Int.J.Curr.Microbiol.App.Sci (2021) *10*(03): 1799-1809

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 10 Number 03 (2021)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2021.1003.225

Antibacterial Potential of Citrus Flavonoid, Naringenin with β-Lactam Antibiotics against Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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Keywords

MRSA, β-Lactam, Antibiotics, Naringenin, Linezolid

Article Info

Accepted:
17 February 2021
Available Online:
10 March 2021

A B S T R A C T

Plants are the main source of drugs nowadays. They provide a high benefit to risk ratio. Naringenin is a plant flavonoid obtained from citrus fruits. It exhibits plethora of effects against a number of diseases. Agar dilution method was used to evaluate minimum inhibitory concentration (MIC) naringenin and three β-lactam drugs amoxicillin, oxacillin and cloxacillin. Checkerboard method was used to determine the fractional inhibitory concentration (FIC) of the drugs in combination with naringenin. Naringenin was reported to have an MIC value of 125µg/ml. The flavonoid showed synergistic effect with both oxacillin (7 strains) and cloxacillin (8 strains) whereas it showed indifference on combination with amoxicillin. Thus from all the results it can be concluded that Naringenin inhibited growth of MRSA and enhanced the anti-microbial effect of the conventional drugs included in the study.

Introduction

Staphylococcus aureus has emerged as a global public health challenge to the world (Munita *et al*., 2016). The micro-organism has shown resistance to almost all the conventional antibiotics related to methicillin and its congeners. The new strain is known as Methicillin resistant *Staphylococcus aureus* (Ferri *et al*., 2017). The affinity of Methicillin sensitive Staphylococcus aureus (MSSA) towards β-lactams has been reduced as it has acquired a special gene i.e. Penicillin Binding Protein (PBP-2a) (Poulsen *et al*., 2014).

Plants are being used as an alternate source of medicine as it has good efficacy and very less or no side-effects. These medicines are being used from ancient history for cure and treatment of diseases. They have been combined with allopathic drugs to have a safer and efficacious treatment strategy. Plant flavonoids contain diverse protective activities like anti-inflammatory, anti-oxidant, anti-proliferative, nephron-protective etc. (Wright, 2016).

The citrus flavonoid taken in this study is Naringenin. This flavonoid is obtained mainly

from tomatoes and/or figs. This flavonoid has been reported to possess various positive effects on human health such as anti-diabetic (Wang *et al*., 2015), anti-oxidant (Erlund *et al*., 2001), anti-inflammatory (Pinho-Ribeiro *et al*., 2016), immunity enhancing (Ke *et al*., 2017), anti-atherogenic (Karim *et al*., 2018), antipyretic (Ahmadi *et al*., 2016; Frabasile *et al*., 2017), anti-viral (Gonçalves *et al*., 2017), anti-cancer and anti-proliferative effects activities (Erlund *et al*., 2001). So, on the basis of the above properties of the citrus flavonoid, it is included in the current study to investigate anti-microbial and anti-bacterial effects alone and in combination with the selected β-lactam drugs.

Materials and Methods

Drugs and chemicals

Amoxicillin, Oxacillin, Cloxacillin, Linezolid, naringenin**,** mueller hinton agar, sodium chloride, blood agar plates

Procurement of bacterial isolates

MRSA positive clinical isolates (13) were obtained from Bacteriology laboratory of local hospital and reference strains MRSA (ATCC 43300) and MSSA (ATCC 11632) were purchased from ATCC, Manassas, USA.

Sample preparation

Different strains (reference strains were reconstituted) were grown in nutrient broth and sub-cultured on blood agar plates (overnight at 37^{o}C) (Figure 1).

Identification test

Coagulase test was performed to interpret the identity of *Staphylococcus aureu*s in all clinical isolates.

Procedure for coagulase test

Human plasma was taken on a glass slide and a single colony of different isolates was added to the plasma drop. The contents on the slide were mixed gently for uniform mixing and left for 10 seconds to react. After that the slides were visualized for any changes in the appearance of the mixture (clumping will be seen in case of *Staphylococcus aureus* strain).

Evaluation of density of the bacterial isolates

Density of different clinical isolates was evaluated and concentration was adjusted to 0.5 McF for each strain (using densiCHEK apparatus).

Stock solution and agar plate preparation for determination of MIC

Concentrations of different antibiotics were selected according to CLSI 2020 guidelines (CLSI M100, 2020) (Table 1). For test drug naringenin, the concentration range taken for evaluation was 2000-0.12µg/ml (Ng'umi et al., 2015). MIC values for all the drugs were determined using agar dilution method (as shown in figure 2).

Determination of FIC and FIC-Index

Checkerboard method was used for evaluation of the fractional concentration of the drugs which can be used to inhibit MRSA growth (Santiago *et al*., 2015).

After evaluation of the MIC values of the clinical isolates, the fractional inhibitory concentration was evaluated after determining the MIC of combination of the test drug with conventional antibiotics used in the current study (A+N, C+N, O+N and L+N) using agar dilution method. Briefly, the agar plates were prepared (Figure 2) and the drugs are added

to the molten agar. After solidification of the agar, spotting with the clinical isolates of MRSA was done and MIC of the combination was evaluated after overnight incubation of the plates at optimum temperature. The FIC and FIC-Index were calculated using following formulae (Hu et al., 2002):

$$FIC\ of\ Naringenin\ (N)(\mu g/ml) = \frac{MIC\ of\ N\ (used\ in\ combination)}{MIC\ of\ N\ alone}$$

$$FIC\ of\ Antibiotic\ (An)\ (\mu g/ml) = \frac{MIC\ of\ An\ used\ in\ combination}{MIC\ of\ An\ alone}$$

$$FIC\ Index = FIC\ of\ An + FIC\ of\ N$$

Results and Discussion

MRSA has been reported to be resistant to almost all β-lactam antibiotics and thus there is a need to search for a better option for treatment of infections caused by this deadly microbe. Extensive studies have been performed in the past and research is still going on finding such a treatment strategy which can provide more benefit to risk ratio. Plant based medicine has emerged as a boon in this regard due to high therapeutic potential against diseases. This *in vitro* study has been designed to investigate growth inhibitory potential of plant flavonoid naringenin against MRSA. β-lactam antibiotics amoxicillin, cloxacillin, oxacillin and linezolid were added to the agar plates and then clinical isolates were grown (overnight incubation at 37ºC) and MIC was calculated for each strain (Table 2-5).

Effect of β-lactam drugs on MRSA

β-lactam antibiotics act by inhibiting the cell wall synthesis and thus trans-peptidase activity of the gram positive micro-organisms (Brudzynski and Sjaarda, 2014; Mun *et al*., 2015).

MIC of reference MRSA strain was found to be 256µg/ml, 64µg/ml, 64µg/ml, 128µg/ml and 125µg/ml for amoxicillin, cloxacillin, oxacillin, linezolid and naringenin respectively whereas for MSSA, the MIC was observed as 2µg/ml (amoxicillin), 0.25µg/ml (cloxacillin), 1µg/ml (oxacillin), 0.5µg/ml (linezolid) and 15.6µg/ml (naringenin) for all the tested antibiotics and test drug naringenin (Table 2-5). The high MIC values of clinical isolates authenticated the resistance of the MRSA isolates against the antibiotics. The test drug showed good anti-bacterial action against the MRSA isolates (Table 2-5).

Table.1 Concentration ranges of drugs according to CLSI 2020

S. No.	Drugs	Resistant (R)	Sensitive (S)	Selected concentration range
1.	Amoxicillin	≥ 16µg/ml	≤ 4µg/ml	256-1µg/ml
2.	Cloxacillin	≥ 4µg/ml	≤ 2µg/ml	64-0.25µg/ml
3.	Oxacillin	≥ 4µg/ml	≤ 2µg/ml	64-0.25µg/ml
4.	Linezolid	≥ 8µg/ml	≤ 4µg/ml	128-0.5µg/ml

Table.2 MICs, FIC and FICI of amoxicillin and test drug naringenin against MRSA reference strain, MSSA reference strain and MRSA clinical isolates. S: Synergy; A: Additive; I: Indifference

S. No.	Strain No.	MIC (µg/ml)		MIC of combination (µg/ml)		FIC (µg/ml)		FICI
		Naringenin (2000-0.12 µg/ml)	**Amoxicillin (256-1µg/ml)**	**Naringenin**	**Amoxicillin**	**Naringenin**	**Amoxicillin**	**N+A**
*****	**MRSA Ref.**	**125**	**256**	**125**	**16**	**1**	**0.0625**	**1.0625 (I)**
******	**MSSA Ref.**	**15.6**	**2**	**3.9**	**0.25**	**0.25**	**0.125**	**0.375 (S)**
1	**101**	**2000**	**32**	**2000**	**2**	**1**	**0.0625**	**1.0625 (I)**
2	**29**	**0.12**	**64**	**0.12**	**4**	**1**	**00625**	**1.0625 (I)**
3	**34**	**0.12**	**128**	**0.12**	**8**	**1**	**0.0625**	**1.0625 (I)**
4	**145**	**0.12**	**16**	**0.12**	**1**	**1**	**0.0625**	**1.0625 (I)**
5	**140**	**3.9**	**16**	**3.9**	**1**	**1**	**0.0625**	**1.0625 (I)**
6	**103**	**125**	**128**	**125**	**8**	**1**	**0.0625**	**1.0625 (I)**
7	**118**	**2000**	**256**	**2000**	**16**	**1**	**0.0625**	**1.0625 (I)**
8	**109**	**0.97**	**256**	**0.97**	**16**	**1**	**0.0625**	**1.0625 (I)**
9	**47**	**0.97**	**256**	**0.97**	**16**	**1**	**0.0625**	**1.0625 (I)**
10	**181**	**31.25**	**256**	**31.25**	**16**	**1**	**0.0625**	**1.0625 (I)**
11	**179**	**0.97**	**256**	**0.97**	**16**	**1**	**0.0625**	**1.0625 (I)**
12	**147**	**0.12**	**32**	**0.12**	**2**	**1**	**0.0625**	**1.0625 (I)**
13	**124**	**0.12**	**32**	**0.12**	**2**	**1**	**0.0625**	**1.0625 (I)**

Table.3 MICs, FIC and FICI of cloxacillin and test drug naringenin against MRSA reference strain, MSSA reference strain and MRSA clinical isolates. S: Synergy; A: Additive; I: Indifference

S. No.	Strain No.	MIC (µg/ml)		MIC of combination (µg/ml)		FIC (µg/ml)		FICI
		Naringenin (2000-0.12µg/ml)	**Cloxacillin (64-0.25 µg/ml)**	**Naringenin**	**Cloxacillin**	**Naringenin**	**Cloxacillin**	**N+C**
*	**MRSA Ref.**	**125**	**64**	**62.5**	**32**	**0.5**	**0.5**	**1 (A)**
**	**MSSA Ref.**	**15.6**	**0.25**	**1.95**	**0.031**	**0.125**	**0.125**	**0.25 (S)**
1	**101**	**2000**	**64**	**500**	**16**	**0.25**	**0.25**	**0.5 (S)**
2	**29**	**0.12**	**2**	**0.03**	**0.5**	**0.25**	**0.25**	**0.5 (S)**
3	**34**	**0.12**	**2**	**0.06**	**0.5**	**0.5**	**0.25**	**0.75 (A)**
4	**145**	**0.12**	**2**	**0.03**	**0.5**	**0.25**	**0.25**	**0.5 (S)**
5	**140**	**3.9**	**2**	**1.95**	**1**	**0.5**	**0.5**	**1 (A)**
6	**103**	**125**	**4**	**31.25**	**1**	**0.25**	**0.25**	**0.5 (S)**
7	**118**	**2000**	**32**	**500**	**8**	**0.25**	**0.25**	**0.5 (S)**
8	**109**	**0.97**	**64**	**0.24**	**16**	**0.24**	**0.25**	**0.49 (S)**
9	**47**	**0.97**	**64**	**0.48**	**32**	**0.49**	**0.5**	**0.99 (A)**
10	**181**	**31.25**	**64**	**7.87**	**16**	**0.25**	**0.25**	**0.5 (S)**
11	**179**	**0.97**	**64**	**0.48**	**32**	**0.49**	**0.5**	**0.99 (A)**
12	**147**	**0.12**	**64**	**0.015**	**8**	**0.125**	**0.125**	**0.25 (S)**
13	**124**	**0.12**	**16**	**0.06**	**8**	**0.5**	**0.5**	**1 (A)**

Table.4 MICs, FIC and FICI of oxacillin and test drug naringenin against MRSA reference strain, MSSA reference strain and MRSA clinical isolates. S: Synergy; A: Additive; I: Indifference

S. No.	Strain No.	MIC (µg/ml)		MIC of combination (µg/ml)		FIC (µg/ml)		FICI
		Naringenin (2000-0.12 µg/ml)	**Oxacillin (64-0.25 µg/ml)**	**Naringenin**	**Oxacillin**	**Naringenin**	**Oxacillin**	**N+O**
*	**MRSA Ref.**	**125**	**64**	**62.5**	**16**	**0.5**	**0.25**	**0.75 (A)**
**	**MSSA Ref.**	**15.6**	**1**	**1.95**	**0.125**	**0.125**	**0.125**	**0.25 (S)**
1	**101**	**2000**	**8**	**1000**	**4**	**0.5**	**0.5**	**1 (A)**
2	**29**	**0.12**	**0.25**	**0.015**	**0.031**	**0.125**	**0.125**	**0.25 (S)**
3	**34**	**0.12**	**64**	**0.03**	**16**	**0.25**	**0.25**	**0.5 (S)**
4	**145**	**0.12**	**8**	**0.015**	**1**	**0.125**	**0.125**	**0.25 (S)**
5	**140**	**3.9**	**4**	**0.48**	**0.5**	**0.12**	**0.125**	**0.245 (S)**
6	**103**	**125**	**4**	**62.5**	**2**	**0.5**	**0.5**	**1 (A)**
7	**118**	**2000**	**16**	**250**	**2**	**0.125**	**0.125**	**0.25 (S)**
8	**109**	**0.97**	**16**	**0.48**	**8**	**0.49**	**0.5**	**0.99 (A)**
9	**47**	**0.97**	**16**	**0.48**	**8**	**0.49**	**0.5**	**0.99 (A)**
10	**181**	**31.25**	**8**	**3.93**	**1**	**0.125**	**0.125**	**0.25 (S)**
11	**179**	**0.97**	**16**	**0.48**	**8**	**0.49**	**0.5**	**0.99 (A)**
12	**147**	**0.12**	**32**	**0.06**	**16**	**0.5**	**0.5**	**1 (A)**
13	**124**	**0.12**	**32**	**0.03**	**8**	**0.25**	**0.25**	**0.5 (S)**

Table.5 MICs, FICs and FICI of Linezolid and test drug naringenin against MRSA reference strain, MSSA reference strain and MRSA clinical isolates. S: Synergy; A: Additive; I: Indifference

S. No.	Strain No.	MIC (µg/ml)		MIC of combination (µg/ml)		FIC (µg/ml)		FICI
		Naringenin (2000-0.12 µg/ml)	Linezolid (128-0.5 µg/ml)	Naringenin	Linezolid	Naringenin	Linezolid	N+L
*	MRSA Ref.	125	128	62.5	64	0.5	0.5	1 (A)
**	MSSA Ref.	15.6	0.5	1.95	0.125	0.125	0.25	0.375 (S)
1	101	2000	2	1000	0.5	0.5	0.25	0.75 (A)
2	29	0.12	0.5	0.06	0.125	0.5	0.25	0.75 (A)
3	34	0.12	0.5	0.06	0.125	0.5	0.25	0.75 (A)
4	145	0.12	0.5	0.06	0.25	0.5	0.5	1 (A)
5	140	3.9	0.5	0.0975	0.0625	0.025	0.125	0.15 (S)
6	103	125	0.5	31.25	0.125	0.25	0.25	0.5 (S)
7	118	2000	8	1000	1	0.5	0.125	0.625 (A)
8	109	0.97	8	0.48	4	0.49	0.5	0.99 (A)
9	47	0.97	2	0.48	1	0.49	0.5	0.99 (A)
10	181	31.25	1	15.62	0.5	0.49	0.5	0.99 (A)
11	179	0.97	0.5	0.48	0.25	0.49	0.5	0.99 (A)
12	147	0.12	1	0.06	0.5	0.5	0.5	1 (A)
13	124	0.12	8	0.06	4	0.5	0.5	1 (A)

Figure.1 MRSA, MSSA reference strain and clinical isolates sub-cultured on blood agar plate

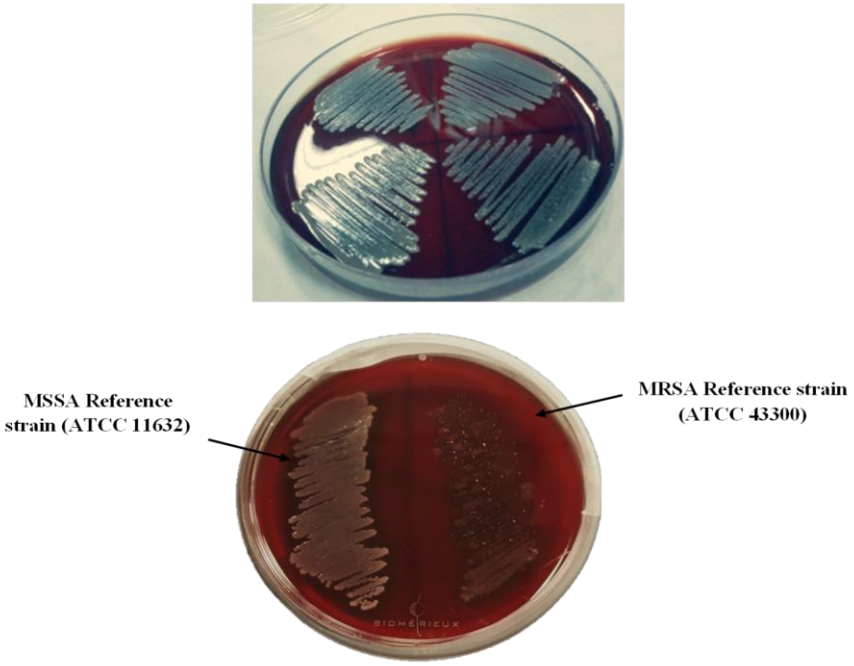
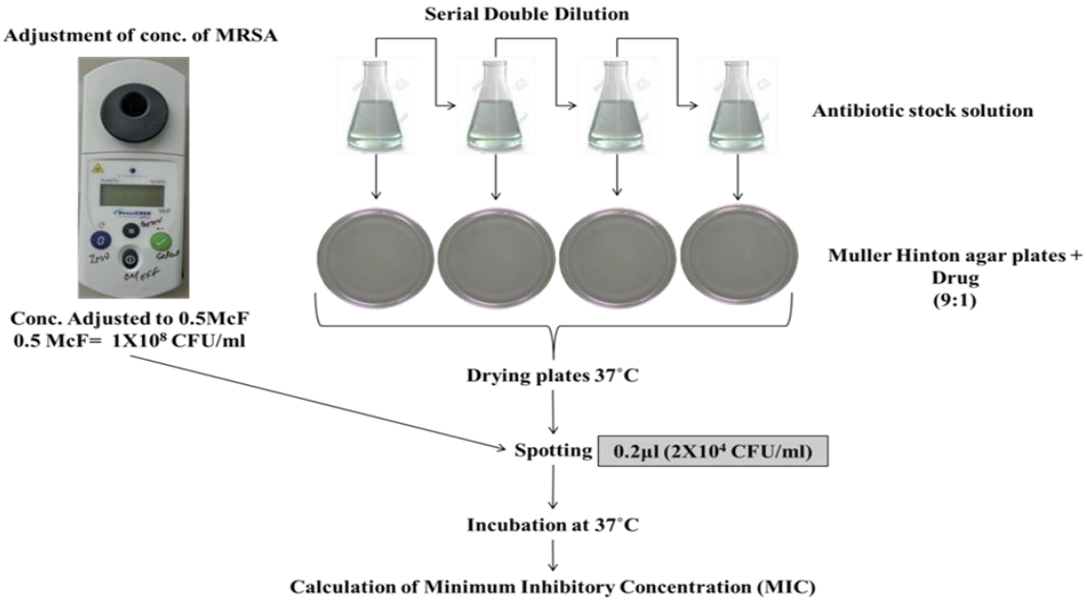


Figure.2 Preparation of stock solutions of drugs and calculation of MIC of different clinical isolates



Many researchers have investigated the anti-bacterial activity of naringenin against gram positive organisms (*S. aureus*) (Kozłowska *et al*., 2019). On further evaluation of the FIC and FIC index of the antibiotics in combination with test drug naringenin, all the antibiotics except amoxicillin showed synergistic/additive effect for most of the clinical isolates (Table 2-5). Indifference effect was observed when the clinical isolates were treated with amoxicillin and naringenin (A+N) (Table 2).

Naringenin molecule and it's derivatives have been reported as anti-microbial agents by many researchers owing to active groups present at two sites C-7 and C-40 (Sakoda *et al*., 2016; Nobakht *et al*., 2014, Lee *et al*., 2014).

This study has shown that naringenin has good anti-microbial activity and it is capable of enhancing the activity of conventional antibiotics against gram positive bacteria MRSA.

From the above results and discussion, it can be concluded that naringenin has anti-MRSA properties which can be further enhanced when it is combined with other antibiotics. So, it can be an efficacious treatment option with less or no side effects and increased compliance. However, the plant based drug may further be investigated for molecular basis behind its anti-microbial activity against MRSA using in-vivo studies in order to have a better translatability in human diseases

Acknowledgments

We are thankful to Mr. Deepak Sharma for his valuable technical assistance.

Ethical approval

The study was ethically approved from the ethics committee of SGT University, Gurugram, Haryana, India and the Ethical approval no. is SGTU/FMHS/MICRO/341.

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How to cite this article:

Ameesha Tomar, Shobha Broor, Swati Kaushik, Tanisha Bharara and Arya, D. S. 2021. Antibacterial Potential of Citrus Flavonoid, Naringenin with β-Lactam Antibiotics against Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Int.J.Curr.Microbiol.App.Sci.* 10(03): 1799-1809. doi: https://doi.org/10.20546/ijcmas.2021.1003.225