Antimicrobial and Anti-Biofilm Activities of Citrus sinensis and Moringa oleifera Against the Pathogenic Pseudomonas aeruginosa and Staphylococcus aureus

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Abstract

Context

The plant Moringa oleifera Lam (Moringaceae), generally termed as drumstick tree, and Citrus sinensis Linn (Rutaceae) fruit have the ability to treat multiple human infections. A biofilm is none other than a complicated microbial community whose nature is greatly resistant to antimicrobial elements. The development of biofilms in abiotic and biotic surfaces has a connection with higher levels of mortality and morbidity. Along with that, it is regarded as a vital element of bacterial pathogenicity.

Aim

The present study evaluated the inhibitory effect and anti-biofilm activity of Moringa oleifera (M. oleifera) and Citrus sinensis (C. sinensis) extracts against those of pathogenic Pseudomonas aeruginosa (P. aeruginosa) and Staphylococcus aureus (S. aureus).

Materials and methods

Two plant materials were collected from the local market of Tabuk city and two human pathogenic microbial strains were used in the study: S. aureus and P. aeruginosa. Further, a series of morphological, physiological, and conventional biochemical tests were performed to identify the selected microorganisms. In addition to this, the study conducted the following tests: antibiotic sensitivity test, extended-spectrum $\beta$-lactamase (ES$\beta$L), and methicillin-resistant Staphylococcus aureus (MRSA) production, biofilm formation in 96-well microtiter plates, minimum inhibitory concentration (MIC) determination, the effect of sub-MICs of C. sinensis extract and M. oleifera extract on the viability of test bacteria, and finally, measurement of the inhibition of biofilm.

Results

A remarkable result of the research is that the peel extract of C. sinensis and the flesh extract of M. oleifera efficiently inhibited biofilm formation by the addition of sub-inhibitory concentrations of (1/16 x MIC - 1/2 x MIC) MRSA and ESBL, respectively. P. aeruginosa shows high resistance to piperacillin (85.0%). Similarly, the resistance of MRSA was also high (65%) against gentamycin and amikacin antibiotics. Regarding ESBL, 12 (60%) isolates showed confirmed positive and 45% of S. aureus showed MRSA activity. On observing the 12 ES$\beta$L-positive P. aeruginosa, it was found that five strains (PS1, PS4, PS6, PS8, and PS11) have formed strong biofilm, methicillin-resistant S. aureus while four strains showed strong biofilm activity (SA2, SA4, SA5, and SA8). The MIC of C. sinensis extract and M. oleifera extract against strong biofilm producers had a range of 50-2000 µg/ml concentration after overnight incubation. The study results revealed that the antibiofilm activity comparatively showed the extract of M. oleifera was better than C. sinensis against the mixed culture (PS1+SA8, PS6+SA2, and PS8+SA4). Hence, it is recommended to use M. oleifera as an option to monitor the development of microbial biofilms or as a model for looking for better medicines.

Conclusion

The presence of antimicrobial activity found in M. oleifera and C. sinensis extracts offers convincing evidence of their likely action as antimicrobial metabolites against the studied microorganism. Anti-biofilm assay findings have shown that M. oleifera and C. sinensis extracts have effectively blocked MRSA and ESBL development in the biofilm matrix.

Categories: Infectious Disease, Environmental Health, Epidemiology/Public Health
Keywords: moringa oleifera, citrus sinensis extracts, s. aureus, pseudomonas species, tabuk city, es$\beta$L and mrsa production, biofilm formation
Introduction
Antibiotics are being utilized for treating infections, in both animals and humans all over the world. Along with the therapeutic usage in animals, antibiotics are generally added in small quantities to animal feeds like prophylaxis and for the purpose of promoting growth [1]. A consistent increase has been noted in terms of microbial resistance to antimicrobials. In addition to that, a decreasing ability is found in the antimicrobials that are available for the purpose of treating general infections.

Antimicrobial resistance (AMR) is a vital risk when it comes to the health and welfare of the people as well as animals, which ends up with a great effect in terms of food security. Anyway, there is a dilemma all across the globe regarding the reduction of new therapeutic elements, which helps in treating different kinds of diseases that affect both animals as well as humans. There are chances for society to go through a post-antibiotic period with present antibiotics getting ineffective slowly because of resistance. This contains big threats in terms of health and national security as well; for instance, bioterrorism and pandemics [2]. The estimation taken at the present time discloses a death toll of about 700,000 people annually. This is mainly because of the antibiotic resistance and a forecast that by 2050, there are higher chances for about 10 million people to be at threat, provided no measures are taken in stopping the drift of rising AMR [3]. In addition to that, it’s presumed that people who live in developing nations that are subjected to mortality and morbidity due to the impact of infectious diseases shall be the ones who would be deadly affected by this condition.

The formation of biofilm is one among the strategies of resistance by several pathogens that indeed makes them even more complicated as compared to the platonic counterparts [4]. A biofilm is none other than a complicated matrix of microorganism communities that consist of proteins, polysaccharides, and other organic elements, wherein cells get fixed together, forming powerful attachments to abiotic and biotic surfaces [5]. Biofilms allow microbes that bind to a surface in order to persist despite harsh states like antimicrobial agents and host defenses [6]. Thus, the formation of biofilm is one of the indirect sources of action through which bacteria act resistant in terms of antibiotics and this is where even resistance genes get transferred among biofilm micro-community members [7]. P. aeruginosa and S. aureus are two, vital, opportunistic-natured pathogens all over the world, which cause nosocomial and community-acquired infections. Biofilm-related infections are caused by P. aeruginosa and S. aureus, and methicillin-resistant S. aureus (MRSA) has evolved to be a pathogen (clinically appropriate) due to its nature of acting resistant toward antibiotics along with the potential of forming biofilms as examined by Alamri et al. [8]. Nearly 60% of microbial infections involve biofilms whilst 2/3rds of bacterial infections in humans are formed due to the biofilms [9].

M. oleifera Lam (Moringaceae) is a tree that has a fast-growing nature, and it is called drumstick tree or horseradish tree. A 4-[(β-D-Glucopyranosyl-1-1-α-1-rhamnopyranosyl)- benzyl thiocarboxamide, which are separated from the seed has displayed the potential activity of antimicrobials as stated by Oladuwo et al. [10]. In the traditional aspect, the report says that when equal parts of C. sinensis Linn (Rutaceae) fruit rind (orange peel) and M. oleifera roots are blended, it is beneficial for the bowels as stated by Gholap et al. [11]. Therefore, this work was planned to study the inhibitory effect and anti-biofilm activity of M. oleifera and C. sinensis extracts against the most common clinical isolates (P. aeruginosa and S. aureus).

Materials And Methods
Plant samples
Two plant materials were collected on the basis of traditional medicinal history from the local market of Tabuk city (Table 1).

| Botanical Name | Common name | Family name | Part used | Ethnomedicinal use |
|---------------|-------------|-------------|-----------|-------------------|
| Citrus sinensis | Egyptian malta | Rutaceae | Peel | Treatment of cold, anorexia, and cough |
| Moringa oleifera | Horseradish tree, Radish tree, Drumstick tree | Moringaceae | Flesh | Curing of fever, infections in the ear, reduction of blood sugar and pressure |

TABLE 1: The botanical name, family, parts used, and ethnomedicinal use under this study

Collection of a bacterial sample
Forty bacterial samples (20 S. aureus and 20 P. aeruginosa) were used in the study. To be doubly sure, basic biochemical tests were performed. The antibiotic assay was performed using the Kirby Bauer disc diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guideline.

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Extraction of test samples (a) \textit{C. sinensis} (Egyptian malta), (b) \textit{M. oleifera}

The methodology of powder preparation of \textit{C. sinensis} and \textit{M. oleifera} was adopted from previously published reports [12]. The details are presented in the supplementary file.

Antibiotic sensitivity testing of \textit{P. aeruginosa}. and \textit{S. aureus}

Antibiotic sensitivity was performed on Mueller-Hinton agar by the Kirby-Bauer method. Amikacin (30 µg), cefazidime (30 µg), cefepime (5 µg), tobramycin (10 µg), piperacillin (100 µg), "imipenem (10 µg), cefoperazone (75 µg), cefoperazone/sulbactam (75/10 µg), cefotaxime (30 µg), cefotaxime/clavulanic acid (50/10 µg), piperacillin/tazobactam (100/10 µg)," cefepime clavulanic acid (30/10 µg), sparfloxacin (5 µg), tobramycin (10 µg), "erythromycin (15 µg), gentamicin (10 µg), oxacillin (1 µg), ciprofloxacin (5 µg), cefoxitin (30 µg), and vancomycin (30 µg)," were used in this study (Hi-Media Labs, Mumbai, India). Interpretation of results as suggested by the manufacturer’s recommendation (Hi-Media Labs).

ESβL and MRSA detection among isolated strains

ESβL producers were detected by using cefazidime and cefotaxime alone and in combination with clavulanic acid (10 mg), as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines. For MRSA detection, the swab (sterile) was dipped in the \textit{S. aureus} suspension (0.5 McFarland) and plated onto Mueller Hinton agar (MHA). Oxacillin discs (1 µg) were used with an overnight incubation at 30°C. When the zone of inhibition was ≤14 mm in diameter, it was considered resistant to oxacillin. The control strains used in this study were "\textit{E. coli} ATCC 25922 (non-ESBL-producer), \textit{K. pneumoniae} 700603 (ESBL-producer), \textit{S. aureus} (ATCC 25923)."

Biofilm formation in 96-well microtiter plates

Biofilm formation was examined by the quantitative determination of biofilm formation in 96-well flat-bottom plates by Coffey and Anderson [13]. For each clinical strain tested, biofilm assays were performed in triplicate and the mean biofilm absorbance value was determined. The biofilms formed were classified as weak (OD590 0.1 to ≤0.400), moderate (OD590 > 0.400 and ≤0.800), and strong (OD590 > 0.800).

Minimum inhibitory concentration (MIC) determination

The MIC of \textit{C. sinensis} extract and \textit{M. oleifera} extract against resistant biofilm-forming strains of \textit{P. aeruginosa} and \textit{S. aureus} was estimated using the standard micro-broth dilution method recommended CLSI guidelines at 37°C at 600 nm after 24 h incubation.

Effect of \textit{C. sinensis} extract and \textit{M. oleifera} extract on mono and mixed-species biofilms

In inhibition assays, bacteria inoculated in microtitre plates were treated with 1/18-1/2 x MICs of \textit{C. sinensis} extract and \textit{M. oleifera} extract and incubated at 37 °C for 48 h. The inhibition of biofilm was measured as described in the previous section. The mixed biofilm formation was quantified as described by Zhang et al. [14].

Statistical analysis

Data represented in the manuscripts were mean value, experiments were performed in triplicate, and tests were analyzed using the student’s t-test.

Results

Overall, 40 bacterial strains (20 \textit{S. aureus} and 20 \textit{P. aeruginosa}) were collected from various hospitals in the city of Tabuk, Kingdom of Saudi Arabia. The collected bacteria were allowed to get subjected to the process of drug resistance observation (tabulated results are shown in Table 2).
Antibiotic resistance pattern

*P. aeruginosa* shows high resistance to piperacillin (85.0%) followed by amikacin, levofloxacin, and sparfloxacin, which show 75%, respectively. Similarly, the resistance of *S. aureus* was also high (65%) against gentamycin and amikacin antibiotics, respectively. Regarding ESBL, 12 (60%) isolates showed confirmed positive and 45% of *S. aureus* showed MRSA activity. For the antibiofilm activity of the *C. sinensis* extract and the *M. oleifera* extract, MRSA and ESβL-positive *P. aeruginosa* were selected for biofilm activity.

Biofilm activity of *P. aeruginosa* and *S. aureus*

The mono species biofilm formation among MRSA and ESβL-positive *P. aeruginosa* were classified as strong, moderate, weak, and negative (Table 3). On observing the 12 ESβL-positive *P. aeruginosa* strains, it is found that five strains (PS1, PS4, PS6, PS8, and PS11) have formed strong biofilms, two strains as moderate (PS2 and PS9), and two as weak biofilms (PS7 and PS10) (Figure 1A). In the case of methicillin-resistant *S. aureus*, four strains show strong biofilm activity (SA2, SA4, SA5, and SA8), whereas two strains (SA1, SA6) were moderate formers and SA9 was a weak biofilm producer (Figure 1B). For the antibiofilm activity of *C. sinensis* extract and *M. oleifera* extract, only the strong biofilm-positive methicillin-resistant *S. aureus* (SA2, SA4, SA5, and SA8) and *P. aeruginosa* (PS1, PS2, PS6, PS12, and PS18) were selected for further experiments.
| Biofilm producers | Strong n (%) | Moderate n (%) | Weak n (%) | Negative n (%) |
|-------------------|--------------|----------------|------------|----------------|
| P. aeruginosa (n=12) | 5 | 2 | 3 | 2 |
| S. aureus (n=9) | 4 | 2 | 1 | 2 |

**TABLE 3:** Classification of P. aeruginosa and S. aureus for biofilm activity as strong, moderate, and weak (data are n (%)) unless otherwise indicated

*P. aeruginosa: Pseudomonas aeruginosa; S. aureus: Staphylococcus aureus*

**FIGURE 1:** Mono-species biofilm formation among isolated strains

[A] Methicillin-resistant *Staphylococcus aureus* (MRSA); [B] extended-spectrum beta-lactamase (ESβL)-producing *Pseudomonas sp.* ***, strong biofilm; **, Moderate biofilm; *, weak biofilm, # negative

**MICs of C. sinensis extract and M. oleifera extract**

The MIC of *C. sinensis* extract and *M. oleifera* extract against strong biofilm producers with a range from 50-2000 µg/ml concentrations after overnight incubation are depicted in Table 4.
### TABLE 4: MIC of Citrus sinensis extract and Moringa oleifera extract strong biofilm-forming ESBL-producing Pseudomonas and methicillin-resistant (MRSA) strains.

| Strains | MIC of Citrus sinensis extract (µg/ml) | Sub-MIC of Citrus sinensis extract (µg/ml) | MIC of Moringa oleifera extract (µg/ml) | Sub-MIC of Moringa oleifera extract (µg/ml) |
|---------|----------------------------------------|------------------------------------------|----------------------------------------|------------------------------------------|
|         | 1/16xMIC | 1/8xMIC | 1/4xMIC | 1/2xMIC | 1/16xMIC | 1/8xMIC | 1/4xMIC | 1/2xMIC |
| ESBL    | PS1 200 | 12.5 | 25 | 50 | 100 | 1200 | 75 | 150 | 300 | 600 |
|         | PS4 300 | 18.7 | 37.5 | 75 | 150 | 1000 | 62.5 | 125 | 250 | 500 |
|         | PS6 100 | 6.25 | 12.5 | 25 | 50 | 800 | 50 | 100 | 200 | 400 |
|         | PS8 400 | 25 | 50 | 100 | 200 | 1600 | 100 | 200 | 400 | 800 |
|         | PS11 350 | 21.8 | 43.7 | 87.5 | 175 | 1000 | 62.5 | 125 | 250 | 500 |
| MRSA    | SA2 100 | 6.25 | 12.5 | 25 | 50 | 800 | 50 | 100 | 200 | 400 |
|         | SA4 400 | 25 | 50 | 100 | 200 | 1600 | 100 | 200 | 400 | 800 |
|         | SA5 600 | 37.5 | 75 | 150 | 300 | 2000 | 125 | 250 | 500 | 1000 |
|         | SA8 200 | 12.5 | 25 | 50 | 100 | 1200 | 75 | 150 | 300 | 600 |

The sub-lethal concentrations dose were selected for biofilm inhibition assay (Table 4). The adding of extracts of *C. sinensis* and *M. oleifera* at respective 1/2 x MIC at the beginning of the growth showed no change in the growth of strong biofilm-positive MRSA (SA2, SA4, SA5, and SA8) (Figure 2A). A similar pattern was also observed for ESBL-positive, strong biofilm-producing *P. aeruginosa* (PS1, PS2, PS6, PS12, and PS18) for *C. sinensis* and *M. oleifera* at respective 1/2 x MIC (Figure 2B). To avoid the reduction of biofilm formation activity, this sub-MIC was performed.
FIGURE 2: Inhibition of biofilm formation by sub-inhibitory concentrations of [A] C. sinensis extract; [B] M. oleifera extract. The data represent the mean values of three independent experiments.

*C. sinensis*: *Citrus sinensis*; *M. oleifera*: *Moringa oleifera*

### Inhibition of mono-culture biofilm by *C. sinensis* extract and *M. oleifera* extract

Figure 2A bar graphs indicate the inhibition of biofilm formation by sub-inhibitory concentrations of *C. sinensis* extract (1/16 x MIC - 1/2 x MIC). The 14%-68% reduction of biofilm formation by ESBL-producing *P. aeruginosa* (PS11); 16%-68% by PS1; 17%-62% by PS6; 61%-10% by PS4; and 12%-56% by PS8 as compared to control. The 12%-59% of MRSA (SA2); followed by SA8 (17%-58%); SA4 (11%-57%), and SA5 (6%-57%) (Table 5, panel A).
### TABLE 5: Reduction percentage of sub-inhibitory concentrations (for monoculture at 1/16xMIC-1/2xMIC; mixed culture at 1/16xMIC-1/8xMIC-1/4xMIC-1/2xMIC) of C. sinensis extract and M. oleifera extract against biofilm formation

*C. sinensis*: *Citrus sinensis*; *M. oleifera*: *Moringa oleifera*; *MIC*: minimum inhibitory concentration

| Isolate Name | Reduction percentage at 1/16xMIC | Reduction percentage at 1/2xMIC | Reduction percentage at 1/16xMIC | Reduction percentage at 1/2xMIC |
|--------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| PS1          | 16%                             | 68%                             | 31%                             | 86%                             |
| PS4          | 10%                             | 61%                             | 30%                             | 84%                             |
| PS6          | 17%                             | 62%                             | 30%                             | 76%                             |
| PS8          | 12%                             | 56%                             | 36%                             | 80%                             |
| PS11         | 14%                             | 68%                             | 24%                             | 77%                             |
| SA2          | 12%                             | 59%                             | 28%                             | 75%                             |
| SA4          | 11%                             | 57%                             | 25%                             | 72%                             |
| SA5          | 6%                              | 57%                             | 28%                             | 73%                             |
| SA8          | 17%                             | 58%                             | 31%                             | 72%                             |

The bar graphs in Figure 2B graphs indicate the inhibition of biofilm formation by sub-inhibitory concentrations of *M. oleifera* extract (1/16 x MIC - 1/2 x MIC). The 31%-86% reduction of biofilm formation by ESBL-producing *P. aeruginosa* (PS1); 30%-84% by PS4; 36%-80% by PS8; 24%-77% by PS11; and 30%-75% by PS6 as compared to control. Then 28%-75% by MRSA (SA2); followed by SA5 (28%-73%); SA4 (25%-72%) and SA8 (31%-72%) (Table 5).

**Inhibition of mixed biofilm**

Figure 3 (panels A-B) illustrates the comparative antibiofilm activity of *C. sinensis* extract and *M. oleifera* extract against the mixed culture (PS1+SA8, PS6+SA2, and PS8+SA4). The overall antibiofilm activity of *M. oleifera* was reported better as compared with *C. sinensis* (Table 5 panel B) when compared with controls at 1/16xMIC-1/8xMIC-1/4xMIC-1/2xMIC, respectively.
Discussion

Biofilms are being identified as crucial in human disease, and the number of infections associated with biofilms is growing [6]. S. aureus, for example, has been shown to be among the most difficult pathogens implicated in a number of infections [15] such as indwelling medical device (IMD) infections associated with it. It has been noted that it is getting impossible to remove Staphylococcus spp biofilm infections when most of the drugs on the market have to be used as combination therapies [16]. Likewise, P. aeruginosa has arisen within immune-compromised individuals as one of the chief reasons for nosocomial infections [17].

The ESBL detection test showed 60% of P. aeruginosa were ESβL producers and the maximum of them showed high resistance to piperacillin (85.0%), which resembles the findings outlined by Harris et al. [18]. Piperacillin-tazobactam is commonly used in seriously ill patients to treat P. aeruginosa infections. The new U.S. medications are ceftazidime-avibactam and ceftolozane-tazobactam. Food and Drug Administration (FDA)-approved combinations of cephalosporin-β-lactamase inhibitors to treat Gram-negative bacilli-induced infections, including P. aeruginosa [19]. Similarly, the resistance of MRSA was also high (65%) against gentamycin and amikacin antibiotics, respectively, and 45% of S. aureus shows MRSA activity. The maximum isolates of S. aureus (84.5%) were comparable to other studies in terms of resistance to gentamycin [20].

The mono species biofilm formation among MRSA S. aureus and ESβL-positive P. aeruginosa were classified as strong, moderate, weak, and negative. On observing the 12 ESBL-positive Pseudomonas strains, it was found that five strains (PS1, PS4, PS6, PS8, and PS11) formed strong biofilms. In the case of methicillin-resistant S. aureus, four strains show strong biofilm activity (SA2, SA4, SA5, and SA8). Strong biofilm (referring to ++++) showed a significantly higher likelihood of tolerance or resistance to antibiotics that will probably result in therapeutic failure in MRSA infections [21]. There is no prevailing opinion to date on the categorization of S. aureus isolates predicated on their biofilm-forming capability. The concept of a strong, medium, weak, and non-biofilm producer consequently varies widely between studies [22].
The MIC of *C. sinensis* extract and *M. oleifera* extract against strong biofilm producers is in the range of 50-2000 μg/ml concentrations after overnight incubation. Similar to our study, the study by Ahmad and Aqil [23] showed the MICs measured from 64 to 1024 μg/ml for cefotaxime, cefuroxime, ampicillin, and penicillin.

Adding the extracts of *C. sinensis* and *M. oleifera* at respective 1/2 x MIC at the beginning of the growth showed no change in the growth of strong biofilm-positive methicillin-resistant *S. aureus* (SA2, SA4, SA5, and SA8). A similar pattern was also observed for ESBL-positive, strong biofilm-producing *P. aeruginosa* (PS1, PS2, PS6, PS12, and PS18) for *C. sinensis* and *M. oleifera* at respective 1/2 x MIC. The formation of biofilms depends on several variables, including the environment, nutrient availability, geographical origin, specimen forms, and properties of surface adhesion and genetic composition of the species [24]. The data may have been influenced by these variables and led to the high prevalence found in the current research. It is not, however, understood as to how these variables are concerned.

Biofilms include MRSA and ESBL with a defensive barrier to withstand antibiotic therapy. A remarkable result of the research is that the peel extract of *C. sinensis* and a flesh extract of *M. oleifera* efficiently inhibited the biofilm-formation by the addition of sub-inhibitory concentrations of (1/16 x MIC - 1/2 x MIC) MRSA and ESBL, respectively. This indicates the acquired effect was dose-dependent. The better biofilm reduction by ESBL-producing *P. aeruginosa* is observed at higher concentrations of *C. sinensis* extracts at 14%-68% in PS11. Similar findings have been observed by Abraham et al. [25], who documented that methanolic caper extraction substantially inhibited biofilm formation and extracellular polymeric substance (EPS) development in *Proteus mirabilis*, *P. aeruginosa*, *Serratia marcescens*, and *E. coli*. Likewise, the better biofilm reduction of MRSA is observed at higher concentrations at 12%-59% in SA2. A similar pattern was also observed in *M. oleifera* extracts (1/16 x MIC - 1/2 x MIC). The 31%-86% % reduction of biofilm formation by ESBL-producing *Pseudomonas* (PS1); 30%-84% by PS4; 36%-80% by PS8; 24%-77% by PS11; and 30%-75% by PS6 as compared to controls. The 28%-75% by MRSA (SA2); followed by SA5 (28%-73 %), SA4 (25%-72%), and SA8 (31%-72%). The existence of previously identified flavonoids, such as queretin, kaempferol, naringenin, and apigenin, which are capable of reducing biofilm synthesis because they can suppress the activity of the autoinducer-2 responsible for cell-to-cell contact, can explain the inhibition of biofilm formation [26]. The inhibitory activity demonstrated by *C. sinensis* and *M. oleifera* could be derived from its ability to synthesize metabolites that can prevent the formation of biofilms. This result, however, needs to be further investigated through sophisticated qualitative and quantitative studies. A literature survey reveals that the antibiotic activity by sub-inhibitory concentrations of *C. sinensis* and *M. oleifera* against MRSA and ESBL is scanty. Though, the antibiotic perspective of flavonoids extracted from *M. oleifera* seed coat in contradiction to *S. aureus* is stated from India [27].

Our findings concerning the function of antibiofilms agree with previous work carried out in other terrestrial plant species from various parts of the world. Research from India, for example, reported that the Vetiveria zizanioides root extract showed an inhibition reduction in MRSA biofilm formation [28]. Similarly, another study conducted in Brazil found that *Piper regnellii*’s dichloromethane extract weakens biofilm formation [29].

The mono and mixed-species biofilm activity was quantified against *M. oleifera* and *C. sinensis*. The findings of the antibiofilm activity comparatively showed that the extract of *M. oleifera* was reported better as compared with *C. sinensis* against the mixed culture (PS1+SA8, PS6+SA2, and PS8+SA4). Hence, it is recommended to use *M. oleifera* as an option to monitor the development of microbial biofilms or as a model for looking for better medicines. Clinically, biofilm infections are significant, whereas bacteria show recalcitrance to antimicrobial compounds. Large concentrations of antimicrobials may be required to eliminate biofilm producers. Owing to the possibility of toxicity and the associated side effects, this might not always be feasible in vivo, however, low-concentration combination therapies can, therefore, be effective in eradicating staphylococcal biofilm-related infections, including those induced by MRSA [30]. For the selection of a suitable antimicrobial agent, the early screening and detection of biofilm producers followed by their antimicrobial susceptibility tests is essential.

**Conclusions**

In the present study, according to my knowledge, this was the first report of the antimicrobial and antibiofilm activity of *M. oleifera* and *C. sinensis* in the Kingdom of Saudi Arabia. This study also provides better efficacy of *M. oleifera* over *C. sinensis* in its antibiofilm activity in both mono and mixed culture in vitro experiments. Further studies are required on the molecular mechanism involved in controlling the resistance by *M. oleifera*. It could, therefore, be concluded that new groups of anti-biotic leads will be provided by the bioassay-guided fractionation and purification of *M. oleifera* and *C. sinensis*.

**Appendices**

**Preparation of *C. sinensis* peel powder**

*C. sinensis* (oranges) were procured from the local fruit market and any extraneous materials were first by washing. The orange peel was resized into 1x1 inch and dried at 40°C till its moisture content reduced to <5%. It was finely ground into powder. The 60 gram of fine powder of peel powder was dissolved in 160 ml of...
absolute ethanol at room temperature for three successive days. Using Whatman paper, the supernatant was filtered, and residues were used for a second and third extraction. For three days, dissolved parts were filtered and stored in a glass bottle. After the third extraction, the filtrates were then evaporated under reduced pressure at 50°C using a rotary evaporator to yield the crude extract [12].

Percentage Yield (%) = \( \frac{\text{Dry weight of the extract}}{\text{Dry weight of plant material}} \times 100 \)

The crude extract was collected in a vial for further use.

**Preparation of M. oleifera (fruit flesh) powder**

*M. oleifera* was procured from the local fruit market and any materials were first by washing. The flesh was resized into 1x1 inch and dried at 40°C till its moisture content reduced to <5%. It was finely ground into a powder. The 60 gram of fine powder of peel powder was dissolved in 160 ml of absolute ethanol at room temperature for three successive days. Using Whatman paper, the supernatant was filtered and residues were used for a second and third extraction. For three days, dissolved parts were filtered and stored in a glass bottle. After the third extraction, the filtrates were then evaporated under reduced pressure at 50°C using a rotary evaporator to yield the crude extract [12].

Percentage Yield (%) = \( \frac{\text{Dry weight of the extract}}{\text{Dry weight of plant material}} \times 100 \)

The crude extract was collected in a vial for further use.

**Effect of C. sinensis extract and M. oleifera extract on mono and mixed-species biofilm**

In brief, each strain (*S. aureus* and *P. aeruginosa*) was grown overnight at 37°C in tryptic soy broth (TSB) and diluted to 1 x 106 CFU/ml in the TSB. Equal numbers (1:1) of each bacterium were mixed together and the mixed bacterial suspension (100 µl) was added to each well of the polystyrene 96-well tissue-culture plates. Each well was filled with 100 µl of fresh TSB containing different concentrations of *C. sinensis* extract (1/16-1/2 x MIC). Negative control wells contained TSB only, and wells with no additives were used as positive controls. After incubation for 48 h at 37°C, plates were gently washed with 1X phosphate-buffered saline (PBS; pH 7.4) and stained with 100 µl of 0.1% crystal violet (CV) (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature. Excess CV was removed by washing, and biofilm was quantified by measuring the corresponding OD590 nm of the supernatant following the solubilization of CV in 95% ethanol. The experiment was repeated with *M. oleifera* extract effect on mono and mixed biofilm study [14].

**Additional Information**

**Disclosures**

**Human subjects:** All authors have confirmed that this study did not involve human participants or tissue.

**Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue.

**Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: Payment/services info: All authors have declared that no financial support was received from any organization for the submitted work. Financial relationships: All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. Other relationships: All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

**Acknowledgements**

The author is very thankful to all the associated personnel in any reference that contributed to/for this research.

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