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RESEARCH ARTICLE

Antibiofilm Effect of *Moringa oleifera* Leaf Extract Against *Staphylococcus aureus*, Cytotoxicity, Biochemical aspects, Anti-Inflammatory potential, and Interference on the Activity of Antimicrobial Drugs

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Antibiofilm Effect of Moringa oleifera Leaf Extract Against Staphylococcus aureus, Cytotoxicity, Biochemical aspects, Anti-Inflammatory potential, and Interference on the Activity of Antimicrobial Drugs

SUMMARY

There is a growing technical difficulty in treating infectious diseases due to bacterial resistance to antimicrobial drugs, such as biofilm formation. Here we provide evidence of the antimicrobial potential of the hydroethanolic extract of Moringa oleifera leaves, traditionally used to treat disorders like cardiovascular and endocrine diseases, on clinical isolates of Staphylococcus aureus. The plant extract was chemically characterized using classic techniques and by ultra-performance liquid chromatography (UPLC). We carried out minimum inhibitory concentration tests, minimal bactericidal concentration and minimal biofilm eradication concentration tests. Moreover, we tested the anti-inflammatory potential and assessed the toxicity of the extract on buffalo green monkey (BGM) cells. We also investigated the effects of combining the extract with clinically relevant antimicrobial drugs (i.e., synergistic or antagonistic interactions). The extract was active at 8 μg/mL and 16 μg/mL for planktonic cells and biofilms, respectively. Its anti-inflammatory potential was confirmed, and it lacked cytotoxicity. No significant interference of the extract on antimicrobial drugs was observed. Flavonoids, tannins, proteins, carbohydrates and vitamin C were detected in the extract. Our data open doors for further studies with isolated molecules of the extract in order to conduct in vivo antimicrobial tests.

Key Words: *Moringa oleifera, Staphylococcus aureus, antimicrobial, biofilms.*

Moringa oleifera Yaprağı Ekstresinin Staphylococcus aureus'a Karşı Antibiyofilm Etkisi, Sitotoksisitesi, Biyokimyasal Yönleri, Anti-inflamatuar Potansiyeli ve Antimikrobiyal İlaçların Aktivitesi Üzerinde Etkileşimi

ÖZ

Biyofilm oluşumu gibi antimikrobiyal ilaçlara karşı bakteriyel direnç nedeniyle infeksiyon hastalıklarının tedavisinde teknik zorluklar artış göstermektedir. Burada, geleneksel olarak kardiyovasküler ve endokrin hastalıklar gibi bozuklukları tedavi etmek için kullanılan Moringa oleifera yapraklarının sulu etanol ekstresinin Staphylococcus aureus'un klinik izolatları üzerindeki antimikrobiyal potansiyeline dair kanıtlar sunuyoruz. Bitki ekstresi, klasik teknikler ve ultra performanslı sıvı kromatografisi (UPLC) kullanılarak kimyasal olarak karakterize edilmiştir. Minimum inhibitör konsantrasyon testleri, minimum bakterisidal konsantrasyon ve minimum biyofilm eradikasyon konsantrasyon testleri gerçekleştirilmiştir. Ayrıca, anti-inflamatuar potansiyeli test ettik ve ekstrenin bufalo yeşil maymunu (BGM) hücreleri üzerindeki toksisitesini değerlendirdik. Ekstreyi klinik olarak ilgili antimikrobiyal ilaçlarla kombine etmenin etkilerini (yani sinerjistik veya antagonistik etkileşimler) de araştırdık. Ekstre, planktonik hücreler ve biyofilmler için sırasıyla 8 μg/mL ve 16 μg/mL'de aktif bulundu. Anti-inflamatuar potansiyeli doğrulandı ve sitotoksik değildi. Ekstrenin antimikrobiyal ilaçlar üzerinde önemli bir etkileşimi gözlenmedi. Ekstrede flavonoidler, tanenler, proteinler, karbonhidratlar ve C vitamini tespit edildi. Verilerimiz, in vivo antimikrobiyal testler yapmak için ekstraktın izole edilmiş molekülleri ile daha ileri çalışmalar için kapılar açmaktadır.

Anahtar Kelimeler: *Moringa oleifera, Staphylococcus aureus, antimikrobiyal, biyofilmler.*

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INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterial species that can be both commensal on the mucosa and skin of humans and an opportunistic pathogen (Krismer et al., 2017). It causes foodborne infections and diseases such as otitis, osteomyelitis, endocarditis, septicemia and mastitis, which also affect milking animals (Abril et al., 2020; Miller et al., 2020). *S. aureus* is among the most common pathogens detected in hospital and community infections, and is associated to elevated mortality rates due to antimicrobial resistance (Kwiecinski et al., 2020; Guo et al., 2020). The main mechanisms of resistance found in this species include enzymatic inactivation of antimicrobials, biochemical alterations that decrease the affinity of microbial molecular targets to the drugs, and biofilm formation (Guo et al., 2020; Miller et al., 2020).

Biofilms are microbial microcolonies that develop in an adhesive matrix of extracellular polymeric substances (EPS), generally composed of carbohydrates, proteins and lipids (Penesyan et al., 2021). Biofilms are polymicrobial in humans, animals, and in most ecosystems. The biochemical composition of the EPS is influenced by factors that comprise the amount and diversity of adhered microbial species, physical and chemical characteristics of the surface where the biofilm is developing, and nutrient availability (Rumbaugh and Sauer, 2020; Penesyan et al., 2021). Biofilms act as physical and chemical barriers to the diffusion and action of antimicrobials (Koo et al., 2017; Brito, 2021). As a result, large amounts of drugs are necessary for prolonged periods, posing risks of adverse reactions such as nephrotoxicity, ototoxicity, and extended bacterial resistance (Rumbaugh and Sauer, 2020; Maslova et al., 2021). Curiously, despite the exposed, the antibiofilm effect of potential antimicrobials of natural sources are not explored as for planktonic (free) cells.

Natural products are relevant sources of antimicrobial molecules, for which reports of microbial resistance are rare. *Moringa oleifera* is traditionally used to treat different disorders such as hypertension and diabetes (Mabrouki et al., 2020). The leaves of the plant are the most consumed part in several countries, due to their high levels of carotenoids, vitamins and minerals like calcium and iron (Leone et al., 2015; Stohs and Hartman, 2015; Dhakad et al., 2019). Different biological properties of *M. oleifera* have been described, including immunomodulatory, hepatoprotective and neuroprotective (Dhakad et al., 2019; Fernandes et al., 2021). Nevertheless, some questions remain unanswered concerning the antimicrobial potential of *M. oleifera* extracts, such as antibiofilm potential and effects when combined to antimicrobial drugs.

Here we show that *M. oleifera* leaf extract (MOLE) is effective against planktonic cells and biofilms of clinical isolates of *S. aureus* and investigated its anti-inflammatory potential. We also investigated its cytotoxicity and its influences on the activity of antimicrobial drugs. Biochemical and ultraperformance liquid chromatography (UPLC) analysis confirmed the presence of polyphenols, proteins, carbohydrates and vitamin C. Our study opens doors for more investigations towards the use of *M. oleifera* for staphylococcal diseases.

MATERIALS AND METHODS

Preparation of the extract

We used a fresh powder (20 g) of Brazilian *M. oleifera* fresh leaves (obtained by spray drying) produced and provided by RVN (MG, Brazil). An authenticity certificate supplied by the manufacturer confirmed the botanical identity of the plant. The leaves are collected during the early hours in the city of Carlos Chagas (Minas Gerais state, Brazil).

The powder was sifted before being suspended in 70% cold ethanol (150 mL), with slow maceration. The powder remained in the solvent for 48 h at 4 °C. Following, this system was centrifuged (5000 *g*, 10 min.), and the supernatant was concentrated by a rota evaporator (40 °C) until a dark paste was obtained. The

final product was weighed (275.38 mg) and stored at 4 °C until used. A stock solution of 4 mg/mL of MOLE was prepared using sterile 0.9% saline (pH 7.2).

Biochemical characterization assays

We conducted the following biochemical analyzes for the leaf powder and MOLE in triplicate. For the leaf powder, the American Association of Cereal Chemists (AACC) Micro-Kjeldahl 46-13.01 method was used to detect and quantify total protein content (AACC, 2010). Calcium, iron and vitamin C levels were determined using the titration methods standardized by the Adolfo Lutz Institute (2008). Ashes and total fibers were analyzed following the Association of Official Analytical Chemists (AOAC) 923.03 method (AOAC, 2005), and the ISO 5498 method (ISO, 1981), respectively.

For MOLE (at 4 mg/mL), we used the biuret reaction to quantify total proteins (Gornall et al., 1949), with a calibration curve prepared using bovine serum albumin (BSA, Thermo Fisher, USA). Total carbohydrates were quantified by using the phenol-sulphuric method (Dubois et al., 1956), with a calibration curve prepared with glucose. The presence of vitamin C was confirmed using the molybdenum blue reaction (Bajaj and Kaur, 1981). Flavonoids and tannins were qualitatively detected in the extract using the Shinoda method and the ferric chloride reaction, respectively (Harborne, 1988).

Ultra-performance liquid chromatography

The stock solution of the extract was diluted to 1 mg/mL in ultra-purified Mili-Q water and filtered through a 220 nm PVDF membrane. An aliquot of 1 µL of the filtrate was injected on a Shimadzu Shim-Pack XR-ODS-III column (C18, 2.2 µm, 2.0×150 mm) of a Nexera UPLC-system (Shimadzu). The analysis was conducted at 220 nm, with a flow rate of 400 µL/min, at 40 °C. The mobile phase A consisted of a 0.1% formic acid aqueous solution, and phase B was a 0.1% solution of formic acid prepared in acetonitrile. An isocratic run was performed using 5% B and 95% A for 5 min, followed by a linear gradient to 100% B in 40 min and a hold at 100% B for 5 min.

Bacterial strains

Ten strains of *S. aureus* were used in this study. They are part of the microorganisms collection from Pitágoras College and were isolated from hemodialysis catheter tips. Their identity was confirmed using Gram-positive cards for VITEK 2 system (version R04.02, bioMérieux) following the manufacturer's instructions. Following, we used two different approaches to assess the isolates' ability to produce β-lactamase: the zone-edge method described by Gill et al. (1981), which uses antimicrobial disks of penicillin and oxacillin, and the iodometric test described by Jarløv and Rosdahl (1986), to confirm the zone-edge results.

Antimicrobial assays

Minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal biofilm eradication concentration (MBEC) of the extract were determined in triplicate following CLSI standards and a protocol standardized by our group (Dias-Souza et al., 2017; CLSI, 2018). The extract was prepared as an aqueous solution to reach final concentrations ranging from 1024 to 8 µg/mL, and the final concentration of the bacterial suspensions was $5x10^4$ CFU/mL. Before the MBEC assay, biofilm formation was induced overnight as described (Dias-Souza et al., 2013a). We used resazurin staining (0.1 g/L, 50 µL) in MIC and MBEC assays to analyze the results. MBC was determined by the direct plate count of colonies.

Interference of the extract on the activity of antimicrobial drugs

We investigated the effect of combining the extract to antimicrobial drugs against *S. aureus* isolates using the Dias-Souza method (Dias-Souza et al., 2013b) in duplicate. MOLE was prepared in MIC and MBEC values in sterile saline, and was added to antimicrobial disks (all from Sensifar, Brazil) of cephalothin (30 µg), ceftriaxone (30 µg) and chloramphenicol (30 µg). Interpretation parameters for statistically significant

(or the tendency of) synergism or antagonism were applied to the results as described (Dias-Souza et al., 2013b). Disks without the addition of MOLE were used as controls.

Anti-inflammatory assay

We used the BSA denaturation assay in triplicate to assess the anti-inflammatory potential of MOLE. This method is based on the ability of a determined compound to inhibit molecular events that unfold upon protein denaturation by heat, such as the formation of aggregates and exposure of chromophore groups. We followed the method and calculations described by Marius et al. (2020), with slight modifications. BSA (ThemoFisher, USA) denaturation was conducted for 15 minutes at 70 °C, using Tenoxicam (Sigma) as a standard non-steroidal anti-inflammatory drug (NSAID) (positive control, 500 µg/mL). Pure BSA exposed to heat was used as a negative control. MOLE was tested at the same concentrations used for antimicrobial activity assays.

Cytotoxicity assay

MOLE was tested for cytotoxic effects in triplicate using BGM cells as described by our group (Dias-Souza et al., 2018). Cells were exposed to the extract in concentrations ranging from 600 to 4.687 µg/mL for 18h. Neutral red uptake was used to assess cell viability as described (Siqueira et al., 2018).

Statistics

Homocedacisty of data was assessed using the Bartlett test. Normality was assessed using the Shapiro-Wilk test. For data with parametric distribution, we used ANOVA followed by the Tukey test. For non-parametric data, we used Kruskal-Wallis followed by the Dunn test. All analyses were carried out using Bioestat 5.0 for Windows.

RESULTS and DISCUSSION

Biochemical characteristics of the extract

The main characteristics of the leaf powder are shown in Table 1. The levels of vitamin C and proteins were higher than the other nutrients. Total ashes content corresponded to approximately 9%. Iron levels were superior to calcium levels.

Table 1: Biochemical parameters of the leaf powder

Total proteins	Total fibers	Ashes	Total iron	Total calcium	Vitamin C
31.74 mg/100g	7.19 g/100g	9.11 g/100g	17.62 mg/100g	1.32 mg/100g	293 mg/100g

The characteristics of MOLE are presented in Table 2. The qualitative data suggested the presence of flavonols in the extract. The UPLC chromatogram indicated the presence of flavonoids in the extract (Figure 1). Retention times of the proeminent peaks were suggestive of gallic acid (~1 min), caffeic acid (~10 min), rutin (~12 and 52 mins), quercetin (~14, 15 min), kaempferol (16 min), isoquercetin (~22 min), and apigenin (~26 min), as described elsewhere (Makita et al., 2016; Zhu et al., 2021).

Table 2: Biochemical parameters of MOLE (Flavonoids, tannins, and Vitamin C were analyzed using qualitative assays)

Total proteins	Total carbohydrates	Flavonoids	Tannins	Vitamin C
6.83 µg/mL	415.15 µg/mL	Positive	Positive	Positive

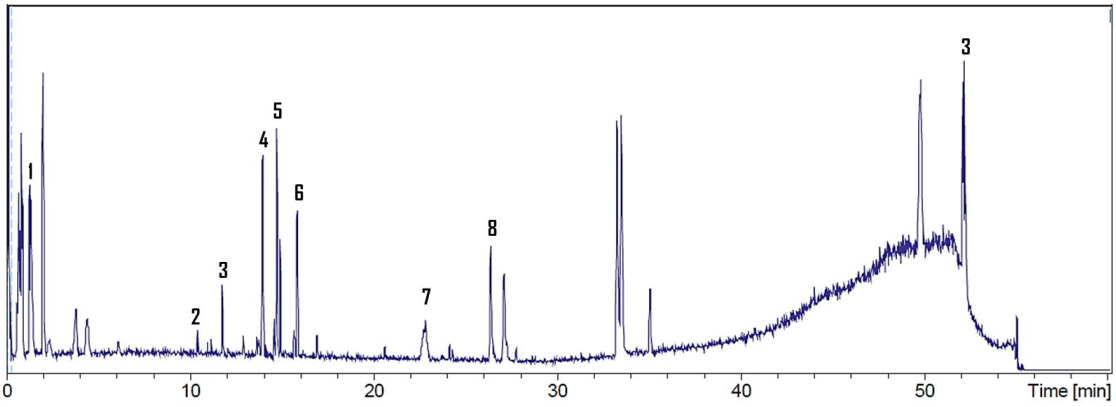

Figure 1. UPLC chromatogram for *M. oleifera* leaf extract (C18 column, 220 nm) for polyphenolics. Peaks: gallic acid (1), caffeic acid (2), rutin (3), quercetin (4, 5) kaempferol (6), isoquercetin (7), apigenin (8). Y-axis represent arbitrary units of readings.

Antimicrobial activity of MOLE

Using the zone-edge and iodometric tests, we confirmed that from the 10 tested isolates, the isolates n#1 to 7 are producers of β-lactamases. MOLE was active against all the bacterial isolates as planktonic cells and biofilms (Table 3). Given that the MIC and MBC values were equally the same for the isolates, we suggest that the extract presents a bactericide effect (Levinson et al., 2009).

Table 3. Results of the antimicrobial assays

MIC	8 µg/mL
MBC	8 µg/mL
MBEC	16 µg/mL

Results are referent to all tested strains

We investigated if combinations of MOLE to clinically relevant antimicrobials in disks would result in synergistic or antagonistic interactions. When comparing the antimicrobials without the addition of MOLE, ceftriaxone was the most effective drug (29.2±1.22 mm, $p<0.05$). Surprisingly, the interference of MOLE on the antimicrobials was very discrete (Table 4, $p>0.05$), and did not meet the criteria to be characterized as synergistic or antagonistic (i.e., sizes of the inhibition zones did not change ±2mm when compared to control). This suggests its safety in an eventual simultaneous use with these drugs, but further *in vivo* experiments are still necessary to confirm this observation.

Table 4. Inhibition zones of antimicrobials with or without addition of *M. oleifera* extract.

Isolates	CEPH	CEPH +E	CEFT	CEFT +E	CHLO	CHLO +E
S1	23	24	30	31	25	25
S2	22	21	28	29	23	24
S3	20	21	30	30	24	25
S4	25	26	29	28	25	25
S5	24	23	27	28	26	27
S6	22	23	28	29	28	29
S7	21	22	29	30	23	24
S8	22	22	31	31	25	24
S9	25	25	30	29	24	25
S10	24	25	30	31	27	27

Data are expressed as averages of duplicates in mm. CEPH: cephalothin. CEFT: ceftriaxone. CHLO: chloramphenicol. +E: addition of the extract at the MIC. *S. aureus* isolates are identified as S+number.

Anti-inflammatory potential and cytotoxicity

The extract presented anti-inflammatory effect at MIC and MBEC values, with no statistical difference between them (Figure 2). Thus, antimicrobial and anti-inflammatory properties of MOLE might be observed simultaneously. Tenoxicam (500 µg/mL) and the highest tested concentration of MOLE (1024 µg/mL) were significantly (although numerically slightly) less effective than MOLE in MIC (8 µg/mL) and MBEC (16 µg/mL) in preventing BSA denaturation ($p<0.05$). The extract had no cytotoxic effect on BGM cells in any tested concentration (data not shown).

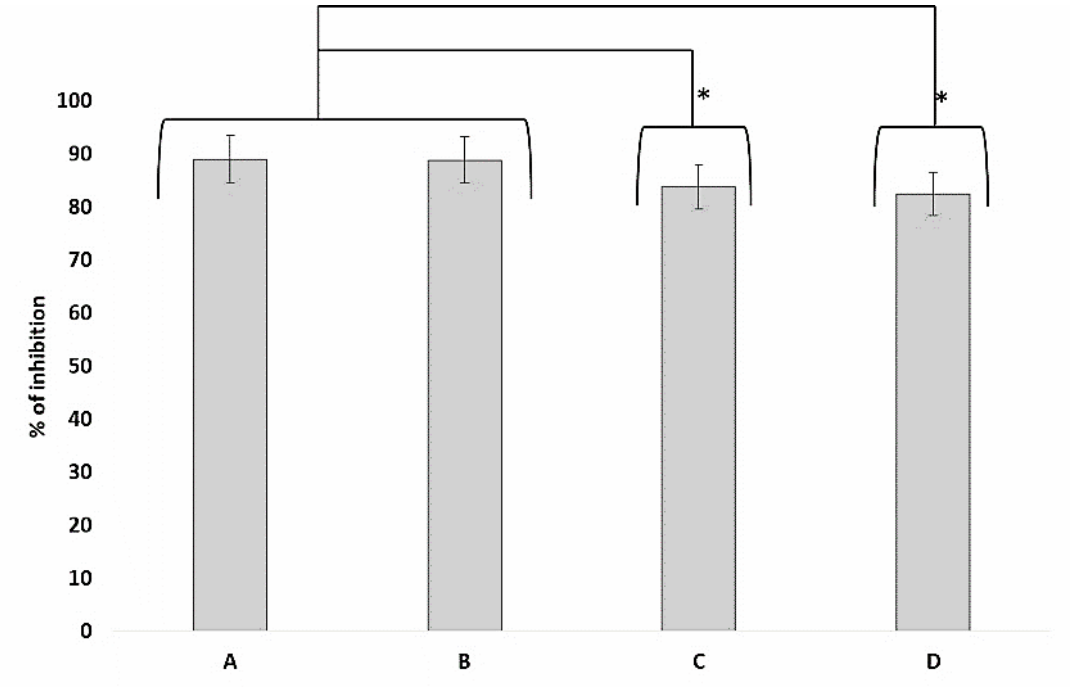

Figure 2. Anti-inflammatory potential of the extract was assessed as inhibition of BSA denaturation by heat. A: extract at MIC (8 µg/mL). B: extract at MBEC (16 µg/mL). C: extract at 1000 µg/mL. D: Tenoxicam (500 µg/mL). *: $p<0.05$

Discussion

M. oleifera leaves are explored as plant food for their varied nutritional benefits (Leone et al., 2015), but data concerning its antimicrobial and antibiofilm effects remain scarce. In the present study, the hydroethanolic extract of *M. oleifera* leaves was active at 8 µg/mL against planktonic cells of *S. aureus* isolates, and at 16 µg/mL against overnight-formed biofilms. This antibiofilm potential is especially relevant as MBEC might be more than 1000 times superior to MIC, due to biofilms' ability to hamper the diffusion and action of antimicrobials (Dias-Souza et al., 2017).

To the best of our knowledge, the MIC, MBC and MBEC values reported here are the lowest described for *M. oleifera* leaf extract against *S. aureus*. MOLE was prepared with a fine particles leaf powder obtained by spray drying. Compared to leaves used in laboratory scale experiments, the powder used in this study has an increased content of phytomolecules per unit of mass. Most of the available works on the antimicrobial activity of *M. oleifera* tested aqueous extracts of parts such as leaves and seeds. However, water is not as efficient as organic solvents like alcohols in extracting bioactive molecules from plants (Jwa, 2019). Furthermore, variations on soil nutrition,

endophytic microbiota, frequency and intensity of the rains (or irrigation), collection and storage procedures, and of methods of harvest and extraction, may all profoundly impact the diversity and level of phytomolecules in plant extracts (Cock, 2011; Masota et al., 2021). Moreover, the sources of bacterial strains are variable and their susceptibility to antimicrobial drugs and plant extracts is poorly predictable. Taken together, these reasons help to explain our remarkable results on the antimicrobial activity of MOLE, which are consistent with the existing studies on the activity of different types of *M. oleífera* leaf extracts against bacterial planktonic cells and biofilms.

The acetone extract prepared with *M. oleifera* leaves was active at 420 µg/mL against planktonic (free) cells of a *S. aureus* reference strain (Ratshilivha et al., 2014). Flavonoids of *M. oleifera* seed coat were active at 50 µg/mL against planktonic cells and biofilms of a reference *P. aeruginosa* strain, and at 80 and 240 µg/mL against planktonic cells and biofilms of *S. aureus,* respectively (Onsare and Arora, 2015). More recently, a study found that the MIC of the leaf extract against clinical isolates of *S. aureus* ranged from 800 to 2000 µg/mL (Zubair, 2020). Biofilms of an environmental strain of *S. aureus* were susceptible to the aqueous leaf extract at 20, 40, and 60 mg/mL (Nasr-Eldin et al., 2017). MIC and MBC of an aqueous leaf extract against an environmental strain of *S. aureus* were 250 and 1000 µg/mL, respectively, and inhibition of biofilm formation required concentrations higher than 250 µg/mL (De Oliveira et al., 2020).

A common (and often dangerous) practice among patients in several countries seeking to increase or accelerate antimicrobial therapy outcomes is combining antimicrobials to phytoextracts. This practice is supported by an inaccurate assumption that combining antimicrobial drugs and phytoextracts with antimicrobial activity results in synergistic interactions (Dias-Souza et al., 2013b). Here we combined MOLE to chloramphenicol, which is widely used in topical medication for infected wounds (Livingston et al., 2013), and to cephalosporins, which are naturally more resistant to enzymatic hydrolysis (Bush et al., 2016). These drugs were selected considering that β-lactamase production was confirmed in most of the isolates. MOLE did not interfere significantly with the activity of these drugs (Table 4), as observed using the method standardized by our group (Dias-Souza et al., 2013b). Likewise, a previous study described that *Vaccinium myrtillus* extract, caused no interference on the activity of antimicrobial drugs against *S. aureus* isolates (Costa et al., 2017). We also described synergistic behavior (i.e., increased activity of the drugs) for combinations of antimicrobial drugs to black tea (*Camellia sinensis*) and açaí (*Euterpe oleracea*) extracts (Dias-Souza et al., 2018; Dos Santos et al., 2018), and antagonistic behavior for cashew (*Anacardium occidentale*) pulp and stembark extracts, and for some carotenoids and flavonoids against Gram-negative bacterial species (Dias-Souza et al., 2013; Dos Santos et al., 2015; Dias-Souza et al., 2016). Therefore, synergistic or antagonistic behaviors of such interactions are poorly predictable, can only be determined experimentally. Patients should be advised to avoid such combinations.

We used the BSA denaturation assay to investigate the anti-inflammatory potential of MOLE. The extract at MIC and MBEC values was more effective than the highest concentration of the extract and tenoxicam, a potent NSAID that inhibits cyclooxygenase (COX) 1 and COX 2 (Valentovic, 2007; Barbosa et al., 2021). Flavonoids are major compounds in *M. oleifera* leaves and are the main anti-inflammatory molecules of the plant (Luetragoon et al., 2021). Additionally, minerals and proteins were found in the powder, and they can interfere with the anti-inflammatory and antioxidant potentials of flavonoids by complexing to them (Selvaraj et al., 2013; Swieca et al., 2013). The level of such interferents in concentrations of MOLE such as MIC was expected to be very low, due to their removal by serial dilutions. Thus, biological properties of flavonoids remained active in MOLE. *In vivo* anti-inflammatory mechanisms described for *M. oleifera* extracts

include decreasing the expression of pro-inflammatory cytokines and blockage of toll-like receptors (Luetragoon et al., 2021; Wuryandari et al., 2021).

The biochemical characteristics of the extract are in agreement with the observation of others, specially concerning the presence of polyphenolics, mostly flavonoids, which are major compounds in the leaves (Hassan et al., 2021). UPLC analysis suggested that quercetin, kaempferol and rutin are among the polyphenolics detected in MOLE. There is evidence indicating that these are the main flavonoids present in the leaves, and have a role in both antimicrobial and anti-inflammatory properties of the plant extract (Abd et al., 2018; Dhakad et al., 2019; Zhu et al., 2021). Here, quercetin was detected in two peaks, possibly due to the different glycone (sugar group) to which they might be bounded to. Similarly, a study found these same molecules in a 80% methanolic extract of the leaves (Makita et al., 2016). Also in line with our observations, quercetin, kaempferol and chlorogenic acid, bounded to different glycones, as well as glucosinolates, where found in leaves and other parts of *M. oleifera*, prepared as 70% methanolic extracts (Bennett et al., 2003). A series of compounds, including phytol, hexadecanoic acid and 4-(1-aminoethyl)-3,3-dimethylazetidin-2-one, were found in the leaves of *M. oleifera* by gas chromatography coupled to mass spectrometry (Bhalla et al., 2021). These compounds are described to be anti-inflammatory as some of the flavonoids detected in the leaves (Bhalla et al., 2021; Zhu et al., 2021). Thus, it is possible flavonoids and these compounds had a role in protecting BSA from denaturation in this study.

The presence of vitamin C, iron and calcium in leaf extracts is well described, what supports their safe use as nutritional supplements (Abd et al., 2018; Fernandes et al., 2020). Such elements are extremely valuable for human nutrition, and could be of interest for use as a nutritional supplement, due to the low cost of the plant compared to traditional (non-phytotherapeutic) products. Recent clinical trials have provided evidence of varied benefits of *M. oleifera* leaves as a complementary treatment for bone healing (Singh et al., 2011), control of blood glucose levels (Leone et al., 2018), management of HIV patients (Gambo et al., 2021), and even as a mouthwash (Buakaew et al., 2021). Thus, more clinical trials considering laboratorial parameters and health benefits would be of interest.

There are no reference values in the Brazilian legislation for the biochemical parameters assessed in *M. oleifera* powder and hydroethanolic extract at the moment (proteins, carbohydrates, Vitamin C and minerals). However, they were within acceptable ranges adopted by international standards of quality control and of recommended daily intake of nutrients such as the codex nutrient reference values of the Food and Agriculture Organization of the United Nations, and dietary reference intake values from the USA Food and Nutrition Board of the National Academies of Sciences Engineering and Medicine (Caballero, 2012; Raymon and Morrow, 2021).

CONCLUSIONS

In this study we investigated the antimicrobial and antibiofilm activities of MOLE, and its anti-inflammatory potential and its cytotoxicity. MOLE was effective against planktonic cells and biofilms of β-lactamase producing *S. aureus* clinical isolates. To the best of our knowledge, this is the lowest effective concentration described to date. MOLE also presented anti-inflammatory potential and lacked cytotoxicity. Taken together, our results open doors for developing formulations to treat topical staphylococcal diseases, and anti-inflammatory and antimicrobial effects can be expected. Further studies with *in vivo* models of staphylococcal diseases are necessary to determine safe concentrations for clinical use and assess the effectiveness of the extract considering metabolic and immunological issues.

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CONFLICT OF INTEREST

The authors declare that there is no conflicts of interest.

AUTHOR CONTRIBUTION STATEMENT

LKSA: performed antimicrobial and anti-inflammatory activities experiments, drafted the manuscript. **LFCM:** performed antimicrobial activity and interference assays. **IPC:** performed cytotoxicity experiments, drafted the manuscript. **RMDS:** co-supervision of the research, critical review of the manuscript. **MVDS:** conceptualization, project administration, supervision, statistical analysis, preparation of the final version of the manuscript.

REFERENCES

AACC – American Association of Cereal Chemists, 2010. *Method 08-01.01*, 30-20, 44-15-A, 46-13.01, 54-60.01, 56-0.

Abd, N.Z.R., Husain, K., Kumolosasi, E. (2018). Moringa Genus: A Review of Phytochemistry and Pharmacology. *Frontiers in Pharmacology,* 2018(*9*),108. doi:10.3389/fphar.2018.00108

Abril, A.G., Villa, T.G, Barros-Velázquez, J., Cañas, B., Sánchez-Pérez, A., Calo-Mata, P., Carrera, M. (2020). *Staphylococcus aureus* Exotoxins and Their Detection in the Dairy Industry and Mastitis. *Toxins*, 12(*9*), 537. https://doi.org/10.3390/toxins12090537

Adolfo Lutz Institute (2008). Métodos físico-químicos para análise de alimentos. 1. Ed. Digital. São Paulo: Instituto Adolfo Lutz, 83-158

AOAC – Association of Official Analytical Chemists. (2002). *Official Method 923.03: Ash of Flour*. Gravimetric. In *Official Methods of Analysis of AOAC International*; AOAC International: Washington, DC, USA.

Bajaj, K. L., Kaur, G. (1981). Spectrophotometric determination of L-ascorbic acid in vegetables and fruits. *The Analyst*, 106(*1258*), 117–120. https://doi.org/10.1039/an9810600117

Barbosa, F.A.S., Castro, E.S., Dias-Souza, M.V. (2021). Pharmacology of non-steroidal anti-inflammatory drugs: a prescription-based overview. *Journal of Applied Pharmaceutical Sciences* (7), 11-20.

Bennett, R. N., Mellon, F. A., Foidl, N., Pratt, J. H., Dupont, M. S., Perkins, L., Kroon, P. A. (2003). Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees *Moringa oleifera* L. (horseradish tree) and *Moringa stenopetala* L. *Journal of Agricultural and Food Chemistry*, 51(*12*), 3546–3553. https://doi.org/10.1021/jf0211480

Bhalla, N., Ingle, N., Patri, S. V., Haranath, D. (2021). Phytochemical analysis of *Moringa oleifera* leaves extracts by GC-MS and free radical scavenging potency for industrial applications. *Saudi Journal of Biological Sciences*, 28(*12*), 6915–6928. https://doi.org/10.1016/j.sjbs.2021.07.075

Brito I. L. (2021). Examining horizontal gene transfer in microbial communities. *Nature Reviews Microbiology*, 19(*7*), 442–453. https://doi.org/10.1038/s41579-021-00534-7

Buakaew, W., Sranujit, R. P., Noysang, C., Sangouam, S., Suphrom, N., Thongsri, Y., Potup, P., Usuwanthim, K. (2021). Evaluation of Mouthwash Containing Citrus hystrix DC., Moringa oleifera Lam. and Azadirachta indica A. Juss. Leaf Extracts on Dental Plaque and Gingivitis. Plants (Basel, Switzerland), 10(*6*), 1153. https://doi.org/10.3390/plants10061153

Bush, K., Bradford, P. A. (2016). β-Lactams and β-Lactamase Inhibitors: An Overview. *Cold Spring Harbor Perspectives in Medicine*, 6(*8*), a025247. https://doi.org/10.1101/cshperspect.a025247

Caballero, B. [Ed]. Encyclopedia of Human Nutrition. 3.ed, Academic Press, 2012.

CLSI (2018). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. 11th ed. CLSI standard M07. Wayne, PA: Clinical and Laboratory Standards Institute.

Cock, I. (2011) Problems of Reproducibility and Efficacy of Bioassays Using Crude Extracts, with reference to *Aloe vera*. *Pharmacogenetics Communications,*1, 52–62.

Costa, G.J., Dos Santos, R.M., Figueiredo, F.J.B., Dias-Souza, M.V. (2017). *Vaccinium myrtillus* extract is effective against *Staphylococcus aureus* and does not interfere on the activity of antimicrobial drugs. *Journal of Applied Pharmaceutical Sciences*, 4(*1*), 6-8

De Oliveira, A. M., de Abreu Filho, B. A., de Jesus Bassetti, F., Bergamasco, R., Gomes, R. G. (2020). Natural Extract of *Moringa oleifera* Leaves Promoting Control of *Staphylococcus aureus* strains biofilm on PVC surface. *Food and Bioprocess Technology*. doi:10.1007/s11947-02002521-x

Dhakad, A.K., Ikram, M., Sharma, S., Khan, S., Pandey, V.V., Singh, A. (2019). Biological, nutritional, and therapeutic significance of *Moringa oleifera* Lam. *Phytotherapy research: PTR*, 33 (*11*), 2870–2903. https://doi.org/10.1002/ptr.6475

Dias-Souza, M. V., Andrade, S., Aguiar, A. P., Monteiro, A. (2013 [a]). Evaluation of Antimicrobial and Anti-biofilm activities of *Anacardium occidentale* stem bark extract. *Journal of Natural Products*, 6(*2013*), 198-205.

Dias-Souza, M.V., Caldoncelli, J.L., Monteiro, A.S. (2013[b]). *Annacardium occidentale* stem bark extract can decrease the efficacy of antimicrobial drugs. *Journal of Medical and Biological Sciences*; 12, 161e5.

Dias-Souza, M.V., Dos Santos, R. M., Cerávolo, I. P., Cosenza, G., Ferreira Marçal, P. H., Figueiredo, F. (2018). *Euterpe oleracea* pulp extract: Chemical analyses, antibiofilm activity against *Staphylococcus aureus*, cytotoxicity and interference on the activity of antimicrobial drugs. *Microbial Pathogenesis*, 114, 29–35. https://doi.org/10.1016/j.micpath.2017.11.006.

Dos Santos, L.D.R., Dos Santos, A.E.S., Cerávolo, I.P., Figueiredo, F.J.B., Dias-Souza, M.V. (2018). Antibiofilm activity of black tea leaf extract, its cytotoxicity and interference on the activity of antimicrobial drugs. *Biointerface Research in Applied Chemistry*, 8(*5*), 3565–3569.

Dias-Souza, M.V., Dos Santos, R.M., de Siqueira, E.P., Ferreira-Marçal, P.H. (2017). Antibiofilm activity of cashew juice pulp against *Staphylococcus aureus*, high performance liquid chromatography/diode array detection and gas chromatography-mass spectrometry analyses, and interference on antimicrobial drugs. *Journal of Food and Drug Analysis*, 25(*3*), 589–596. https://doi.org/10.1016/j.jfda.2016.07.009.

Dos Santos, R., Pimenta-Freire, G., Dias-Souza, M.V. (2015). Carotenoids and flavonoids can impair the effectiveness of some antimicrobial drugs against clinical isolates of *Escherichia coli* and *Staphylococcus aureus*. *International Food Research Journal*, 22(*5*), 1777–1782.

DuBois, M., Gilles, K.A., Hamilton, J.K., et al. Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry* 1956 28(*3*), 350-356, DOI: 10.1021/ac60111a017

Fernandes, Â., Bancessi, A., Pinela, J., Dias, M. I., Liberal, Â., Calhelha, R. C., Ćirić, A., Soković, M., Catarino, L., Ferreira, I., Barros, L. (2021). Nutritional and phytochemical profiles and biological activities of *Moringa oleifera* Lam. edible parts from Guinea-Bissau (West Africa). *Food chemistry*, 341(*1*), 128229. https://doi.org/10.1016/j.foodchem.2020.128229

Gambo, A., Moodley, I., Babashani, M., Babalola, T. K., Gqaleni, N. (2021). A double-blind, randomized controlled trial to examine the effect of Moringa oleifera leaf powder supplementation on the immune status and anthropometric parameters of adult HIV patients on antiretroviral therapy in a resource-limited setting. *PloS One*, 16(*12*), e0261935. https://doi.org/10.1371/journal.pone.0261935

Gill, V. J., Manning, C. B., Ingalls, C. M. (1981). Correlation of penicillin minimum inhibitory concentrations and penicillin zone edge appearance with staphylococcal beta-lactamase production. *Journal of Clinical Microbiology*, 14(*4*), 437–440. https://doi.org/10.1128/jcm.14.4.437-440.1981

Gornall, A. G., Bardawill, C. J., David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *The Journal of Biological Chemistry*, 177(*2*), 751–766.

Guo, Y., Song, G., Sun, M., Wang, J., Wang, Y. (2020). Prevalence and Therapies of Antibiotic-Resistance in *Staphylococcus aureus*. *Frontiers in Cellular and Infection Microbiology*, 10, 107. https://doi.org/10.3389/fcimb.2020.00107

Harborne, J. B. (Ed.). (1988). The Flavonoids. doi:10.1007/978-1-4899-2913-6

Hassan, M. A., Xu, T., Tian, Y., Zhong, Y., Ali, F., Yang, X., Lu, B. (2021). Health benefits and phenolic compounds of Moringa oleifera leaves: A comprehensive review. *Phytomedicine: international journal of phytotherapy and phytopharmacology*, 93, 153771. https://doi.org/10.1016/j.phymed.2021.153771

ISO 5498. (1981). Animal Feeding Stuffs-Determination of Crude Fibre Content (ISO 5498).

Jarløv, J. O., Rosdahl, V. T. (1986). Quantitative determination of beta-lactamase production in *Staphylococcus aureus* strains compared to qualitative testing by a microbiological clover leaf test, a chromogenic cephalosporin test and a iodometric test. *Acta pathologica, microbiologica, et immunologica Scandinavica. Section B, Microbiology*, 94(*6*), 415–421. https://doi.org/10.1111/j.1699-0463.1986.tb03077.x

Jwa S. K. (2019). Efficacy of *Moringa oleifera* Leaf Extracts against Cariogenic Biofilm. *Preventive Nutrition and Food Science*, 24(*3*), 308–312. https://doi.org/10.3746/pnf.2019.24.3.308

Koo, H., Allan, R. N., Howlin, R. P., Stoodley, P., Hall-Stoodley, L. (2017). Targeting microbial biofilms: current and prospective therapeutic strategies. *Nature Reviews Microbiology*, 15(*12*), 740–755. https://doi.org/10.1038/nrmicro.2017.99

Krismer, B., Weidenmaier, C., Zipperer, A., Peschel, A. (2017). The commensal lifestyle of *Staphylococcus aureus* and its interactions with the nasal microbiota. *Nature Reviews Microbiology*, 15(*11*), 675–687. https://doi.org/10.1038/nrmicro.2017.104

Kwiecinski, J. M., Horswill, A. R. (2020). *Staphylococcus aureus* bloodstream infections: pathogenesis and regulatory mechanisms. *Current opinion in microbiology*, 53, 51–60. https://doi.org/10.1016/j.mib.2020.02.005

Leone, A., Bertoli, S., Di Lello, S., Bassoli, A., Ravasenghi, S., Borgonovo, G., Forlani, F., & Battezzati, A. (2018). Effect of Moringa oleifera Leaf Powder on Postprandial Blood Glucose Response: In Vivo Study on Saharawi People Living in Refugee Camps. Nutrients, 10(*10*), 1494. https://doi.org/10.3390/nu10101494

Leone, A., Spada, A., Battezzati, A., Schiraldi, A., Aristil, J., Bertoli, S. (2015). Cultivation, Genetic, Ethnopharmacology, Phytochemistry and Pharmacology of *Moringa oleifera* Leaves: An Overview. *International Journal of Molecular Sciences*, 16(*6*), 12791–12835. https://doi.org/10.3390/ijms160612791

Levison, M. E., Levison, J. H. (2009). Pharmacokinetics and pharmacodynamics of antibacterial agents. *Infectious Disease Clinics of North America*, 23(*4*), 791–vii. https://doi.org/10.1016/j.idc.2009.06.008

Livingston, R. J., Butterworth, J. W., Belt, P. (2013). Reaction or infection: topical chloramphenicol treatment. *Annals of the Royal College of Surgeons of England*, 95(*1*), e20–e21. https://doi.org/10.1308/003588413x13511609955418

Luetragoon, T., Sranujit, R. P., Noysang, C., Thongsri, Y., Potup, P., Somboonjun, J., Maichandi, N., Suphrom, N., Sangouam, S., Usuwanthim, K. (2021). Evaluation of Anti-Inflammatory Effect of *Moringa oleifera Lam*. and *Cyanthillium cinereum* (Less) H. Rob. Lozenges in Volunteer Smokers. Plants (Basel, Switzerland), 10(*7*), 1336. https://doi.org/10.3390/plants10071336

Mabrouki, L., Rjeibi, I., Taleb, J., Zourgui, L. (2020). Cardiac Ameliorative Effect of *Moringa oleifera* Leaf Extract in High-Fat Diet-Induced Obesity in Rat Model. BioMed research international, 2020, 6583603. https://doi.org/10.1155/2020/6583603

Makita, C., Chimuka, L., Steenkamp, P., Cukrowska, E., Madala, E., (2016). Comparative analyses of flavonoid content in *Moringa oleifera* and *Moringa ovalifolia* with the aid of UHPLC-qTOF-MS fingerprinting. *South African Journal of Botany*, 105, 116-122.

Marius, M., Amadou, D., Donatien, A. A., Gilbert, A., William, Y. N., Rauf, K., Arif, M., Adeline, F., Saint, N. I., Dar, H., Rehman, N. U., Ahmad, I. (2020). *In Vitro* Antioxidant, Anti-inflammatory, and *In Vivo* Anticolitis Effects of Combretin A and Combretin B on Dextran Sodium Sulfate-Induced Ulcerative Colitis in Mice. *Gastroenterology Research and Practice*, 2020, 4253174. https://doi.org/10.1155/2020/4253174

Maslova, E., Eisaiankhongi, L., Sjöberg, F., McCarthy, R. R. (2021). Burns and biofilms: priority pathogens and *in vivo* models. *NPJ Biofilms and Microbiomes*, 7(*1*), 73. https://doi.org/10.1038/s41522-021-00243-2

Masota, N. E., Vogg, G., Ohlsen, K., Holzgrabe, U. (2021). Reproducibility challenges in the search for antibacterial compounds from nature. *PloS One*, 16(*7*), e0255437. https://doi.org/10.1371/journal.pone.0255437

Miller, L. S., Fowler, V. G., Shukla, S. K., Rose, W. E., Proctor, R. A. (2020). Development of a vaccine against *Staphylococcus aureus* invasive infections: Evidence based on human immunity, genetics and bacterial evasion mechanisms. *FEMS Microbiology Reviews*, 44(*1*), 123–153. https://doi.org/10.1093/femsre/fuz030

Nasr-Eldin, M., Abdelhamid, A., Baraka, D. (2017). Antibiofilm and Antiviral Potential of Leaf Extracts from *Moringa oleifera* and Rosemary (*Rosmarinus officinalis* Lam.). *Egyptian Journal of Microbiology*, 52(*1*), 129-139. doi: 10.21608/ejm.2017.1439.1027

Onsare, J. G., Arora, D. S. (2015). Antibiofilm potential of flavonoids extracted from *Moringa oleifera* seed coat against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans*. *Journal of Applied Microbiology*, 118(*2*), 313–325. https://doi.org/10.1111/jam.12701

Penesyan, A., Paulsen, I. T., Kjelleberg, S., Gillings, M. R. (2021). Three faces of biofilms: a microbial lifestyle, a nascent multicellular organism, and an incubator for diversity. *NPJ Biofilms and Microbiomes*, 7(*1*), 80. https://doi.org/10.1038/s41522-021-00251-2

Ratshilivha, N., Awouafack, M. D., du Toit, E. S., Eloff, J. N. (2014). The variation in antimicrobial and antioxidant activities of acetone leaf extracts of 12 *Moringa oleifera* (Moringaceae) trees enables the selection of trees with additional uses. *South African Journal of Botany*, 92, 59–64. https://doi.org/10.1016/j.sajb.2014.02.002.

Raymon JL, Morrow K. Krause and Mahan's Food the Nutrition Care Process. 15.ed, Elsevier, 2021.

Rumbaugh, K. P., Sauer, K. (2020). Biofilm dispersion. *Nature Reviews Microbiology*, 18(*10*), 571–586. https://doi.org/10.1038/s41579-020-0385-0

Selvaraj, S., Krishnaswamy, S., Devashya, V., Sethuraman, S., Krishnan, U. M. (2013). Flavonoid-Metal Ion Complexes: A Novel Class of Therapeutic Agents. *Medicinal Research Reviews*, 34(*4*), 677–702. doi:10.1002/med.21301

Singh, V., Singh, N., Pal, U. S., Dhasmana, S., Mohammad, S., & Singh, N. (2011). Clinical evaluation of cissus quadrangularis and moringa oleifera and osteoseal as osteogenic agents in mandibular fracture. *National Journal of Maxillofacial Surgery*, 2(*2*), 132–136. https://doi.org/10.4103/0975-5950.94466

Siqueira, E.P., Ceravolo, I.P., Kohlhoff, M., Krettli, A.U., Zani, C.L. (2018). Synthesis and Antiplasmodial Activity of 2-Methyl-3- Carboxyl-Naphtho [2, 3-B] Furan Quinone Derivatives. *J Med Chem Drug Des* 1(*2*). dx.doi.org/10.16966/2578-9589.108

Stohs, S. J., Hartman, M. J. (2015). Review of the Safety and Efficacy of *Moringa oleifera*. *Phytotherapy research*: PTR, 29(*6*), 796–804. https://doi.org/10.1002/ptr.5325

Swieca, M., Gawlik-Dziki, U., Dziki, D., Baraniak, B., Czyż, J. (2013). The influence of protein–flavonoid interactions on protein digestibility *in vitro* and the antioxidant quality of breads enriched with onion skin. *Food Chemistry*, 141(*1*), 451–458.

Valentovic M. Tenoxicam. In: Enna S.J., Bylund D.B. xPharm: The Comprehensive Pharmacology Reference, Elsevier, 2007, 1-4. https://doi.org/10.1016/B978-008055232-3.62726-6

Wuryandari, M., Atho'illah, M. F., Laili, R. D., Fatmawati, S., Widodo, N., Widjajanto, E., Rifa'i, M. (2021). *Lactobacillus plantarum* FNCC 0137 fermented red *Moringa oleifera* exhibits protective effects in mice challenged with *Salmonella typhi* via TLR3/TLR4 inhibition and down-regulation of pro-inflammatory cytokines. *Journal of Ayurveda and Integrative Medicine*, 13(*2*), 100531. Advance online publication. https://doi.org/10.1016/j.jaim.2021.10.003

Zhu, Y., Yin, Q., Yang, Y. (2021). A comparative study of HPLC-DAD and UPLC-UV methods for simultaneous determination of 11 polyphenols in *Moringa oleifera* leaves. *Tropical Journal of Pharmaceutical Research*, 20(*11*), 2371-2379

Zubair, M. (2020). Antimicrobial and Anti-Biofilm Activities of *Citrus sinensis* and *Moringa oleifera* Against the Pathogenic *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Cureus*, 12(*12*), e12337. https://doi.org/10.7759/cureus.12337