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Synergistic Effect between Fucoidan and Antibiotics against Clinic Methicillin-Resistant *Staphylococcus aureus*

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Abstract

Fucoidan is a sulfated polysaccharide that is primarily extracted from brown seaweeds which has been broadly studied in recent years due to its numerous biological properties, including anticoagulant, antithrombotic, antitumor, and antiviral activities. In this study, fucoidan was evaluated against clinic isolated methicillin-resistant *Staphylococcus aureus* (MRSA) 1 - 20, either alone or with antibiotics, via broth dilution method and checkerboard and time kill assay. Minimum inhibitory concentrations (MICs)/Minimum bactericidal concentrations (MBCs) values for the fucoidan against all the tested bacteria ranged between 64 - 512/256 - 2048 microg/mL, for ampicillin 32 - 1024/64 - 1024 microg/mL and for oxacillin 8 - 64/16 - 256 microg/mL respectively. Furthermore, the MIC and MBC were reduced to one half-eighth as a result of the combination of the fucoidan with antibiotics. 2 - 6 hours of treatment with 1/2 MIC of fucoidan with 1/2 MIC of antibiotics resulted from an increase of the rate of killing in units of CFU/mL to a greater degree than was observed with alone. These results suggest that fucoidan could be employed as a natural antibacterial agent against multi-drug bacteria.

Keywords

Fucoidan, Antibacterial Activity, Methicillin-Resistant *Staphylococcus aureus*, Synergistic Effect, Minimum Inhibitory Concentrations (MICs), Minimum Bactericidal Concentrations (MBCs)

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1. Introduction

Staphylococcus aureus is known to be a versatile pathogen causing a side variety of community and hospital acquired infections that are associated with high morbidity and mortality rates [1] [2]. Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are becoming increasingly widespread [3]. MRSA normally possesses a multidrug-resistant genotype that causes it to be resistant to β-lactams, aminoglycosides, fluoroquinolones, and macrolides [3]-[5]. As a result, there is an urgent need to develop anti-MRSA agents with novel mechanisms of action [6]-[8]. Many studies have been conducted to evaluate natural products as novel antibiotic substances against MRSA and to develop antibiotics that would be advantageous for combating the therapeutic problems associated with MRSA [8]-[11].

Several species of the brown algae have been reported to contain biologically active compounds, which have demonstrated a variety of biological activies, including anti-coagulant, antithrombotic, antiviral, antitumor, antioxidant, anti-inflammatory, antifungal, and antibacterial effects [12]-[18]. Antibacterial activity of red, brown and green algae against both Gram-positive and Gram-negative bacteria has been established by several scientists [18] [19]. Fucoidan is a complex sulfated polysaccharide that is found in the cell walls of several edible brown algae, including *Fucus vesiculosus* [14] [20]. The structures and compositions of fucoidan vary among different brown seaweed species, but generally the compound consists primarily of L-fucose and sulfate, along with small quantities of D-galactose, D-mannose, D-xylose, and uronic acid [14] [20] [21]. The recent interest has mainly focused on the potentially beneficial biological activities of fucoidan in humans including antitumor, immunomodulatory, anti-inflammatory, antiviral, antithrombotic, anticoagulant, and antioxidant effects as well as specific activities against kidney, liver, and urinary system disorders [17] [21]-[24]. We reported previously that fucoidan exerts antibacterial effects against cariogenic bacteria and periodontopathogenic bacteria [25]. Drug synergism between known antibiotic and bioactive plant extracts is a novel concept and could be benificial (synergistic or addition interaction) or deleterious (antagonistic or toxic outcome) [26] [27]. Although a broad range of biological and pharmacological activities of fucoidan have been reported, the mechanism(s) behind its antibacterial effects are not fully understood.

In this study, the antibacterial activities of fucoidan were observed when used alone and in combination with oxacillin and ampicillin against clinic isolated methicillin-resistant *Staphylococcus aureus*.

2. Materials and Methods

2.1. Preparation of Bacterial Strains

20 isolates of methicillin-resistant *Staphylococcus aureus* isolated from the Wonkwang University Hospital, as well as standard strains of methicillin-sensitive *S. aureus* (MSSA) ATCC 25923 and methicillin-resistant *S. aureus* (MRSA) ATCC 33591 were used. Antibiotic susceptibility was determined from the size of the inhibition zone, in accordance with the guidelines established by the National Committee for Clinical Laboratory Standards (NCCLS, 1997), and the strains utilized were defined as MRSA on the basis of the occurrence of the *mecA* gene and their resistance to oxacillin (Kim *et al*., 2004). Briefly, the growth of bacteria was examined at 37˚C in 0.95 mL of BHI broth containing various concentrations of fucoidan. These tubes were inoculated with 5×10^5 colony-forming units (CFU)/mL of an overnight culture grown in BHI broth, and incubated at 37˚C. After 24 h of incubation, the optical density (OD) was measured spectrophotometrically at 550 nm. Three replicates were measured for each concentration of tested drugs.

2.2. Minimum Inhibitory Concentration/Minimum Bactericidal Concentration Assay

The antimicrobial activities of fucoidan against clinical isolates MRSA 20 and reference strains were determined *via* the broth dilution method [28]. The minimum inhibitory concentration (MIC) was recorded as the lowest concentration of test samples resulting in the complete inhibition of visible growth. The minimum bactericidal concentration (MBC) was determined based on the lowest concentration of the extracts required to kill 99.9% of bacteria from the initial inoculum as determined by plating on agar.

2.3. Checkerboard Dilution Test

The synergistic combinations were investigated in the preliminary checkerboard method performed using the

MRSA 1 - 20 of clinical isolate strains via MIC and MBC determination [6] [28]. The fractional inhibitory concentration index (FICI) and fractional bactericidal concentration index (FBCI) are the sum of the FICs and FBCs of each of the drugs, which were defined as the MIC and MBC of each drug when used in combination divided by the MIC and MBC of each drug when used alone. The interaction was defined as synergistic in cases in which the FIC index was less than or equal to 0.5, additive in cases in which the FIC index was greater than 0.5 and less than or equal to 1.0, indifferent when the FIC index was greater than 1.0 and less than or equal to 2.0, and antagonistic in cases in which the FIC index was greater than 2.0 [6] [28].

2.4. Time-Kill Curves

Bactericidal activities of the drugs under study were also evaluated using time-kill curves on the isolated and reference strains. The tubes containing fucoidan with bacteria were incubated at 37˚C and viable counts were conducted at 0, 0.5, 1, 2, 3, 4, 5, 6, 12 and 24 h after the addition of antimicrobial agents, on agar plates incubated for up to 24 hours at 37˚C. The cultures of all bacteria, all of which had a cell density of $\times 10^6$ CFU/ml, were exposed to the MIC of fucoidan alone and with ampicillin or oxacillin, with several repetitions. The colony counts were conducted in duplicate, and the means were determined.

3. Results and Discussion

Many researchers are studying natural products that could be used as antibiotics against MRSA, and are employing novel dosing regimens and antimicrobials that would be advantageous for combating the therapeutic problems associated with *S. aureus* [8] [9] [11] [29] [30]. The results of the antibacterial activity showed that the fucoidan exhibited inhibitory activities against isolates MRSA 1 - 20 and reference stains, MRSA and MSSA. In **Table 1**, the MICs/MBCs for fucoidan were determined to be either 64/128 or 512/2048 μg/mL; for ampicillin, either 32/64 or 1024/2048 μg/mL; for oxacillin, either 8/16 or 64/256 μg/mL against reference strains and MRSA 1 - 20 isolates. MRSA is resistant to not only methicillin and other *β*-lactams, but also to a host of other antibacterial agents, including macrolide [5] [6]. As MRSA evidences multidrug resistance, it has been emerging worldwide as one of the most important hospital and community pathogens [3]. Thus, new agents are required for the treatment of MRSA-associated infections. Some natural products have been identified as candidates for novel antibiotic substances [8] [26] [28] [31].

The more susceptibility of Gram-positive bacteria to the algal extract was due to the differences in their cell wall structure and their composition [18]. In Gram-negative bacteria, the outer membrane acts as a barrier to many environmental substances including antibiotics [32]. Combinations of some herbal materials and different antibiotics might affect the inhibitory effect of these antibiotics [11] [27] [28] [30] [33]. The combination of oxacillin and fucoidan resulted in a reduction in the MICs/MBCs for tested all bacteria, with the MICs/MBCs of 16/32 or 128/256 μg/mL and for oxacillin becoming 2 - 128/4 - 256 μg/mL and reduced by ≥4-fold in most of tested MRSA, evidencing a synergistic effect as defined by a FICI of ≤0.5 except MRSA 1, 5, 7, 16, and 19 and by a FBCI of ≤0.5 except MRSA 5, 7, 9, 16, and 19 (**Table 2**). In combination with fucoidan, the MICs/MBCs for ampicillin were reduced by ≥4-fold in most of *S. aureus* tested, evidencing a synergistic effect as defined by a FICI of ≤0.5 except MRSA 2, 8, 9, 16, and 20 and by FBCI of ≤0.5 except MRSA 2, 5, 6, 10, 12, and 17 (**Table 3**). The effects of fucoidan administered in combination with oxacillin or ampicillin against standard (MSSA and MRSA) and clinical isolates of MRSA (MRSA 1 - 20) were confirmed by time-kill curve experiments (**Figures 1-4**). Cultures of each strain of bacteria with a cell density of 10^6 CFU/mL were exposed to the MIC of fucoidan alone or fucoidan (1/2 MIC) with oxacillin (1/2 MIC) or ampicillin (1/2 MIC). We observed that 3 hours of fucoidan treatment with ampicillin or oxacillin resulted in an increased rate of killing as compared to that observed with fucoidan (MIC) alone (**Figures 1-4**). A profound bactericidal effect was exerted when a combination of drugs was utilized. The growth of the tested bacteria was completely attenuated after 2 - 5 h of treatment with the 1/2 MIC of fucoidan, regardless of whether it was administered alone or with oxacillin (1/2 MIC) or ampicillin (1/2 MIC).

Fucoidan is a complex sulfated polysaccharide that is found in the cell walls of several edible brown algae, including *Fucus vesiculosus* [20]. Sulfated polysaccharide fractions from *Sargassum fulvellum*, *S. kjellmanianum*, *L. angustata*, *L. angustata* var. *longissima*, *L. japonica*, *Ecklonia cava*, and *Eisenia bicyclis* have been evaluated for their bioactivities and attributed that to the high concentration of polysaccharides in these species which are known to have antimicrobial properties [14] [16] [34]. The more susceptibility of Gram-positive bac-

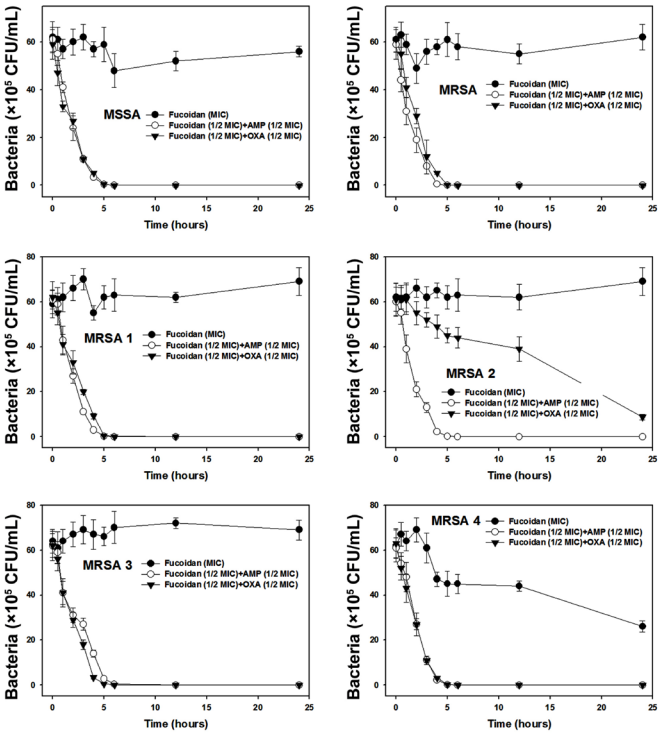

Figure 1. Time-kill curves of MIC of the fucoidan alone and 1/2 MIC of fucoidan with 1/2 MIC of oxacillin or ampicillin against isolates MRSA (1 - 4) and methicillin-sensitive *S. aureus* (MSSA) ATCC 25923 and methicillin-resistant *S. aureus* (MRSA) ATCC 33591 strains. Bacteria were incubated with the fucoidan alone (●) and with ampicillin (○) or with oxacillin (▼) over time. CFU, colony-forming units.

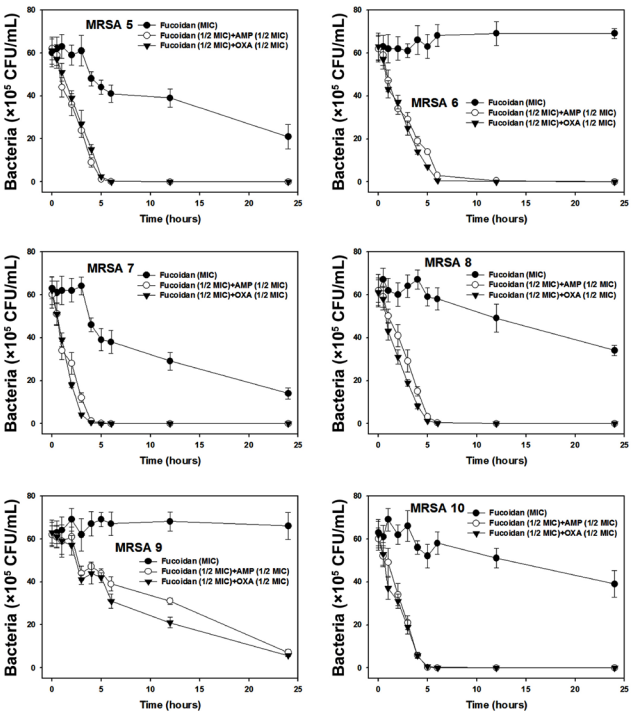

Figure 2. Time-kill curves of MIC of the fucoidan alone and 1/2 MIC of fucoidan with 1/2 MIC of oxacillin or ampicillin against isolates MRSA (5 - 10). Bacteria were incubated with the fucoidan alone (●) and with ampicillin (○) or with oxacillin (▼) over time. CFU, colony-forming units.

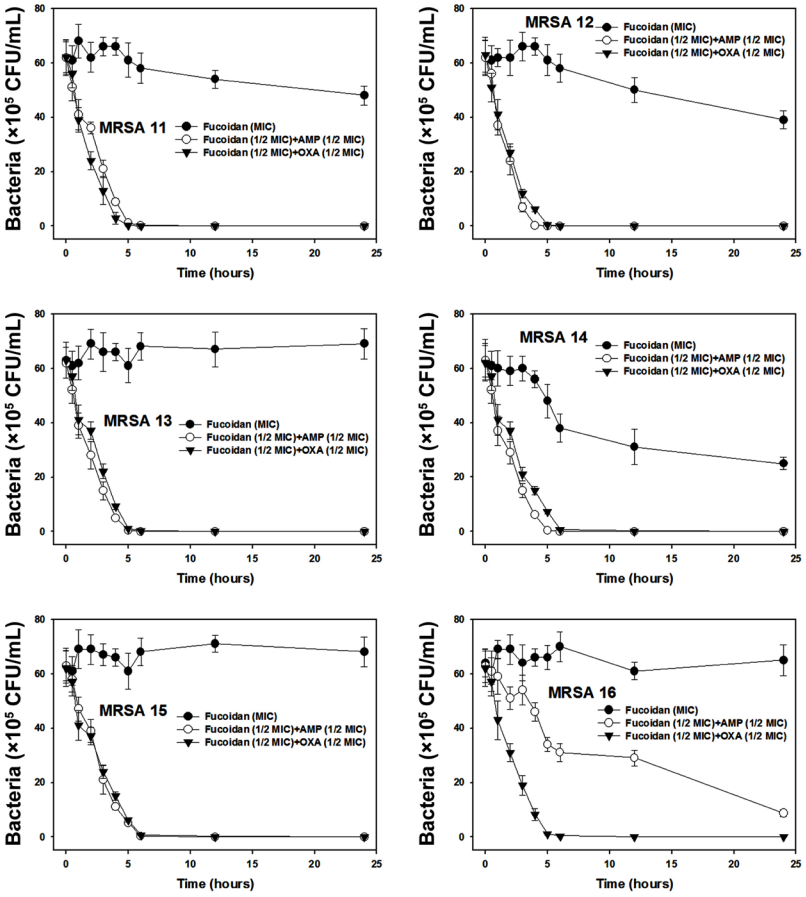

Figure 3. Time-kill curves of MIC of the fucoidan alone and 1/2 MIC of fucoidan with 1/2 MIC of oxacillin or ampicillin against isolates MRSA (11 - 16). Bacteria were incubated with the fucoidan alone (●) and with ampicillin (○) or with oxacillin (▼) over time. CFU, colony-forming units.

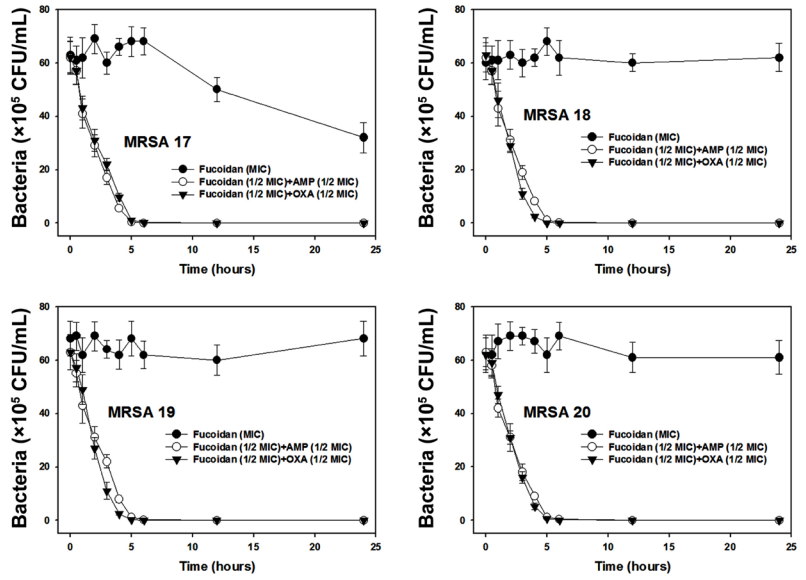

Figure 4. Time-kill curves of MIC of the fucoidan alone and 1/2 MIC of fucoidan with 1/2 MIC of oxacillin or ampicillin against isolates MRSA (17 - 20). Bacteria were incubated with the fucoidan alone (●) and with ampicillin (○) or with oxacillin (▼) over time. CFU, colony-forming units.

teria to sulfated polysaccharides was due to the differences in their cell wall structure and their composition [35]-[37].

4. Conclusion

Fucoidan exerted synergistic effects when administered with oxacillin or ampicillin and the antimicrobial effect and resistant regulation of fucoidan against MRSA might be useful for potential application as a natural product agent.

Acknowledgements

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Table 1. Antibacterial activity of fucoidan and antibiotics in isolated MRSA and some of reference bacteria.

Samples	Fucoidan (μg/mL)			Ampicillin	Oxacillin
	$MIC_{50<}$	$MIC_{90<}$	MIC/MBC	MIC/MBC (μg/mL)	
MSSA ATCC 25923[1]	32	128	128/256	8/16	0.25/1
MRSA ATCC 33591[2]	16	128	128/512	1024/2048	8/16
MRSA 1[3]	16	64	64/256	1024/2048	16/32
MRSA 2	32	128	128/512	128/256	8/16
MRSA 3	64	128	256/1024	1024/2048	8/16
MRSA 4	32	128	128/256	256/512	16/32
MRSA 5	16	64	64/128	128/256	16/32
MRSA 6	16	64	64/256	256/256	8/16
MRSA 7	32	64	128/256	128/512	64/256
MRSA 8	8	64	64/128	128/256	8/32
MRSA 9	32	128	128/512	128/512	16/32
MRSA 10	16	64	64/128	64/128	8/16
MRSA 11	32	128	128/512	128/256	16/64
MRSA 12	64	256	256/1024	256/256	32/64
MRSA 13	128	512	512/2048	32/64	32/64
MRSA 14	32	64	128/256	128/256	16/32
MRSA 15	16	64	64/256	64/128	8/16
MRSA 16	16	128	128/512	128/256	16/32
MRSA 17	16	64	64/128	128/256	8/16
MRSA 18	8	64	64/256	64/128	8/32
MRSA 19	16	64	64/256	64/128	8/16
MRSA 20	64	256	256/1024	128/512	16/32

[1]MSSA (ATCC 25923): reference strain Methicillin-sensitive *Staphylococcus aureus*. [2]MRSA (ATCC 33591): reference strain Methicillin-resistant *Staphylococcus aureus*. [3]MRSA (1 - 20): Methicillin-resistant *Staphylococcus aureus* isolated a clinic.

Table 2. Synergistic effects of the fucoidan with oxacillin in isolated MRSA and some of reference bacteria.

Samples	Agent	MIC/MBC (μg/mL)		FIC/FBC	FICI/ FBCI[2]	Outcome
		Alone	Combination[1]			
MSSA ATCC 25923[3]	Fucoidan	128/256	32/64	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Oxacillin	0.25/1	0.0625/0.25	0.25/0.25		
MRSA ATCC 33591[4]	Fucoidan	128/512	32/64	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 1[5]	Fucoidan	64/256	32/64	0.5/0.25	1.0/0.5	**Additive/Synergistic**
	Oxacillin	16/32	8/8	0.5/0.25		
MRSA 2	Fucoidan	128/512	32/64	0.25/0.125	0.5/0.375	**Synergistic/Synergistic**
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 3	Fucoidan	256/1024	64/256	0.25/0.25	0.5/0.5	**Synergistic/ Synergistic**
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 4	Fucoidan	128/256	32/64	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 5	Fucoidan	64/128	16/64	0.25/0.5	0.75/0.75	**Additive/ Additive**
	Oxacillin	16/32	8/8	0.5/0.25		
MRSA 6	Fucoidan	64/256	16/32	0.25/0.125	0.5/0.375	**Synergistic/Synergistic**
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 7	Fucoidan	128/256	64/128	0.5/0.5	0.75/0.625	**Additive/ Additive**
	Oxacillin	64/256	16/32	0.25/0.125		
MRSA 8	Fucoidan	64/128	16/32	0.25/0.25	0.5/0.375	**Synergistic/Synergistic**
	Oxacillin	8/32	2/4	0.25/0.125		
MRSA 9	Fucoidan	128/512	32/128	0.25/0.25	0.5/0.75	**Synergistic/Additive**
	Oxacillin	16/32	4/16	0.25/0.5		
MRSA 10	Fucoidan	64/128	16/32	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 11	Fucoidan	128/512	32/64	0.25/0.125	0.375/0.25	**Synergistic/Synergistic**
	Oxacillin	16/64	2/8	0.125/0.125		
MRSA 12	Fucoidan	256/1024	64/128	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Oxacillin	32/64	8/16	0.25/0.25		
MRSA 13	Fucoidan	512/2048	128/256	0.25/0.125	0.5/0.375	**Synergistic/Synergistic**
	Oxacillin	32/64	8/16	0.25/0.25		
MRSA 14	Fucoidan	128/256	32/64	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 15	Fucoidan	64/256	16/32	0.25/0.125	0.5/0.375	**Synergistic/Synergistic**
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 16	Fucoidan	128/512	64/128	0.5/0.25	0.75/0.75	**Additive/Additive**
	Oxacillin	16/32	4/16	0.25/0.5		
MRSA 17	Fucoidan	64/128	16/32	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 18	Fucoidan	64/256	16/32	0.25/0.125	0.5/0.375	**Synergistic/Synergistic**
	Oxacillin	8/32	2/8	0.25/0.25		
MRSA 19	Fucoidan	64/256	16/32	0.25/0.125	0.75/0.625	**Additive/Additive**
	Oxacillin	8/16	4/8	0.5/0.5		
MRSA 20	Fucoidan	256/1024	64/128	0.25/0.125	0.5/0.375	**Synergistic/Synergistic**
	Oxacillin	16/32	4/8	0.25/0.25		

[1]The MIC and MBC of fucoidan with oxacillin. [2]the FIC index. [3]MSSA (ATCC 25923): reference strain Methicillin-sensitive *Staphylococcus aureus*. [4]MRSA (ATCC 33591): reference strain Methicillin-resistant *Staphylococcus aureus*. [5]MRSA (1 - 20): Methicillin-resistant *Staphylococcus aureus* isolated a clinic.

Table 3. Synergistic effects of fucoidan with ampicillin in isolated MRSA and some of reference bacteria.

Samples	Agent	MIC/MBC (μg/mL)		FIC/FBC	FICI/ FBCI[2]	Outcome
		Alone	Combination[1]			
MSSA ATCC 25923[3]	Fucoidan	128/256	32/64	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	8/16	2/4	0.25/0.25		
MRSA ATCC 33591[4]	Fucoidan	128/512	32/64	0.25/0.125	0.375/0.25	**Synergistic/Synergistic**
	Ampicillin	1024/2048	128/256	0.125/0.125		
MRSA 1[5]	Fucoidan	64/256	16/64	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	1024/2048	256/512	0.25/0.25		
MRSA 2	Fucoidan	128/512	32/128	0.25/0.25	0.75/0.75	**Additive/Additive**
	Ampicillin	128/256	64/128	0.5/0.5		
MRSA 3	Fucoidan	256/1024	64/256	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	1024/2048	256/512	0.25/0.25		
MRSA 4	Fucoidan	128/256	32/64	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	256/512	64/128	0.25/0.25		
MRSA 5	Fucoidan	64/128	16/64	0.25/0.5	0.5/0.75	**Synergistic/Additive**
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 6	Fucoidan	64/256	16/64	0.25/0.25	0.5/0.75	**Synergistic/Additive**
	Ampicillin	256/256	64/128	0.25/0.5		
MRSA 7	Fucoidan	128/256	32/64	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	128/512	32/128	0.25/0.25		
MRSA 8	Fucoidan	64/128	16/32	0.25/0.25	0.75/0.5	**Additive/Synergistic**
	Ampicillin	128/256	64/64	0.5/0.25		
MRSA 9	Fucoidan	128/512	32/128	0.25/0.25	0.75/0.5	**Additive/Synergistic**
	Ampicillin	128/512	64/128	0.5/0.25		
MRSA 10	Fucoidan	64/128	16/32	0.25/0.25	0.5/0.75	**Synergistic/ Additive**
	Ampicillin	64/128	16/64	0.25/0.5		
MRSA 11	Fucoidan	128/512	32/64	0.25/0.125	0.5/0.375	**Synergistic/ Synergistic**
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 12	Fucoidan	256/1024	64/128	0.25/0.125	0.5/0.625	**Synergistic/Additive**
	Ampicillin	256/256	64/128	0.25/0.5		
MRSA 13	Fucoidan	512/2048	128/256	0.25/0.125	0.375/0.375	**Synergistic/Synergistic**
	Ampicillin	32/64	4/16	0.125/0.25		
MRSA 14	Fucoidan	128/256	32/64	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 15	Fucoidan	64/256	16/64	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	64/128	16/32	0.25/0.25		
MRSA 16	Fucoidan	128/512	64/128	0.5/0.25	0.75/0.5	**Additive/ Synergistic**
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 17	Fucoidan	64/128	16/64	0.25/0.5	0.5/0.75	**Synergistic/Additive**
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 18	Fucoidan	64/256	16/64	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	64/128	16/32	0.25/0.25		
MRSA 19	Fucoidan	64/256	16/64	0.25/0.25	0.5/0.5	**Synergistic/ Synergistic**
	Ampicillin	64/128	16/32	0.25/0.25		
MRSA 20	Fucoidan	256/1024	64/128	0.25/0.125	0.75/0.375	**Additive/Synergistic**
	Ampicillin	128/512	64/128	0.5/0.25		

[1]The MIC and MBC of fucoidan with ampicillin. [2]the FIC index. [3]MSSA (ATCC 25923): reference strain Methicillin-sensitive *Staphylococcus aureus*. [4]MRSA (ATCC 33591): reference strain Methicillin-resistant *Staphylococcus aureus*. [5]MRSA (1 - 20): Methicillin-resistant *Staphylococcus aureus* isolated a clinic.

Conflict of Interest Statement

The authors have declared no conflict of interest.

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