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Original Article

Reversing the biofilm-inducing effect of two xanthones from *Garcinia mangostana* by 3-methyl-2(5H)-furanone and N-butyryl-D-L homoserine lactone

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ABSTRACT

Background: According to the WHO, 12 bacteria cause numerous human infections, including Enterobacteriaceae *Klebsiella pneumoniae*, and thus represent a public health problem. Microbial resistance is associated with biofilm formation; therefore, it is critical to know the biofilm-inducing potential of various compounds of everyday life. Likewise, the reversibility of biofilms and the modulation of persister cells are important for controlling microbial pathogens. In this work, we investigated the biofilm-inducing effects of xanthones from *Garcinia mangostana* on *Klebsiella pneumoniae*. Furthermore, we investigated the reversal effect of 3-methyl-2(5H)-furanone and the formation of persister cells induced by xanthones and their role in modulating the biofilm to the antibiotic gentamicin.
Methods: To analyze the biofilm-inducing role of xanthones from *Garcinia mangostana*, cultures of *K. pneumoniae* containing duodenal probe pieces were treated with 0.1–0.001 μM α- and γ-mangostin, and the biofilm levels were measured using spectrophotometry. To determine biofilm reversion, cultures treated with xanthones, or gentamicin were mixed with 3-methyl-2(5H)-furanone or N-butyryl-DL-homoserine lactone. The presence of *K. pneumoniae* persister cells was determined by applying the compounds to the mature biofilm, and the number of colony-forming units was counted.
Results: The xanthones α- and γ-mangostin increased *K. pneumoniae* biofilm production by 40% with duodenal probes. However, 3-methyl-2(5H)-furanone at 0.001 μM reversed biofilm formation by up to 60%. Moreover, adding the same to a culture treated with gentamicin reduced the biofilm by 80.5%. This effect was highlighted when 3-methyl-2(5H)-furanone was administered 6 h later than xanthones. At high concentrations of α-mangostin, persister *K. pneumoniae* cells in the biofilm were about 5 – 10 times more abundant than cells, whereas, with γ-mangostin, they were about 100 times more.
Conclusion: Two xanthones, α- and γ-mangostin from *G. mangostana*, induced biofilm formation in *K. pneumoniae* and promoted persister cells. However, the biofilm formation was reversed by adding 3-methyl-2(5H)-furanone, and even this effect was achieved with gentamicin. In addition, this compound controlled the persister *K. pneumoniae* cells promoted by α-mangostin. Thus, synthetic, and natural biofilm-inducing compounds could harm human health. Therefore, avoiding these substances and looking for biofilm inhibitors would be a strategy to overcome microbial resistance and recover antibiotics that are no longer used.

Introduction

The COVID-19 pandemic has revealed a severe global public health problem related to bacterial resistance to misused and overused antibiotics (CDC, 2022; Iskandar et al., 2022). Antibiotic treatments are long, expensive, have limited effectiveness, and cause a high mortality

List of abbreviations: COVID-19, Coronavirus Disease; WHO, World Health Organization; QS, Quorum Sensing; NMR, Nuclear Magnetic Resonance; CFU, Colony Forming Unit; LB, Luria-Bertani broth; IPS, Institución Prestadora de Salud (Spanish); F, 3-methyl-2(5H)-furanone; HSL, N-butyryl-DL homoserine lactone.

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rate. Besides this, nosocomial infections are frequently increasing. For these reasons, the World Health Organization (WHO) has issued an alert regarding the shortage of antibiotics, estimating that by 2050, there will be around 50 million human deaths due to bacterial infections. Similarly, it has also called for more attention to the search for new antibacterial strategies and molecules (WHO, 2018), calling governments to pay more attention to fostering scientific research in these fields.

Klebsiella pneumoniae is a significant Enterobacteriaceae considered as one of the opportunistic pathogens causing broad spectra of diseases and showing increasingly frequent acquisition of resistance to antibiotics, such as β-lactam and carbapenem molecules (CDC, 2019; Effah et al., 2020; Navon-Venezia et al., 2017; Ventola, 2015). Extended-spectrum beta-lactamase (ESBL)-producing enterobacteria cause about 26,000 healthcare-associated infections and 1700 yearly deaths (CDC, 2019). In addition, it is estimated that each year, enterobacteria cause 140,000 infections in the USA, of which, 9300 are caused by carbapenem-resistant bacteria (Ventola, 2015).

There are several biochemical sources of bacterial resistance, although the usual one relates to forming a biofilm of complex polysaccharides. This structure is a part of the bacterial communication system called quorum sensing (QS), which includes other virulence factors such as the production of an impermeable biofilm and the synthesis of toxins, proteases, siderophores, and motility elements like fimbriae. Therefore, treating nosocomial infections has become a severe drawback, mainly due to reduced antibiotic permeability and a modified host cellular metabolism (Galani et al., 2021; Rémy et al., 2018). Thus, biofilm cells are 100 – 1000 times more antibiotic-resistant than planktonic cells (Olsen, 2015).

Usually, homoserine lactone derivatives and many other synthetic and natural molecules have been reported as inducers and inhibitors of biofilms (Silva et al., 2016). Therefore, it is likely that other factors external to bacterial biochemistry, such as some food molecules, drugs and phytotherapeutics, may also induce responses in the QS and thus indirectly stimulate microbial virulence, all of which pose a risk to human health. Consequently, we analyzed the biofilm-inducing roles of two natural xanthones, α- and γ-mangostins, reported in *Garcinia* species, such as *Garcinia mangostana L.* and *Garcinia canbogia* (Gaertn.) Desr. (Clusiaceae) on a clinical isolate of *K. pneumoniae*. The fruits of these species are widely used in traditional medicine, foods, and nutraceuticals (Chen et al., 2018; Pinto et al., 2021). Furthermore, considering the possibility of using QS inhibitor molecules as adjuvants to currently used antibiotics (Miethke et al., 2021), we studied the possibility of reversing of the biofilm-induced effect and cell persister formation of the main xanthones of *Garcinia mangostana* L. α-mangostin and γ-mangostin and the antibiotic-resistance of gentamicin by the natural product 3-methyl-2(5H)-furanone.

Material and methods

Chemicals and reagents

The compounds 3-methyl-2(5H)-furanone F and N-butyryl-DL-homoserine lactone HSL were obtained from SIGMA-Merck (St. Louis, MO, USA).

α-mangostin and γ-mangostin (Fig. 1) were obtained from the endosperm of *Garcinia mangostana* in a previous work (Upegui et al., 2015) and identified by NMR. A stock solution of 2 mg/ml of all compounds was prepared in methanol; dilutions were made using Luria-Bertani broth (LB medium) until final concentrations of 0.1 – 0.001 μM.

Clinical bacterial isolates

The clinical isolates of *K. pneumoniae* were provided by "*Institución Prestadora de Salud*" (IPS) Universitaria, Medellín, Colombia, grown on solid nutrient agar, and then incubated at 37 °C for 24 h The bacterial inoculum was prepared in sterile saline by surface scraping with a microbiological loop, and the bacterial inoculum was measured spectrophotometrically at a wavelength of 600 nm using sterile saline as a blank. The absorbance of the inoculum was adjusted to an optical density of 0.02 to obtain a final cell density of 1.0×10^6 CFU/ml.

The antibiotic resistance profile was determined using the VITEK 2 automated equipment (BioMérieux ®, Marcy lEtoile, France) at the IPS Universitaria, with the bacterial inoculum adjusted on a MacFarland scale (0.50 – 0.63). It was also determined by using antibiotic discs by quantifying the diameter of the inhibition halo through ImageJ (BD BBL™ Sensi-Disc™). Antibiotic assays were routinely used to determine a resistance profile, gentamicin, ciprofloxacin, tetracycline, rifampicin, clindamycin, erythromycin, penicillin, vancomycin, oxacillin, ampicillin, and ampicillin/sulbactam.

Determining the minimum inhibitory concentration of xanthones

A clinical isolate of *K. pneumoniae* was grown overnight on solid nutrient agar at 37 °C. The standard broth dilution method determined xanthones' minimum inhibitory concentrations (MICs) (CLSI, 2020). A culture of *K. pneumoniae* at a cell density of 1.0×10^6 Colony Forming Unit (CFU/ml) was exposed to different concentrations of xanthones in 96-well microplates (Merck, St Louis, Mo. USA) and subsequently incubated at 37 °C for 20 h Quantification was performed spectrophotometrically at a wavelength of 600 nm. The blank and growth control were the wells without bacteria or xanthones.

Evaluation of biofilm formation

Biofilm formation under static conditions was carried out on 2.5 cm Medex PVC medical-grade FR18 duodenal probes purchased at a local

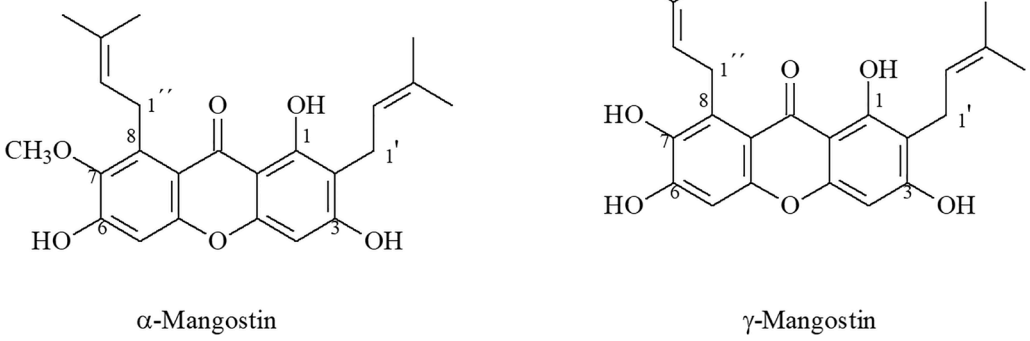

Fig. 1. Structures of α-mangostin and γ-mangostin.

pharmacy; these were placed inside 1.5 ml Eppendorf tubes containing LB medium and LB medium with α-mangostin, γ-mangostin, 3-methyl-2 (5H)-furanone, N-butyryl-DL-homoserine lactone, and gentamicin at concentrations of 0.1 – 0.001 μM, and then the tubes were incubated at 37 °C for 30 h Subsequently, the tubes were washed three times with sterile distilled water. The biofilm associated with the duodenal probe and the Eppendorf tube was treated with 0.05% crystal violet and eluted with 1 ml of 96% ethanol; the crystal violet was quantified by spectrophotometry at a wavelength of 575 nm. Duodenal probes without bacteria or xanthones were used as the blank and growth control, respectively. The results were normalized to the biofilm in the growth control, corresponding to 100%. The percentage of biofilm was measured using the following formula:

$$\text{Biofilm}\% = \left(\frac{Control\ OD\ 575\ nm - treated\ OD\ 575\ nm}{Control\ OD\ 575nm}\right)x100$$

Reversion of biofilm formation

Eppendorf tubes containing *K. pneumoniae*, culture medium, and probes were treated with xanthones at 0.01 μM and 3-methyl-2(5H)-furanone or N-butyryl-DL-homoserine lactone at concentrations ten times lower than that used for xanthones, 0.001 μM, and incubated at 37 °C for 30 h Likewise, in another experiment, these substances were added 6 h after the addition of xanthones and incubated at 37 °C for 24 h The separation and quantification of the biofilm were carried out as above.

Detection of persister K. pneumoniae cells in cultures treated with xanthones

The previously described protocol (Cruz et al., 2018) was followed with some modifications. A 24-hour culture of *K. pneumoniae* formed in polystyrene 96-well microplates was used for persister cell assays. The supernatant was discarded, and the biofilm formed was washed thrice with 200 μl of sterile saline. Then, the two xanthones or gentamicin dissolved in LB medium at 0.1 – 0.001 μM were added to the remaining film and incubated for 6 h at 37 °C. The supernatant containing free bacteria was then collected. The formed biofilm was washed once with 200 μl of sterile saline and suspended in 200 μl of LB medium by vigorous pipetting and scraping.

Both supernatant and biofilm solutions were transferred to a new 96-well microplate and diluted 10-fold in sterile saline. Finally, 5 μl of each dilution was dropped on the agar, and the CFU was counted after 20 h of incubation at 37 °C.

In another experiment, a 24 h biofilm induced by the addition of xanthones or gentamicin at concentrations of 0.01 μM was incubated for 6 h with N-butyryl-DL-homoserine lactone or 3-methyl-2(5H)-furanone at concentrations 10 times lower, 0.001 μM. The procedure described above was then followed. Each experiment was performed in duplicate.

Statistical analysis

Biofilm formation data were presented as mean and standard deviation (SD, experiments were performed in triplicate and repeated two times). All results followed a normal distribution and homoscedasticity, so the differences were evaluated using one-way ANOVA. The experiments of persister cells have been evaluated using an unpaired two-tailed *t*-test. $p < 0.05$ was statistically significant.

Results

MIC of xanthones

Natural xanthones did not appreciably modify the viability of *K. pneumoniae* in the range of 0.09 – 12.5 μM as bacterial growth was 85 – 115% (*See supplementary material*). On the other hand, the antibiogram showed resistance to ampicillin, ampicillin/sulbactam, tetracycline, clindamycin, erythromycin, penicillin, vancomycin, and oxacillin, and susceptibility to ciprofloxacin, cefazolin, ceftriaxone, cefepime, aztreonam, and tigecycline.

Effect of xanthones from G. mangostana on biofilm formation of K. pneumoniae

The ability of xanthones and several molecules to modulate the biofilm formation on *K. pneumoniae* was determined in the first instance. Both xanthones, α-mangostin and γ-mangostin, especially the former, were potent inducers of biofilm in *K. pneumoniae* at all concentrations assayed, reaching levels up to 58.7% (Fig. 2). However, they were generally more effective at low concentrations. At 0.1 μM and 0.01 μM N-butyryl-DL-homoserine lactone, a known QS autoinducer in Gram (-) bacteria, also presented a high biofilm-inducing effect of α-mangostin and γ-mangostin to 40 and 12.7%, respectively ($p < 0.05$). On the other hand, 3-methyl-2(5H)-furanone, whose inhibitory activity was previously demonstrated (Cadavid et al., 2018) at the lowest concentration, remarkably inhibited biofilm formation by 56.4%, but the recognized antibiotic gentamicin at 0.01 μM induced biofilm by 44.3% ($p < 0.05$).

Effect of N-butyryl-DL homoserine lactone on biofilm induction by xanthones

The simultaneous addition of N-butyryl-DL homoserine lactone HSL at a concentration ten times lower than that of xanthones paradoxically reduces their initial biofilm-inducing effect that was previously detected (Fig. 2), even presenting a net inhibition value of 75.3% (effect% of α-mangostin pure - effect% after HSL addition) with α-mangostin at 0.01 μM (Fig. 3). In addition, a slight biofilm inducer effect of 21.8% ($p < 0.05$) was observed in γ-mangostin. However, when the HSL addition was late, 6 h afterwards, these xanthones maintained their biofilm-inducing potential.

Effect of 3-methyl-2(5H)-furanone on biofilm induction by xanthones

The simultaneous addition of the xanthones and 3-methyl-2(5H)-furanone, a potent 56.4% biofilm inhibitor as demonstrated above at 0.001 μM to the *K. pneumoniae* culture, completely inhibited the initial biofilm-inducing effect caused by both α and γ-mangostin (Fig. 4). Furthermore, when this addition was made after 6 h of xanthone addition, the inducing effects of α-mangostin and γ-mangostin were slightly recovered on biofilm formation.

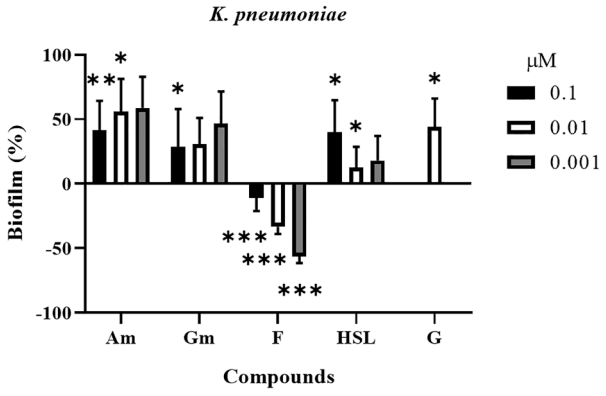

Fig. 2. Effects of compounds on biofilm induction in *K. pneumoniae* at different concentrations. Natural xanthones Am, α-mangostin; Gm, γ-mangostin; F, 3-methyl-2(5H)-furanone; HSL, N-butyryl-DL-homoserine lactone, a compound used in the control experiment; G, gentamicin 0.01 μM. Data represent the mean of two independent experiments (± SD), ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. The dashed line represents growth control without any treatment.

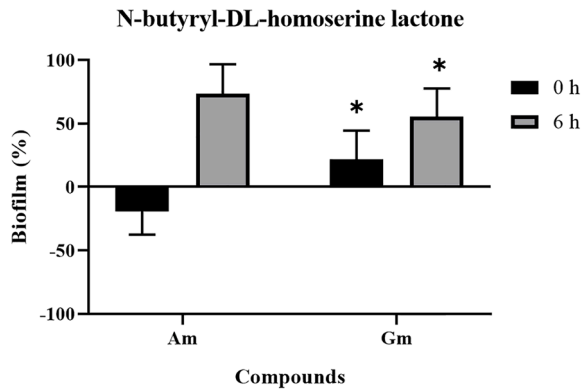

Fig. 3. Effects of N-butyryl-DL-homoserine lactone on xanthones added simultaneously (0 h) and at the sixth hour (6 h) of *K. pneumoniae* culture. Am, α-mangostin; Gm, γ-mangostin. Xanthones were evaluated at 0.01 μM, while N-butyryl-DL-homoserine lactone was evaluated at 0.001 μM. Data represent the mean of two independent experiments (± SD), $*p < 0.05$.

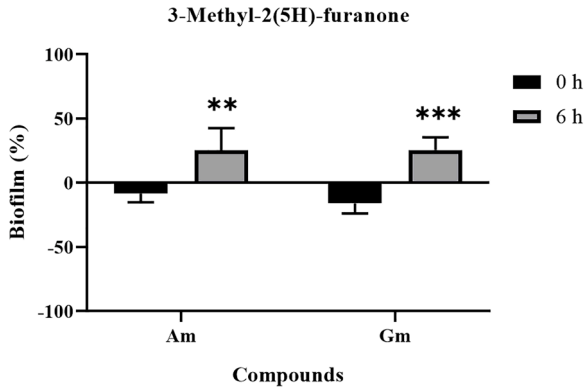

Fig. 4. Effect of 3-methyl-2(5H)-furanone on xanthones added simultaneously (0 h) and at the sixth hour (6 h). Am, α-mangostin; Gm, γ-mangostin. Xanthones were evaluated at 0.01 μM, while 3-methyl-2(5H)-furanone was evaluated at 0.001 μM. Data represent the mean of two independent experiments (± SD), $***p < 0.001$, $**p < 0.01$.

Effect of 3-methyl-2(5H)-furanone and N-butyryl-DL homoserine lactone on biofilm induction by gentamicin

The simultaneous application (time 0 h) of 3-methyl-2(5H)-furanone at only 0.001 μM completely abolished the biofilm-inducing effects of gentamicin (Fig. 5), causing now an inhibitor effect of 64%, and a net change of biofilm by 80.5%. However, when 3-methyl-2(5H)-furanone was applied after the sixth hour of microbial culture, the inducing effect of gentamicin was recovered until 22.5%. The effects of HSL were also drastically reduced with the addition of 3-methyl-2(5H)-furanone, and, as with gentamicin, there was a slight 12.8% biofilm inhibitory effect at 0 h and a net change in the biofilm by 25.5%.

When HSL was applied at the sixth hour, the gentamicin-inducing effect recovered at 12.7%, while the inhibitory effect of 3-methyl-2(5H)-furanone was reversed, causing an induction of 21.8% (Fig. 6).

The complete results related to the modulation of the biofilm using pure and mixed substances are summarized in Table 1. The effects were calculated regarding the variation of the individual net effect and the value obtained by the mixture of substances.

Persister K. pneumoniae cells

The presence of persister cells in the formed biofilm was established by determining the ability of the biofilm formed at 30 h to generate CFU.

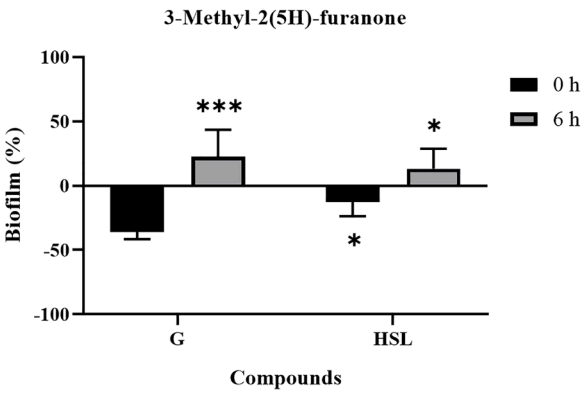

Fig. 5. Effects of 3-methyl-2(5H)-furanone added simultaneously or at 6 h to *K. pneumoniae* cultures treated or gentamicin G and N-butyryl-DL-homoserine lactone HSL. The latter two compounds were evaluated at 0.01 μM while the former was at 0.001 μM. Data represent the mean of two independent experiments (± SD), $***p < 0.001$, $*p < 0.05$.

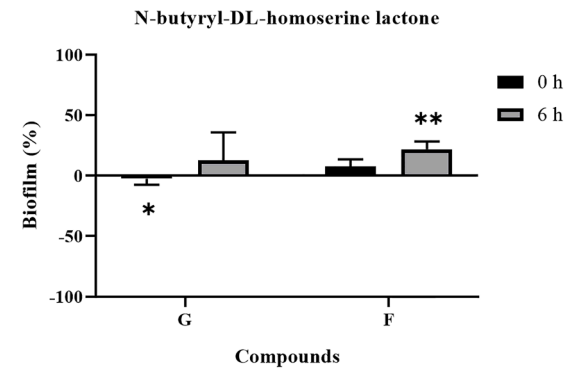

Fig. 6. Effects of N-butyryl-DL-homoserine lactone HSL added simultaneously or at 6 h to *K. pneumoniae* cultures treated with gentamicin G and 3-methyl-2 (5H)-furanone F. The latter two compounds were evaluated at 0.01 μM while the former was at 0.001 μM. Data represent the mean of two independent experiments (± SD), $***p < 0.001$, $*p < 0.05$.

Table 1
Effects of substances on biofilm modulation via xanthones in *K. pneumoniae* when applied simultaneously or 6 h later.

Compound 0.01 μM	Effect of pure compound on *K. pneumoniae* biofilm (%)	Effect of molecule combination (%). Net change			
		3-methyl-2 (5H)-furanone		N-butyril-DL homoserine lactone	
		0 h	6 h	0 h	6 h
α-mangostin	**56.1**↑	64.3↓	30.9↓	75.3↓	**17.4**↑
γ-mangostin	**30.8**↑	46.7↓	15.3↓	9.0↓	**24.8**↑
Gentamicin	**44.3**↑	80.5↓	21.8↓	46.7↓	**31.6**↑

Note: Arrows indicate an increase (↑) or decrease (↓) in biofilm. The specific effect corresponds to the percentage variation of the biofilm for the normal culture at 0.01 μM, while in the combination, the variation is the net effect of the individual value and that obtained with a combination of molecules.

The compound α-mangostin induces biofilm formation but reduces the 10:1 population difference between persister-embedded cells and free *K. pneumoniae* cells observed in the control experiment in a concentration-dependent manner (Fig. 7). This difference is minimal at 0.01 μM until this effect is reversed at 0.001 μM, and there are more free cells than embedded persister cells. However, γ-mangostin caused a marked reduction in the proportion of cells, such that at 0.1 and 0.01 μM, there are more than 100 times as many free cells as in the biofilm,

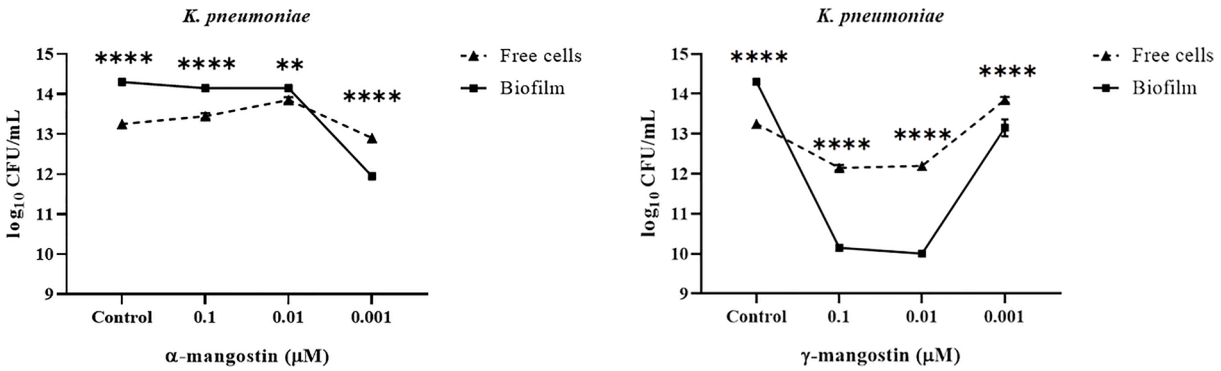

Fig. 7. Effect of xanthones on free cell populations in biofilm. Xanthones were added at 24 h after an established biofilm of *K. pneumoniae*. Data represent the mean of two independent experiments (± SD), $^{****}p < 0.0001$, $^{**}p < 0.01$.

probably indicating the formation of an unstable structure.

The addition of 3-methyl-2(5H)-furanone F and N-butyryl-DL-homoserine (Fig. 8) at 0.001 μM kept the populations of planktonic and *K. pneumoniae* persister-embedded cells almost equal in the biofilm treated with α-and γ-mangostin, but significantly reduced the proportion of embedded persister cells in the biofilm observed in the control, which was about 100. However, the same two molecules caused high biofilm stability induced by γ-mangostin since the persister-embedded and planktonic cells are now practically balanced (Fig. 8).

The effect obtained with gentamicin was like that of 3-methyl-2(5H)-furanone F and N-butyryl-DL-homoserine. Although this antibiotic induces biofilm formation in *K. pneumoniae*, with its application, there are more free cells and, therefore, a less stable biofilm (Fig. 9).

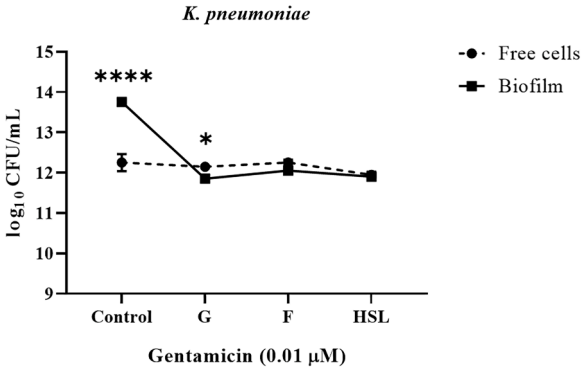

Fig. 9. Effect of 3-methyl-2(5H)-furanone F and N-butyryl-DL-homoserine lactone HSL at 0.001 μM on free and embedded cells in a 24 h biofilm of *K. pneumoniae* induced by gentamicin. Data represent the mean of two independent experiments (± SD), $^{****}p < 0.0001$, $^{*}p < 0.05$.

Discussion

The endosperm of *G. mangostana* is a rich source of xanthones with anti-inflammatory, anticancer, antimicrobial, antiallergic, antiparasite, and anthelmintic activities (Chen et al., 2018; Pinto et al., 2021). However, the best-known application of these compounds is in the nutraceutical diet as an antioxidant. Similarly, *G. canbogia* produces these same compounds and is administered as a dietary supplement to prevent obesity, though this has been controversial.

Microbial resistance is becoming a severe public health problem (CDC, 2019; Navon-Venezia et al., 2017; Ventola, 2015; WHO, 2018). In addition to being life-threatening, resistance to recalcitrant chronic infections, especially those of nosocomial origin, causes a reduction in the patient's quality of life, high costs, a saturation of hospital capacity, and eventually death. Nevertheless, the pharmaceutical industry is uninterested in developing new antibiotics due to the reduced profit margin and the high costs of this process (Miethke et al., 2021). In this sense, QS is a recognized mechanism of bacterial communication through which the synthesis of molecules that favor bacterial virulence, including biofilm formation, is expressed. Several classes of natural products are QS-inhibitory or -inducer compounds (Cadavid et al., 2018; David et al., 2018; Díaz-Nuñez et al., 2021; Silva et al., 2016; Yang et al., 2020). As such, these compounds could also be found in many medicines, foods, beverages, nutraceuticals, and other substances of natural origin used in phytotherapy. Therefore, it is essential to analyze the inducing potential of QS from these compounds since they could be a potentially high risk to human health.

To prevent or eliminate microbial resistance, the modulation of the production and stability of biofilms is a suitable option since this effect does not involve a biocidal effect (Mishra et al., 2020; Pompilio et al.,

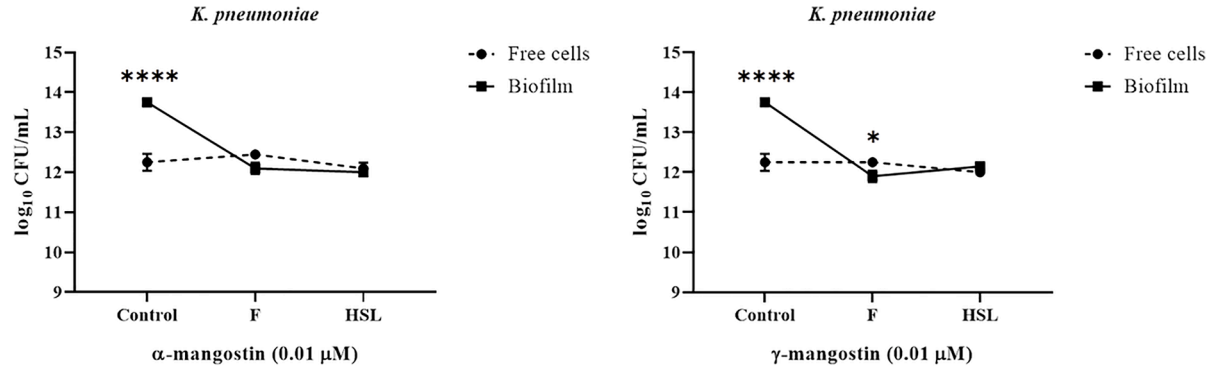

Fig. 8. Effect of 3-methyl-2(5H)-furanone F and N-butyryl-DL-homoserine lactone HSL at 0.001 μM on free and embedded cells in a 24 h biofilm of *K. pneumoniae* induced by α mangostin and γ-mangostin. Data represent the mean of two independent experiments (± SD), $^{****}p < 0.0001$, $^{*}p < 0.05$.

2023). Accordingly, this work evaluated the biofilm-modulating capacity of two xanthones from *G. mangostana* against a clinical strain of *K. pneumoniae*. Previously, these compounds were also analyzed as molecules involved in QS in *Chromobacterium violaceum* based on violacein production (Asfour, 2016; Mohamed et al., 2014), but those results cannot be extrapolated to other pathogenic bacteria such as *K. pneumoniae.*

Thus, we found that α- and γ-mangostin from *G. mangostana* were not antibiotics on the clinical isolate of *K. pneumoniae*, as the MIC detected was > 12.5 μM, above the concentrations used in the biofilm assays, which were in the range of 0.1 to 0.001 μM. At the lowest concentrations, these xanthones showed biofilm-inducing activity on *K. pneumoniae*. Therefore, α-mangostin showed the highest inducing activity (56.1%), while γ-mangostin induced 30.8% (Table 1). Compounds used as controls exhibited the expected response in this essay, and 3-methyl-2(5H)-furanone inhibited the biofilm in a dose-dependent manner by up to 56.4%, while N-butyryl-DL-homoserine lactone induced it by 40% (Fig. 2). However, researchers have proposed the absence of autoinducer synthesis in *K. pneumoniae* and have discovered receptors for these compounds. (Pacheco et al., 2021).

More assays were performed to determine an early or late effect of 3-methyl-2(5H)-furanone and N-butyryl-DL homoserine lactone in the biofilm synthesis by adding to *K. pneumoniae* cultures compounds and xanthones simultaneously or after 6 h of cultures. To avoid influencing the concentration of the compounds, a concentration ten times lower than the natural molecules of *G. mangostana* was applied. The simultaneous application of compounds to xanthones caused a reversal of inducing activity with 3-methyl-2(5H)-furanone at 0.001 μM since α-mangostin reduced the biofilm induction by 64.3% (Table 1). At the same time, the γ-mangostin there was 46.7%. Likewise, N-butyryl-DL-homoserine lactone at 0.001 μM reduced the activity of α-mangostin by 75.3% (Fig. 3) and slightly in γ-mangostin (9.0%). However, when these molecules were applied to the *K. pneumoniae* culture 6 h after the xanthones, the biofilm formation profile underwent another modification (Table 1). Consequently, the inhibitory activity of 3-methyl-2(5H)-furanone was retained but less than in the simultaneous application. The α-mangostin reached 30.9% instead of 64.3% (Fig. 4). In turn, combining with N-butyryl-DL homoserine lactone increased the biofilm-inducing activity in *K. pneumoniae* in all cases (Fig. 2).

The QS inhibitors were proposed to overcome microbial resistance caused by biofilms (Kuo et al., 2015; Melander et al., 2020), reducing the nosocomial infection spreading and eventually recovering the use of old antibiotics. Therefore, it was also possible to establish interactions of compounds used as a control with the antibiotic gentamicin. The behavior of this *K. pneumoniae* clinic isolated to gentamicin was demonstrated by the resistance to eight antibiotics and high biofilm-induction levels, which were close to 20% at 0.01 μM and 44.5% at 0.001 μM, respectively (Fig. 2). The simultaneous application of 3-methyl-5(H)-furanone with gentamicin to *K. pneumoniae* cultures changed the original biofilm-inducing effect of gentamicin (44.5%) to a strong inhibitory effect of 36%, a net change of 80.5% (Table 1, Fig. 5), while N-butyryl-DL-homoserine also caused an appreciable net inhibitory effect, from 46.7% initially to only 9.0%. However, if these two compounds are applied 6 h after culturing, their original effect practically does not change, indicating an irreversible effect in the early stages of biofilm formation.

The observed effects with different types of substances can be interpreted from several biosynthetic points of view. The HSL analysis is complex because it has been reported that *K. pneumoniae* does not produce these compounds but has receptors to its exogenous supply (Pacheco et al., 2021). Therefore, its effect would be more related to possible interactions with the receptor of this type of substance. On the other hand, the early effects (0 h) indicate that the blocking or antagonistic effect may be more related to the biosynthesis of autoinducers, which, in the case of *K. pneumoniae,* is a furanosyl borate diester.

Concerning *K. pneumoniae* persister cells, all compounds, including mangosteens, gentamicin, and 3-methyl-2(5H)-furanone F and N-butyryl-DL-homoserine lactone, caused a population change in cultures compared to the control, in which there were more embedded persister cells in the biofilm than free or planktonic cells, which would indicate a relatively stable biofilm (Figs. 7-9). However, the change in this population difference was more pronounced with the application of gentamicin 3-methyl-2(5H)-furanone F and N-butyryl-DL-homoserine lactone, which was from a 100:1 population difference to an approximate population equilibrium. In this way, the biofilm formed is more unstable, and more free cells achievable by antibiotics are generated.

Our results demonstrate that some natural products pose a health risk because they can induce microbial resistance through biofilm formation. They can also increase the risks of recurrent nosocomial infections by stimulating the presence of persister cells of *K. pneumoniae* in the biofilm. However, several results also indicate that it is possible to reverse biofilm formation using specific molecules and raising the possibility of recovering the effect of antibiotics like gentamicin that currently have reduced activity because of the induced biofilm. Therefore, exploring the concentration effect of reverser substances in-depth is crucial, as they were supplied in quantities ten times lower than those of the inducers, suggesting a potential for more powerful reversal activity.

Conclusion

The xanthones α- and γ-mangostin found in some phytotherapeutics, nutraceutical, and functional foods induce biofilms and persister cells in *K. pneumoniae,* representing a potential risk to human health by generating resistance to antibiotics and increasing the possible spread of microbial infections. This threat is significant for immunosuppressed, older patients or those undergoing surgery and hospitalization. However, 3-methyl-2(5H)-furanone, a known natural product, abolished the induction of the biofilm and that of the antibiotic gentamicin. In addition, this compound also affected the persister cell population. Besides the mentioned risk, all this indicates the possibility of recovering antibiotics that are no longer used.

Finally, other issues besides to MIC must be considered to classify the resistance/susceptibility of a pathogen to an antibiotic since biofilm formation and persister cells are also dangerous factors for human health.

CRediT authorship contribution statement

Maria L. Carmona-Orozco: Investigation, Methodology, Writing – review & editing. **Wiston Quiñones:** Conceptualization, Resources, Writing – review & editing. **Sara M. Robledo:** Conceptualization, Methodology, Supervision, Formal analysis, Validation, Writing – review & editing. **Fernando Torres:** Conceptualization, Resources, Writing – review & editing. **Fernando Echeverri:** Conceptualization, Funding acquisition, Project administration, Supervision, Methodology, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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Supplementary materials

Supplementary material associated with this article can be found, in

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