Preliminary studies regarding antimicrobial effect of various kuwanon G – antibiotic combinations on some MRSA strains

Cristina HORHOGEA[1], Cristina RÎMBU[1], Petruța AELENEI[2,*], Eleonora GUGUIANU[1], Carmen CREȚU[1], Gabriel DIMITRIU[3], Anca MIRON[2]

[1]Microbiology-Immunology Laboratory, Department of Public Health, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Ion Ionescu de la Brad, Iasi, Romania

[2]Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy Grigore T. Popa, Iasi, Romania

[4]Department of Preventive Medicine and Interdisciplinary, Discipline Medical Informatics and Biostatistics, Faculty of Medicine, University of Medicine and Pharmacy Grigore T. Popa, Iasi, Romania

petruta.aelenei@yahoo.com

Abstract

Methicillin-resistant Staphylococcus aureus (MRSA) is a constant therapeutic challenge in humans and animals, due to the limited range of antibiotics that can be used for the management of infections. This preliminary study is based on the assessment of the antibacterial activity of kuwanon G (a prenylated flavonoid present in white mulberry, Morus alba L., Moraceae) and its interactions with various antibiotics (oxacillin, amoxicillin, erythromycin and gentamicin) against four MRSA clinical isolates (MRSA T1 – T4). The sources of all clinical isolates resistant to cefoxitin and oxacillin were infections (recurrent otitis, pyoderma and laryngopharyngitis) in dogs. Minimum inhibitory concentrations (MICs) for kuwanon G and antibiotics were determined by the microdilution method. Interactions between kuwanon G and antibiotics were evaluated by the checkerboard method and time-kill assay. MICs varied between 6.25 and 12.5µg/mL for kuwanon G alone against all four MRSA clinical isolates. According to the calculated fractional inhibitory concentration index, various combinations were synergistic and additive. Microbicidial time has confirmed the synergy as the logarithmic reductions of colony-forming units obtained for the combinations of kuwanon G and some antibiotics were $2\log_{10}$ lower than the logarithmic reductions obtained for the most potent/active component of the combination. The obtained results are promising, taking into account the antibacterial activity of kuwanon G, as well as its synergistic effects with the most used antibiotics. This study reports on the antibacterial activity of kuwanon G and suggests its ability to act synergistically with antibiotics; combinations effective in combating Gram-positive including MRSA infections might be developed.

Key-words*: checkerboard, kuwanon G, MRSA, synergy, time-kill assay*

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a Gram-positive bacterium that developed drug resistance to β-lactam antibiotics through horizontal gene transfer and natural multiple selections. Infections with MRSA are a real problem for humans and animals and the treatment of these infections is challenging due to the limited range of antibiotics that can be used because of antibiotic resistance (1 - 5). Kuwanon G (KG) is a prenylated flavonoid present in white mulberry (*Morus alba* L., *Moraceae*) leaves, fruits and root bark (fig. 1) (6, 7).

The aim of this preliminary study was to investigate the antibacterial activity of kuwanon G and its interactions with four common antibiotics against MRSA strains.

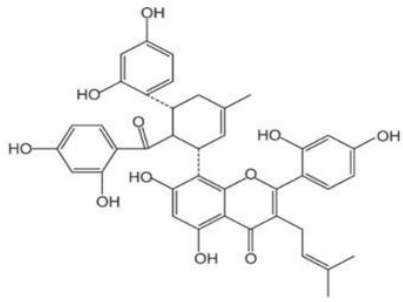

Figure 1. Chemical structure of kuwanon G.

Material and methods

For this study, there were selected four MRSA (MRSA T1 – T4) clinical strains resistant to oxacillin and cefoxitin. The strains were isolated from various infections (recurrent otitis, pyoderma and laryngopharyngitis) in dogs (phenotype being established by the diffusimetric method).

Minimum inhibitory concentrations (MICs) of KG, oxacillin (OX), amoxicillin (Amx), erythromycin (Er) and gentamicin (Gn) against MRSA isolates were determined by the microdilution method according to current Clinical & Laboratory Standards Institute (CLSI) (8) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (9).

Two *in vitro* tests were performed in order to evaluate the interactions between KG and antibiotics: checkerboard method (10) and time-kill assay (11). The experimental design of checkerboard method involves the use of 96-well microtiter plates in order to evaluate the bacterial growth in the presence of the combination of two components (KG and antibiotic) in various concentrations after incubation at 37^0C for 24 hours. The absorbances were determinated spectophotometrically (450/650 nm) before and after incubation. MIC was defined as the concentration that reduced the bacterial growth by 80% compared to the bacterial culture control. Checkerboard method enables the interpretation of the results through fractional inhibitory concentration index (FICI) and isobolograms (12).

$$FICI = FIC_{antibiotic} + FIC_{kuwanon\ G} \text{ where:}$$

$$FIC_{Antibiotic} = \frac{MIC_{antibiotic\ in\ combination\ with\ kuwanon\ G}}{MIC_{antibiotic\ alone}},$$

$$FIC_{kuwanon\ G} = \frac{MIC_{kuwanon\ G\ in\ combination\ with\ antibiotic}}{MIC_{kuwanon\ G\ alone}}.$$

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 (11).

The results obtained the checkerboard method were subjected to Bliss independence–based model interpretation with graphical representation of the experimental dose-response surface and theoretical dose-response surface of interaction. Experimental dose-response surface ($E_{measured}$) represents the experimental percentage of growth in the presence of different concentrations of KG and/or antibiotic. Taking into account the non-interactive process between two components, $E_{predicted}$ is the calculated percentage of growth based on the experimental percentage of growth according to Bliss independence–based model. Theoretical dose-response surface of interaction (ΔE) represents the difference between predicted ($E_{predicted}$) and measured ($E_{measured}$) percentage of

growth with KG and antibiotic at various concentrations. Points of difference surface above zero (positive) indicate synergy and below zero (negative) indicate antagonism (10).

In time-kill assay, the bactericidal effect of the combination of KG (at ½MIC_{KG} concentration) and antibiotic (at ½$MIC_{antibiotic}$ concentration) was compared with the bactericidal effect of the antibiotic alone, KG alone and bacterial culture control. After 0, 4, 24 and 48 hours of incubation at 37^0C, aliquots were withdrawn and the colony forming units (CFU) were determined after incubation at 37˚C. Synergy/antagonism is interpreted if the combination increases/decreases by 100 (or $2log_{10}$) times the bactericidal effect, compared to the most potent/active antibacterial agent of the combination after 24 hours or 48 hours (11).

Results and discussion

MIC values of KG alone against all MRSA clinical isolates varied between 6.25 and 12.50 µg/mL and the bacterial susceptibility of MSRA clinical isolates to tested antibiotics is presented in table 1.

Table 1. MIC (µg/mL) of antibiotics and KG*

MRSA clinical isolates	MIC_{OX}	MIC_{Amx}	MIC_{Er}	MIC_{Gn}	MIC_{KG}
MRSA T1	16 (R)	16 (R)	>170.67 (R)	0.25 (S)	12.50
MRSA T2	128 (R)	128 (R)	10.67 (R)	0.25 (S)	6.25
MRSA T3	256 (R)	256 (R)	>170.67 (R)	0.50 (S)	12.50
MRSA T4	256 (R)	256 (R)	>170.67 (R)	1 (S)	12.50

*European Committee on Antimicrobial Susceptibility - Testing Breakpoint tables for interpretation of MICs and zone diameter Version 7.0. Valid from 2017-01-01; Abbreviation: S – sensible, R – resistant

- **KG – OX combinations**

Checkerboard method showed synergies for the combinations KG – OX (FICI= 0.04-0.5; table 2, fig. 2a) against MRSA T1 – T4 clinical isolates. Time-kill assay did not confirm synergy for the combinations KG – OX against MRSA T1 –T4, but excluded the antagonism, because the combination of KG with antibiotics did not decrease, but also did not increase the viable colony count by more than $2log_{10}$CFU/mL compared to the viable count obtained with the most active/potent agent of combination (KG). These differences between the results obtained by the checkerboard method and time kill assay can be explained by the different measured phenomena – the checkerboard method assesses the inhibitory effect while the time kill assay measures the bactericidal process (13).

Table 2. Effects of KG – OX combinations

Strain	MIC_{OX} (µg/mL)	MIC_{OX-KG} (µg/mL)	FIC_{OX}	MIC_{KG-OX} (µg/mL)	MIC_{KG} (µg/mL)	FIC_{KG}	FICI*	TKA**
MRSA T1	16	0.50	0.01	0.20	12.25	0.03	0.04 (S)	NC
MRSA T2	128	0.50	0.01	0.20	6.25	0.03	0.04 (S)	NC
MRSA T3	256	0.50	0.01	1.56	12.5	0.13	0.14 (S)	NC
MRSA T4	256	0.50	0.01	6.25	12.5	0.50	0.50 (S)	NC

Abbreviation: S – synergy, NC – synergy has not been confirmed, MIC_{OX-KG} – MIC of OX in presence of KG, MIC_{KG-OX} – MIC of KG in presence of OX

*effect of the combination determined through checkerboard method, ** effect of the combination determined through time-kill assay

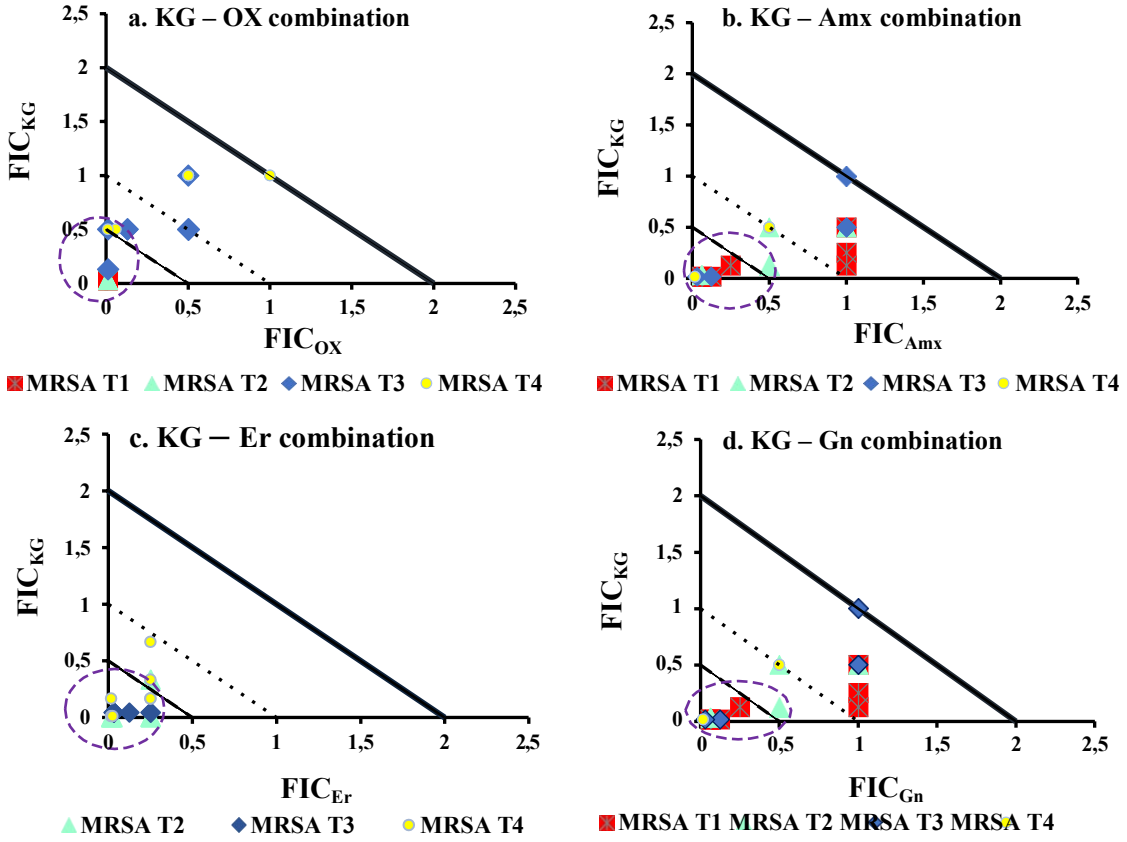

Figure 2. Interactions between KG – OX (a), KG – Amx (b), KG – Er (c) and KG – Gn (d) against MRSA clinical isolates T1 – T4; purple colored dotted circles highlight synergies.

➢ **KG – Amx combinations**

Checkerboard method showed synergy for the combinations KG – Amx (FICI=0.04-0.14; table 3, fig. 2b) against MRSA T1 - T3 clinical isolates and additive effects (FICI=0.51) against MRSA T4. Time-kill assay confirmed synergy for the combinations KG – Amx against MRSA T1 – T2 (fig. 3), but excluded the antagonism against MRSA T3 –T4.

Table 3. Effects of KG – Amx combinations

Strain	MIC_{Amx} (µg/mL)	$MIC_{Amx–KG}$ (µg/mL)	FIC_{Amx}	$MIC_{KG–Amx}$ (µg/mL)	MIC_{KG} (µg/mL)	FIC_{KG}	FICI *	TKA**
MRSA T1	16	0.50	0.01	0.20	12.25	0.03	0.04 (S)	S
MRSA T2	128	0.50	0.01	0.20	6.25	0.03	0.04 (S)	S
MRSA T3	256	0.50	0.01	1.56	12.5	0.13	0.14 (S)	NC
MRSA T4	256	0.50	0.01	6.25	12.5	0.50	0.51 (Ad)	NC

Abbreviation: S – synergy, NC – synergy has not been confirmed, $MIC_{Amx–KG}$ – MIC of Amx in presence of KG, $MIC_{KG–Amx}$ – MIC of KG in presence of Amx

*effect of the combination determined through checkerboard method, ** effect of the combination determined through time-kill assay

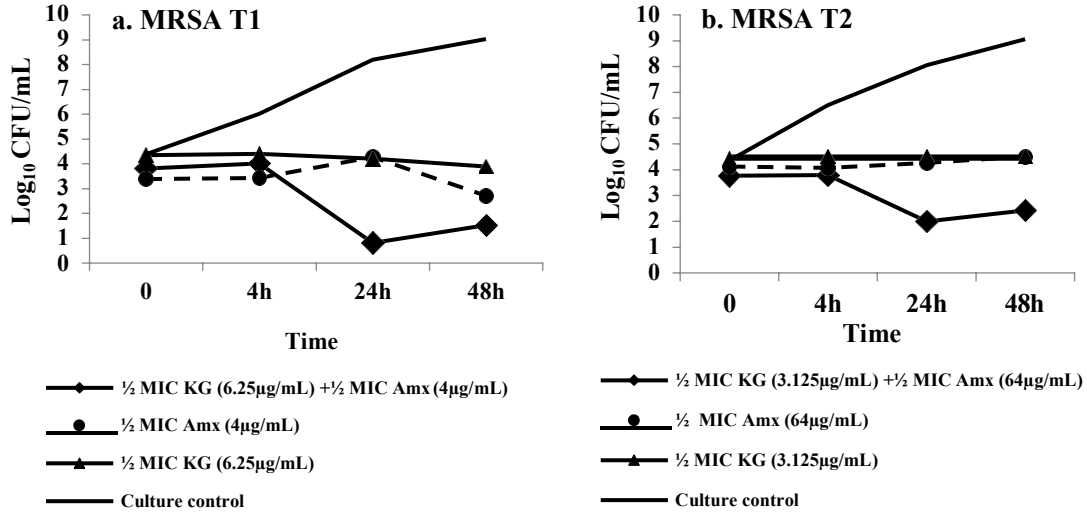

Figure 3. ***Time–kill curves of KG alone, Amx alone and their combination against MRSA T1 (a) and MRSA T2 (b).***

- **KG – Er combinations**

Checkerboard method showed synergies for the combinations KG – Er (FICI=0.03-0.1; table 4, fig. 2c) against MRSA T2 - T4 clinical isolates. Time-kill assay did not confirm synergy for combinations KG – Er against MRSA T2 –T4, but excluded the antagonism. It should be noted that KG did not decrease MIC_{Er} against MRSA T1.

Table 4. Effects of KG – Er combinations

Strain	MIC_{Er} (µg/mL)	$MIC_{Er–KG}$ (µg/mL)	FIC_{Er}	$MIC_{KG–Er}$ (µg/mL)	MIC_{KG} (µg/mL)	FIC_{KG}	FICI*	TKA**
MRSA T1	>170.67¥	>170.67	ND	12.25	12.25	1	ND	NC
MRSA T2	(341.33)	1.00	0.10	0.52	6.25	0.00	0.10 (S)	NC
MRSA T3	10.67	0.33	0.03	0.13	12.5	0.04	0.07 (S)	NC
MRSA T4	>170.67¥ (341.33)	8.00	0.02	12.5	12.5	0.01	0.03 (S)	NC

Abbreviation: S – synergy, NC – synergy has not been confirmed, $MIC_{Er–KG}$ – MIC of Er in presence of KG, $MIC_{KG–Er}$ – MIC of KG in presence of Er, ¥MIC_{Er} >170.67 µg/mL and for calculation of FIC_{Er}, MIC_{Er} was considered as being 341.33 µg/mL

*effect of the combination determined through checkerboard method, ** effect of the combination determined through time-kill assay

- **KG – Gn combinations**

Checkerboard method showed synergies for the combinations KG – Gn (FICI=0.03-0.09; table 5, fig. 2d) against MRSA T1 – T4 clinical isolates.

Table 5. Effects of KG – Gn combinations

Strain	MIC_{Gn} (µg/mL)	$MIC_{Gn–KG}$ (µg/mL)	FIC_{Gn}	MIC_{KG-Gn} (µg/mL)	MIC_{KG} (µg/mL)	FIC_{KG}	FICI*	TKA**
MRSA T1	0.25	0.02	0.06	0.20	12.25	0.02	0.08 (S)	S
MRSA T2	0.25	0.02	0.06	0.20	6.25	0.03	0.09 (S)	S
MRSA T3	0.50	0.02	0.03	0.20	12.5	0.02	0.05 (S)	S
MRSA T4	1	0.02	0.02	0.20	12.5	0.02	0.03 (S)	S

Abbreviation: S – synergy, $MIC_{Gn–KG}$ – MIC of Gn in presence of KG, $MIC_{KG–Gn}$ – MIC of KG in presence of Gn, *effect of the combination determined through checkerboard method, ** effect of the combination determined through time-kill assay

The experimental percentage of growth (fig. 4a) in the presence of different concentrations of KG and/or Gn and the theoretical dose-response surface of interaction (fig. 4b) were represented for KG – Gn combination against MRSA T4 according to Bliss independence–based model interpretation.

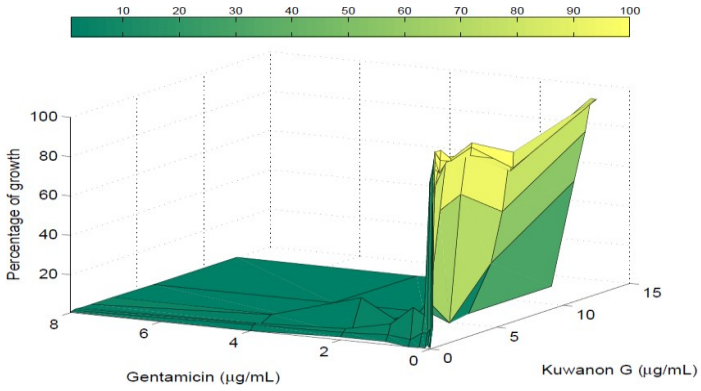

Figure 4a. The three-dimensional plot of the experimental percentage of growth ($E_{measured}$) between KG and Gn against MRSA T4.

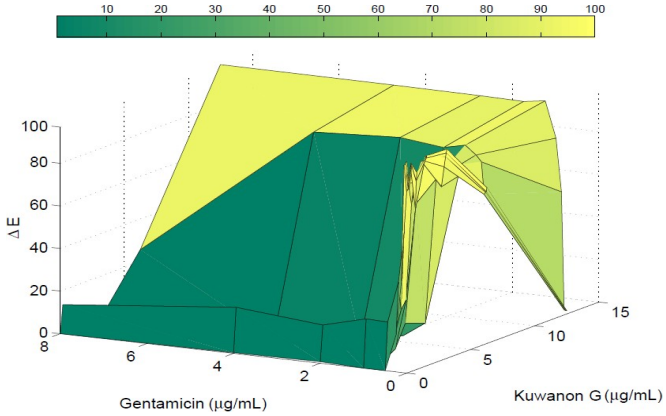

Figure 4b. Theoretical dose-response surface of interaction (ΔE) between KG and Gn against MRSA T4 (ΔE above zero (positive) indicates synergy).

Time-kill assay confirms synergy for the combinations KG – Gn against MRSA T1 (fig. 5a and fig. 6a), MRSA T2 (fig. 5b and fig. 6b), MRSA T3 (fig. 5c and fig. 6c) and MRSA T4 (fig. 5d and fig. 6d).

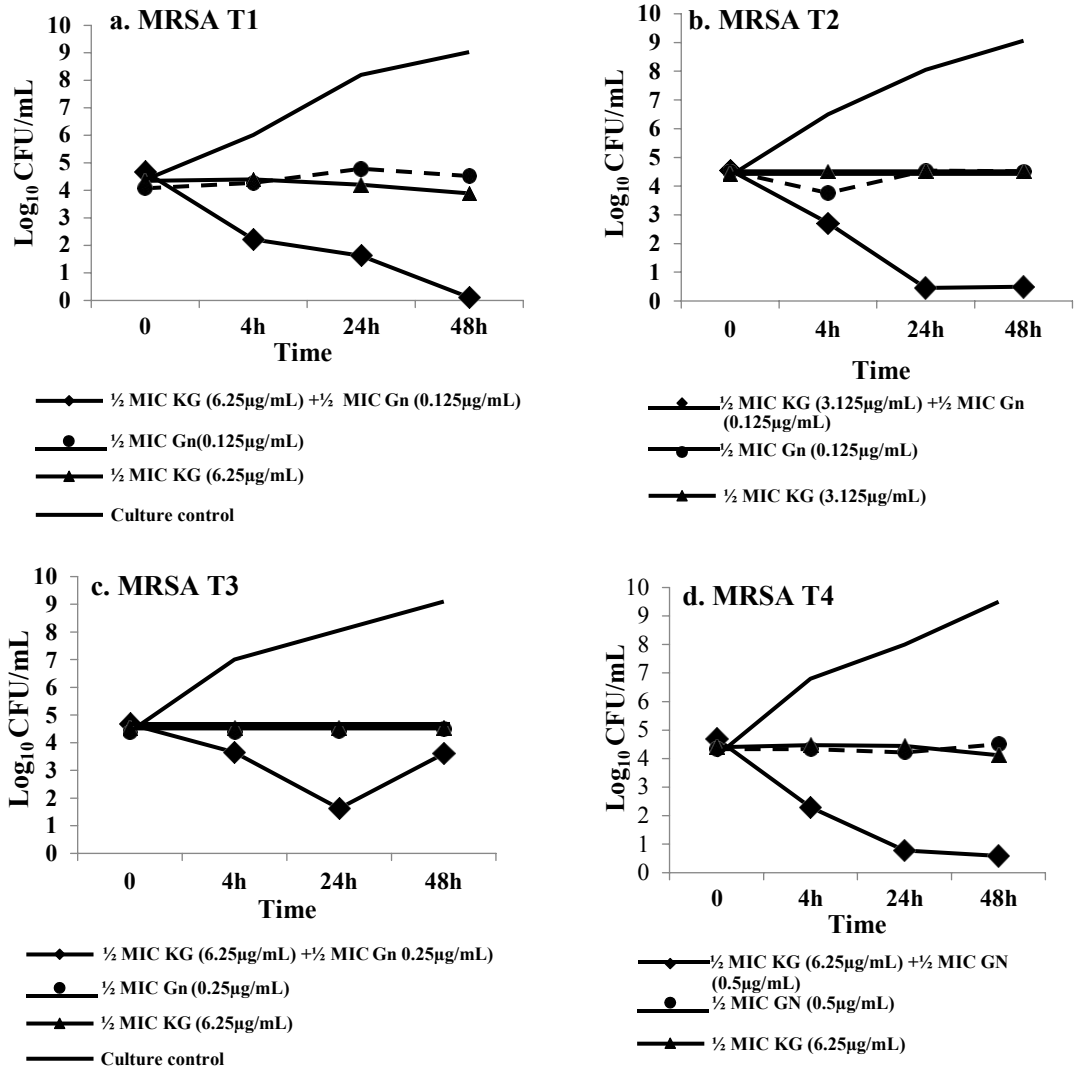

Figure 5. Time–kill curves of KG alone, Gn alone and their combinations against MRSA T1 (a), MRSA T2 (b), MRSA T3 (c), MRSA T4 (d).

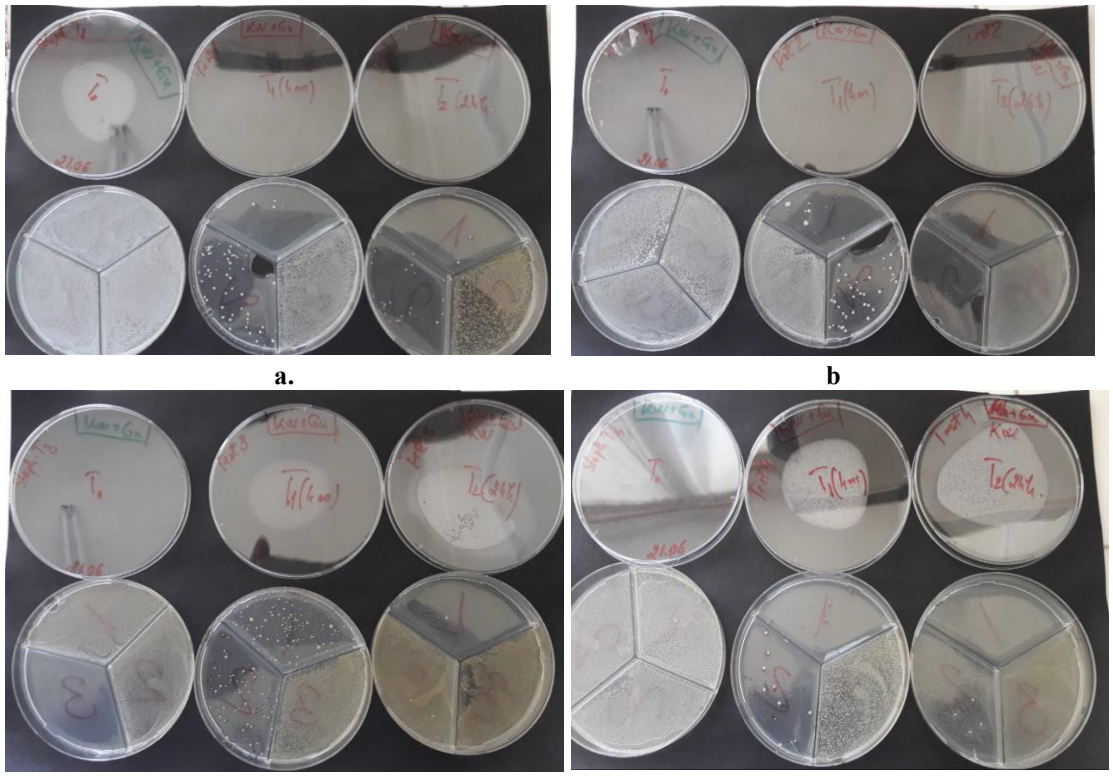

Figure 6. Differences between KG/Gn, Gn, KG against MRSA T1 (a), MRSA T2 (b), MRSA T3 (c), MRSA T4 (d) in time kill-assay determinations.

Conclusion

The results of this preliminary study highlight the antibacterial activity of kuwanon G and its ability to synergize with antibiotics – oxacillin, amoxicillin, erythromycin and gentamicin. The combinations: kuwanon G – oxacillin, kuwanon G – amoxicillin, kuwanon G – erythromycin and kuwanon G – gentamicin tested using the checkerboard method showed synergistic effects against MRSA clinical isolates. The synergistic effects were partially confirmed by the time-kill assay. This study reports on the antibacterial activity of kuwanon G and suggests its ability to act synergistically with antibiotics; combinations effective in combating Gram-positive including MRSA infections might be developed.

References

1. Fair RJ, Tor Y Antibiotics and Bacterial Resistance in the 21st Century. *Perspect Medicin Chem*. 2014; 6: 25–64.
2. Holmes NE, Howden BP. What's new in the treatment of serious MRSA infection? *Curr Opin Infect Dis*. 2014; 27(6): 471-8.
3. Drebes J, Künz M, Pereira CA *et al*. MRSA infections: from classical treatment to suicide drugs. *Curr Med Chem*. 2014; 21(15):1809-19.
4. Tverdek FP, Crank CW, Segreti J. Antibiotic therapy of methicillin-*resistant Staphylococcus aureus* in critical care. *Crit Care Clin*. 2008; 24(2): 249-60.
5. Gurusamy KS, Koti R, Toon CD *et al.* Antibiotic therapy for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in surgical wounds. *Cochrane Database Syst Rev*. 2013; 20 (8): CD009726.
6. Gryn-Rynko A, Bazylak G, Olszewska-Slonina D. New potential phytotherapeutics obtained from white mulberry (*Morus alba* L.) leaves. *Biomed Pharmacother* 2016; 84: 628-636.

7. Jung HW, Kang SY, Kang JS et al. Effect of Kuwanon G isolated from the root bark of Morus alba on ovalbumin-induced allergic response in a mouse model of asthma. *Phytother Res.* 2014; 28(11):1713-9.
8. CLSI. Performance Standards for Antimicrobial Susceptibility Testing 27th Edition, CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institutes; 2017.
9. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.0, 2017.
10. Segatore B, Bellio P, Setacci D *et al.* In vitro interaction of usnic acid in combination with antimicrobial agents against methicillin-resistant *Staphylococcus aureus* clinical isolates determined by FICI and ΔE model methods. *Phytomedicine* 2012; 19(3-4):341-7.
11. Mulyaningsih S, Sporer F, Zimmermann S et al. Synergistic properties of the terpenoids aromadendrene and 1,8-cineole from the essential oil of Eucalyptus globulus against antibiotic-susceptible and antibiotic-resistant pathogens. *Phytomedicine* 2010; 17:1061-6.
12. van Vuuren S, Viljoen A. Plant-based antimicrobial studies--methods and approaches to study the interaction between natural products. *Planta Med* 2011; 77(11): 1168-8.
13. White RL, Burgess DS, Manduru M, Bosso JA. Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. *Antimicrob Agents Chemother* 1996; 40(8):1914.