The antibacterial activity and synergies between morusin and some antibiotics against MRSA strains – preliminary study

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Abstract

Mulberry (Morus alba L., Moraceae) is one of the most valuable and rich in phytochemicals plant. Morusin is a prenylated flavonoid present in mulberry roots and leaves. The in vitro antibacterial activity of morusin and its interactions with conventional antibiotics (oxacillin, amoxicillin and gentamicin) were evaluated against four methicillin resistant Staphylococcus aureus clinical isolates (MRSA T1 – T4) with resistance to oxacillin and cefoxitin which had been isolated from dogs with various pathologies. Minimum inhibitory concentrations (MICs) were determined by the microdilution method. The interactions were assessed by the chequerboard method - with interpretation through fractional inhibitory concentration index (FICI) and isobologram analysis. The interactions were confirmed by the time-kill assay. MICs varied between 3.125 and 6.25µg/mL for morusin alone against all four MRSA clinical isolates. Chequerboard method showed synergies for the combinations: morusin – oxacillin (FICI=0.024 - 0.27), morusin – amoxicillin (FICI=0.024 - 0.27) and morusin - gentamicin (FICI=0.05 - 0.12) against all four tested isolates. Time-kill assay determined synergies for the following combinations: morusin – oxacillin against MRSA T1, morusin – amoxicillin against MRSA T2 and morusin - gentamicin against all four isolates. Our preliminary study evaluated the antibacterial activity of morusin and its ability to act synergistically with antibiotics; these results suggest that morusin might be a promising strategy to overcome antibiotic resistence.

Key-words: *bacterial resistance, chequerboard, morusin, synergy, time-kill assay*

Introduction

Mulberry (*Morus alba* L., Moraceae) is one of the most valuable and rich in phytochemicals plant. Mulberry leaves are used for feeding silkworms due to the high content of proteins (1). Numerous reviews have been published on both *in vitro* and *in vivo* studies that assessed antidiabetic, antioxidant, anticancer, hypolipidemic, antiatherogenic and anti-inflammatory activities of mulberry (2 - 4). Mulberry extracts and their isolated compounds showed antimicrobial potential against harmful pathogens: *Bacilllus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Mycobacterium smegmatis* (5 - 8). Morusin (fig. 1) is a prenylated flavonoid isolated from the root and leaves of mulberry with antibacterial activity against Gram-positive bacteria (9).

The post-antibiotic apocalypse due to the frequent and improper use of antibiotics involves new strategy in overcoming antibiotic resistance (10). Methicillin resistant *S. aureus* (MRSA) is one great concern with challenges because most of the strains are resistant to beta-lactams,

cephalosporins, aminoglycosides, macrolides, fluoroquinolones, but also to other important antibiotics such as glycopeptides (vancomycin and teicoplanin) (11).

A promising strategy in overcoming antibiotic resistance is the synergy between vegetal products and conventional antibiotics (12).

The present preliminary study aimed to assess the antibacterial activity and the interactions between morusin and commonly used antibiotics against MRSA clinical isolates.

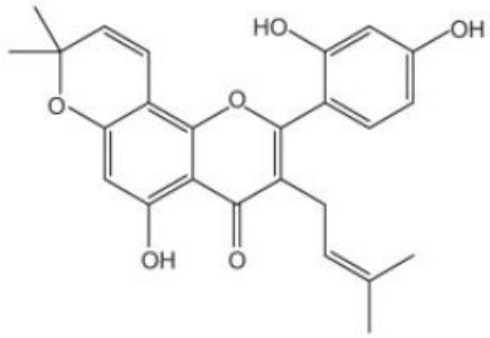


Figure 1. Chemical structure of morusin.

Material and methods

Minimum inhibitory concentrations (MICs) of oxacillin (OX), amoxicillin (Amx), gentamicin (Gn) and morusin (MO) were determined by the microdilution method against four MRSA clinical isolates according to the Clinical & Laboratory Standards Institute (CLSI) (13) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (14). The sources of all clinical isolates resistant to cefoxitin and oxacillin were infections (recurrent otitis, pyoderma and laryngopharyngitis) in dogs.

The interactions between MO and antibiotics were determined using the chequerboard method (15) with interpretation through fractional inhibitory concentration index (FICI) and isobolograms (12).

$$FICI = FIC_{Antibiotic} + FIC_{Morusin} \text{ where:}$$

$$FIC_{Antibiotic} = \frac{M\ IC_{Antibiotic\ in\ combination\ with\ morusin}}{MIC_{Antibiotic\ alone}},$$

$$FIC_{Morusin} = \frac{M\ IC_{Morusin\ in\ combination\ with\ antibiotic}}{MIC_{Morusin\ alone}}.$$

A combination is synergistic if FICI value ≤ 0.5, additive when it is > 0.5 and ≤ 1, indifferent when it is 1 – 4, and antagonistic when it is > 4 (16).

Graphical representation of experimental dose-response surface and theoretical dose-response surface of interaction were performed according to Bliss independence–based model. Experimental dose-response surface ($E_{measured}$) represents the experimental percentage of growth in the presence of different concentrations of MO and/or antibiotics. $E_{predicted}$ is the calculated percentage of growth based on the experimental percentage of growth according to Bliss independence–based model, taking into account the non-interactive process between two components. The difference between predicted ($E_{predicted}$) and measured ($E_{measured}$) dose-response surface is the theoretical dose-response surface of interaction (ΔE). A ΔE value above zero (positive) indicates synergy and below zero (negative) indicates antagonism (15).

Time-kill assay was performed in order to confirm the results obtained in the chequerboard method. According to the time-kill assay, synergy is considered if the decrease in the viable colony count ≥ $2\log_{10}$ CFU/mL; the combination is evaluated in comparison to the count obtained with the most active single component, after 24 or 48 hours. The antagonism is defined as an increase in the colony count of ≥ $2\log_{10}$ CFU/mL, the combination being compared to the count obtained with the most active single component of combination after 24 or 48 hours (16).

Results and discussion

MIC values of MO alone against four MRSA clinical isolates varied between 3.125 and 6.25 µg/mL. The obtained results were in agreement with the already published results. Sohn HY *et al.* have reported MIC values of 5–30 µg/mL for MO against *Streptococcus faecalis, S. aureus, Mycobacterium smegmatis* and *Bacillus subtilis* (9). Our results confirmed the antibacterial activity of MO against Gram-positive bacteria including MRSA strains.

Table 1. *In vitro* interactions between MO and antibiotics determined by the chequerboard method and time-kill assay

	MRSA T1	**MRSA T2**	**MRSA T3**	**MRSA T4**
MIC_{MO} (µg/mL)	6.25	6.25	3.13	6.25
➢ **OX combinations**				
MIC_{OX} (µg/mL) (susceptibility to OX)¥	16 (Resistant)	128 (Resistant)	256 (Resistant)	256 (Resistant)
$MIC_{OX–MO}$; $MIC_{MO–OX}$ (µg/mL)	0.50; 0.10	0.50; 0.10	2; 0.78	4; 1.56
FICI* / TKA**	0.05 (S)/ S	0.024 (S)/ Nc	0.26 (S)/ Nc	0.27 (S)/ Nc
➢ **Amx combinations**				
MIC_{Amx} (µg/mL) (susceptibility to Amx)¥	16 (Resistant)	128 (Resistant)	256 (Resistant)	256 (Resistant)
$MIC_{Amx–MO}$; $MIC_{MO–Amx}$ (µg/mL)	0.50; 0.10	0.50; 0.10	2; 0.78	4; 1.56
FICI* / TKA**	0.05 (S)/ Nc	0.024 (S)/ S	0.26 (S)/ Nc	0.27 (S)/ Nc
➢ **Gn combinations**				
MIC_{Gn} (µg/mL) (susceptibility to Gn)¥	0.25 (Sensible)	0.25 (Sensible)	0.50 (Sensible)	1 (Sensible)
$MIC_{Gn–MO}$; $MIC_{MO–Gn}$ (µg/mL)	0.02; 0.10	0.02; 0.39	0.02; 0.10	0.03; 0.10
FICI* / TKA**	0.08 (S)/ S	0.12 (S)/ S	0.06 (S)/ S	0.05 (S)/ S

Abbreviation: MO – morusin, OX – oxacillin, Amx – amoxicillin, Gn – gentamicin, $MIC_{atb–MO}$ – MIC of antibiotic in presence of MO, $MIC_{MO–atb}$ – MIC of MO in presence of antibiotic; FICI – fractional inhibitory concentration index, S –synergy, Nc– synergy has not been confirmed

*effect of the combination determined through checkerboard method, **effect of the combination determined through time-kill assay, ¥susceptibility to antibiotic according to European Committee on Antimicrobial Susceptibility - Testing Breakpoint tables for interpretation of MICs and zone diameter Version 7.0. Valid from 2017-01-01.

According to the FICI interpretation and isobologram representation (checkerboard method), synergy was observed for combinations MO – OX (FICI = 0.024-0.27; fig. 2a), MO – Amx (FICI = 0.024-0.27; fig. 2b) and MO – Gn (FICI = 0.05-0.12; fig. 2c) against all four MRSA clinical isolates. Fig. 3 describes the experimental design of the checkerboard method and the

synergy obtained for the combinations MO - Gn against MRSA T4. Table 2 summarizes the results of both the checkerboard method and time-kill assay against all MRSA clinical strains.

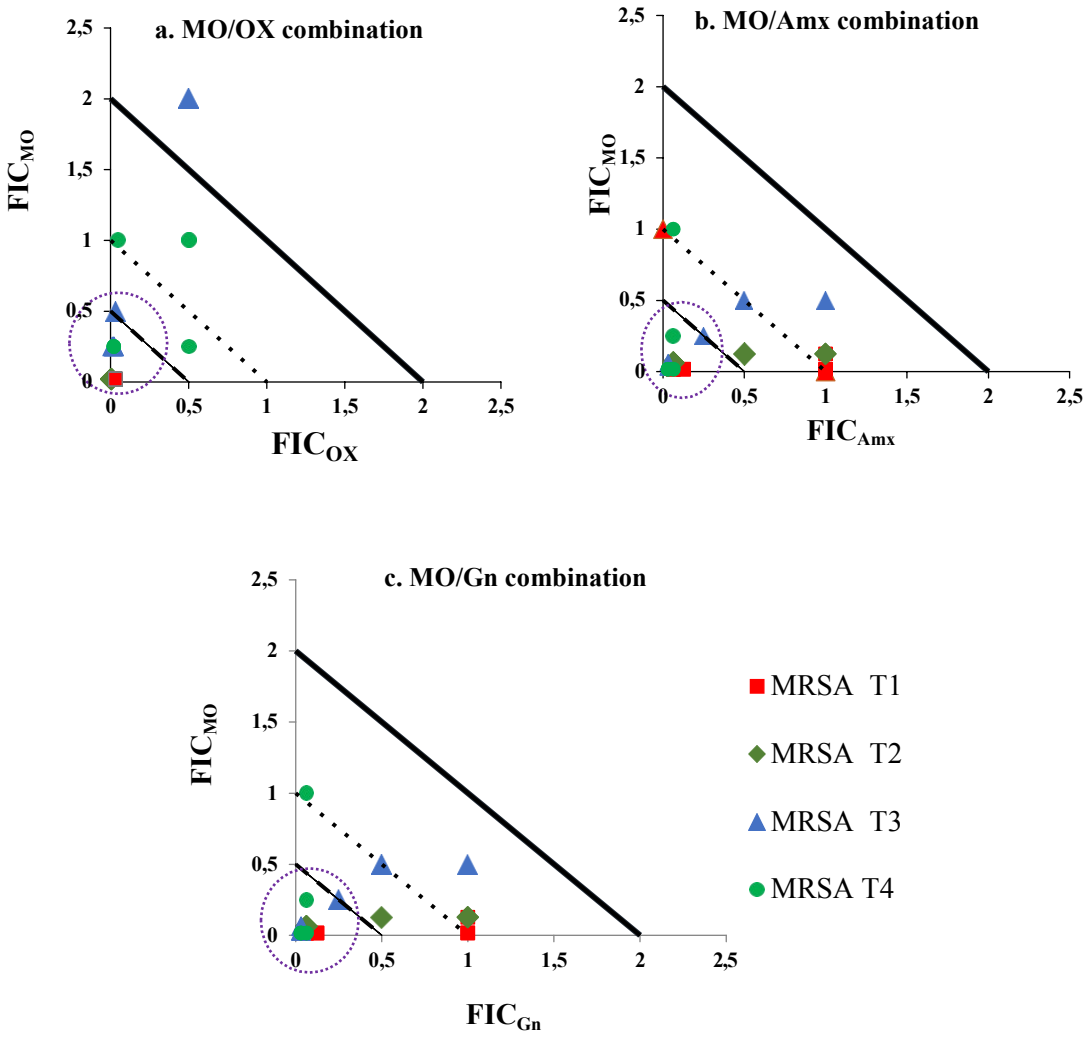


Figure 2. Interactions between MO – OX (a), MO – Amx (b) and MO – Gn (c) against MRSA clinical isolates T1 – T4;
purple colored dotted circles highlight synergies.

Figure 3. Experimental design of the chequerboard method with the exemplification of the results obtained for the combination MO - Gn against MRSA T4.

The experimental percentage of growth (fig. 4a) in the presence of different concentrations of MO and/or antibiotics and theoretical dose-response surface of interaction (fig. 4b) are represented and synergies have been confirmed through Bliss independence–based model interpretation.

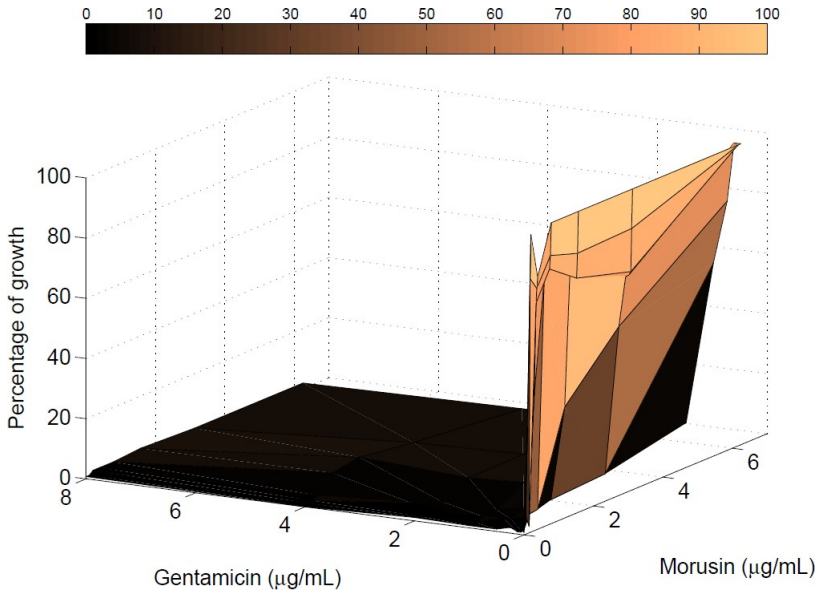


Figure 4a. Three-dimensional plot of the experimental percentage of growth ($E_{measured}$) between MO and Gn against MRSA T4.

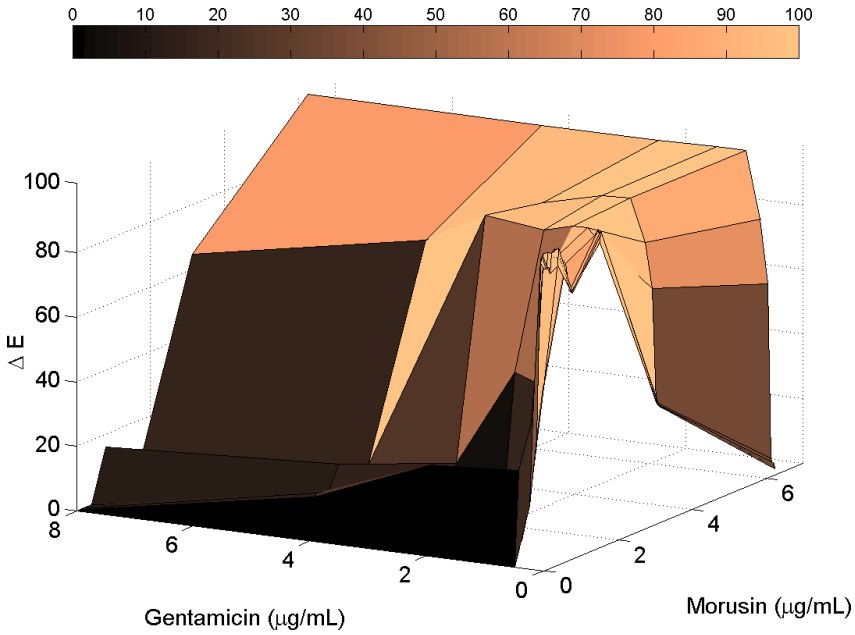


Figure 4b. Theoretical dose-response surface of interaction (ΔE) between MO and Gn against MRSA T4 (ΔE above zero (positive) indicates synergy).

Time-kill assay confirmed the synergy for the combinations MO – OX against MRSA T1 (fig. 5a) and MO – Amx against MRSA T2 (fig. 5b). The results obtained in the time-kill assay method for combinations MO – OX against MRSA T2-T4 and MO – Amx against MRSA T1, MRSA T3 and MRSA T4 were not fully in agreement with those observed when using the checkerboard method because the logarithmic reductions of the colony-forming units obtained for the combinations between MO and antibiotics were not $2\log_{10}$ lower than the logarithmic reductions obtained for the most potent/active component (MO) of the combinations. No increase in the viable colony count of more than $2\log_{10}$ CFU/mL compared to the viable count obtained with the most active single agent of combination (MO) was recorded and the antagonism was excluded for the combinations MO – OX and MO – Amx against MRSA strains.

Differences between the results obtained in the checkerboard method and time-kill assay have been also reported by other authors (12). These differences can be explained by the difference between the measured phenomena - checkerboard method assesses the inhibitory effect while time kill assay measures the bactericidal effect. The concordance between the results given by the two methods has been estimated as being 44-88% (17).

In our study, time-kill assay confirmed the synergy for the combination MO – Gn against all four clinical isolates: MRSA T1 (fig. 6a and fig. 7a), MRSA T2 (fig. 6b and fig. 7b), MRSA T3 (fig. 6c and fig. 7c) and MRSA T4 (fig. 6d and fig. 7d), because the logarithmic reductions of the colony-forming units obtained for the combination MO - Gn were $2\log_{10}$ lower than the logarithmic reductions obtained for the most potent/active component (Gn) of the combination.

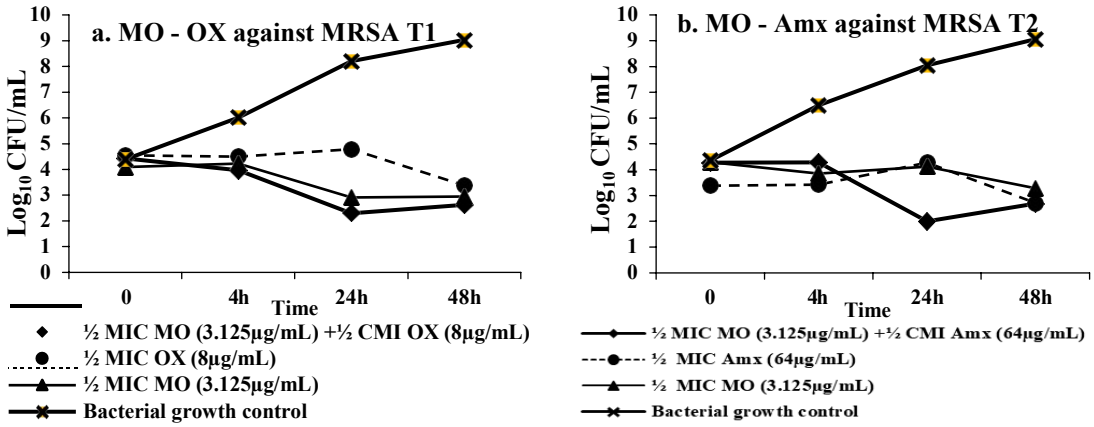


Figure 5. Time–kill curves for the combinations MO – OX against MRSA T1 (a) and MO – Amx against MRSA T2 (b).

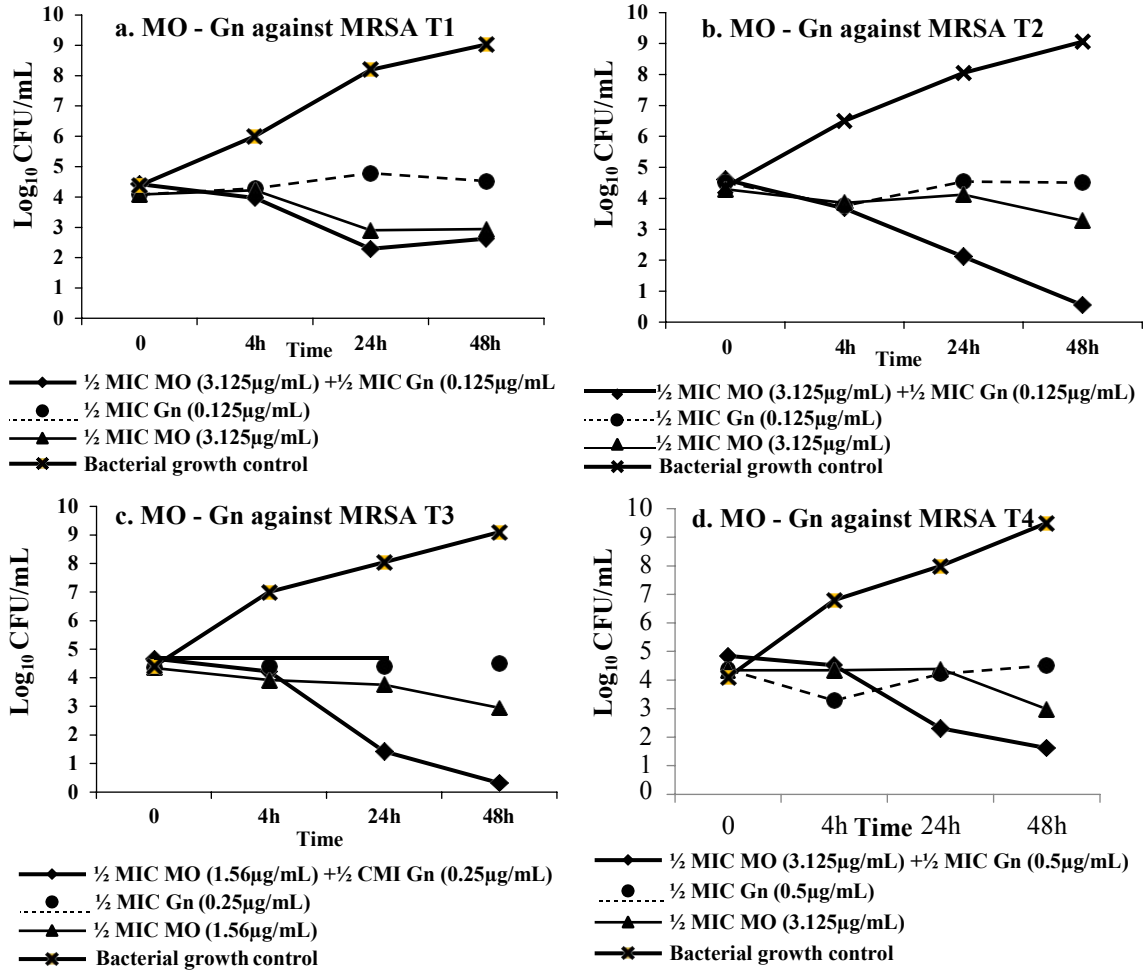
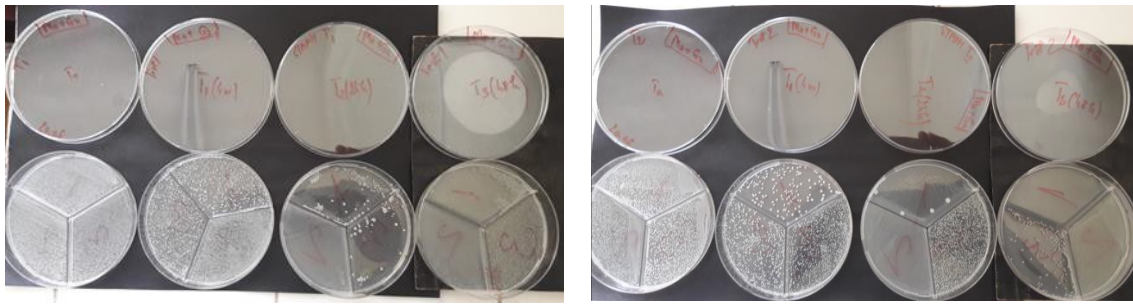
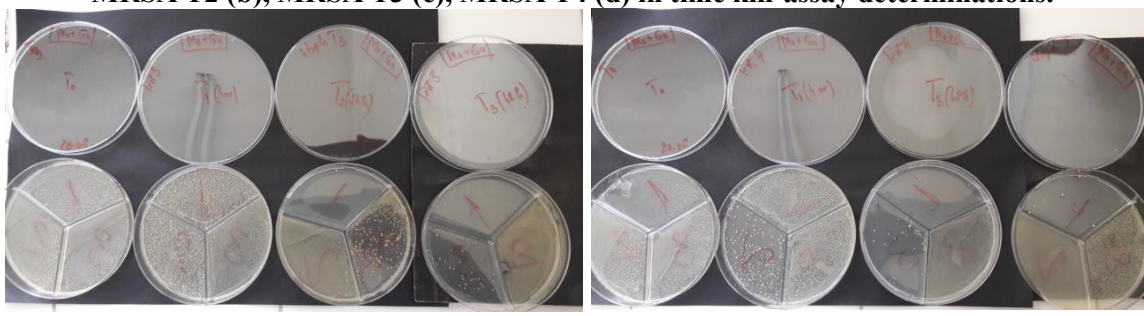


Figure 6. Time–kill curves for the combination MO – Gn against MRSA T1 (a), MRSA T2 (b), MRSA T1 (c) and MRSA T1 (d).



a. **b.**

Figure 7. Differences between MO – Gn (1), Gn (2) and MO (3) against MRSA T1 (a), MRSA T2 (b), MRSA T3 (c), MRSA T4 (d) in time kill-assay determinations.



c. **d.**

Figure 7. Differences between MO – Gn (1), Gn (2) and MO (3) against MRSA T1 (a), MRSA T2 (b), MRSA T3 (c), MRSA T4 (d) in time kill-assay determinations (*cont*.).

Conclusion

Our study reports on the antibacterial activity of morusin alone against four MRSA clinical isolates and its ability to act synergistically with antibiotics. As MRSA has become an increasingly global concern, synergy between phytochemicals and conventional antibiotics is a promising option to overcome antibiotic resistence. This preliminary study showed that morusin has the potential to reverse the bacterial resistence to oxacillin and amoxicillin of MRSA and increase the susceptibility of MRSA strains to gentamicin.

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