds with the bacteria occurs through bacteriostatic mechanisms. That is, even though the MIC values demonstrate an inhibitory potential, only concentrations above those that have been tested may actually be able to kill the bacterial cells. Bajpai *et al*. [56], when testing the essential oil and extracts from *Cleistocalyx operculatus* against *Xanthomonas* species, found MBC concentrations above the values presented by the MIC assay. The different mechanisms through which the bioactive compound interacts with the microorganism can also influence the bactericidal action. Silva *et al*. [57], when evaluating the antimicrobial potential of different essential oils against *S. aureus* and *E. coli*, obtained MIC concentrations that could or might not be bactericidal, depending on the species of bacteria.

4. Conclusions

The antimicrobial activity was shown to be greater as the particle size of the nanoemulsions decreased. Nanoemulsions with a lower z-average are more stable and enhance the bioactivity of natural compounds of EOs. Among the studied compounds, those that showed the best efficiency against the tested bacteria were thymol and eugenol. Although it also

showed low concentrations of MIC, geraniol did not obtain MBC results, which demands the need for new tests using higher concentrations of the compound. Most compounds showed bacteriostatic activity, with only thymol and eugenol being able to prevent bacteria development completely. Satisfactory results of bacterial inhibition were obtained in all low concentrations used, which suggests the use of small volumes of these components in possible large-scale applications. Future tests are needed to assess the *in vivo* toxicity of thymol and eugenol nanoemulsions in citrus fruits and encapsulation studies (spray dryer) for greater protection of the active principle.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the study's design, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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