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Evaluation of Synergistic Antibacterial Effect of Combined S*crophularia striat*a Extract and Antibiotics Against *Pseudomonas aeruginosa* and Methicillin -Resistant *Staphylococcus aureus*

Shabnam Pourmoslemi[*], Shirin Moradkhani[**], Pari Tamri[*,1] and Sahar Foroughinia[*]**

[*]Department of Pharmaceutics, School of Pharmacy, Medicinal Plants and Natural Products Research Center, Hamadan University of Medical Sciences Hamadan, Iran

[**]Department of Pharmacognosy, School of Pharmacy, Medicinal Plants and Natural Products Research Center, Hamadan University of Medical Sciences Hamadan, Iran

[***]Department of Pharmacology &Toxicology, School of Pharmacy, Medicinal Plants and Natural Products Research Center, Hamadan University of Medical Sciences Hamadan, Iran

Abstract

Scrophularia striata from Scrophulariacea family has been used in Iranian folk medicine for the treatment of infectious diseases. In this study we evaluated the synergistic effect of *S. striata* hydroalcoholic extract (SSE) and commercially available antibiotics against *Pseudomonas aeruginosa* and Methicillin- resistant *Staphylococcus aureus* (MRSA) bacteria. The resazurin-based microdilution method was used to determine the minimum inhibitory concentration (MIC) values of plant extract and standard antibiotics. The interaction between standard antibiotics and *Scrophularia striata* extract was evaluated by using the checkerboard method. The results of this study revealed that SSE enhances the antibacterial activity of antibiotics. The combination of SSE and Vancomycin had synergistic to additive effects against MRSA. SSE in combination with Gentamicin had synergistic to additive effects against *P. aeruginosa*. The interaction between Ceftazidime and SSE was additive against *P. aeruginosa*. The best result was the synergistic effect between SSE and Piperacillin-Tazobactam against *P. aeruginosa*. In conclusion this research indicated that *S. striata* has the potential to enhance the antibacterial activity of antibiotics and could be a source to the designing new compounds with synergistic effect in combination with standard antibiotics.

Keywords: *Scrophularia striata, Pseudomonas aeruginosa,* Methicillin resistance *Staphylococcus aureus*, Synergy, Antibiotics

Introduction

Antibiotic resistance has become a serious public health problem worldwide [(1)] . Methicillin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* are two of the more problematic antibiotic- resistant pathogens encountered over the past decade [(2)] .

MRSA infection is the main cause of nosocomial infections and usually is associated with mortality, morbidity and cost burden [(3)]. Resistance to methicillin has occurred in *S. aureus* by penicillin binding protein mutation, a chromosomal mutation[(4)] . The rate of MRSA infections is increasing rapidly throughout the world and more importantly, in the past decades the prevalence of community acquired MRSA infections has notably increased [(5)]. The most common MRSA infections are skin and subcutaneous tissue infections or invasive infections such as meningitis, pneumonia, osteomyelitis, lung abscess, bacteremia and infective endocarditis [(6)]. Several antibiotics including Clindamycin, Co- trimoxazole, Vancomycin and Daptomycin are being used to treat MRSA infections [(7)] . However, the increasing resistance of pathogens to these medicines and their side effects have led to poor therapeutic outcomes and increased mortality [(8)].

Pseudomonas aeruginosa is a gram negative bacillus commonly found in soil, water and the environment [(9)] . *P. aeruginosa*, as an opportunistic pathogen is a major cause of hospital acquired infections, especially in patients with underlying conditions [(10)]. *P. aeruginosa* has the ability to survive on minimum nutritional necessities and to tolerate different environmental conditions, allowing this organism to persist in both hospital and community setting[(9)]. It has become difficult to eradicate *P. aeruginosa* due to its high capacity to resists antibiotics [(11)] . A number of antibacterial agents such as Piperacillin-Tazobactam, Ceftazidime, Cefepime, Ciprofloxacin and Imipenem- Cilastatin are used to treat *P. aeruginosa* infections but a limited number of these agents have reliable activity against *P. aeruginosa* isolates [(9)]. Thus, it is necessary to find new ways to overcome the resistance of MRSA and *P. aeruginosa* to antibiotics. Combination therapy using two or more

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antibacterial agents is an important strategy to overcome antibiotic- resist organisms [12]. However, combining antibiotics result in more antibiotics adverse effects and drug interactions.

Many previous studies have shown the antibacterial activity of plant constituents and some studies have proved the synergistic antibacterial effect of the combination of antibiotics and phytochemicals [13, 14, 15] .

Scrophularia striata (Scrophulariaceae) is an herbaceous plant that grows wild in the west regions of Iran [16]. In traditional medicine, it has been used for the treatment of the inflammatory and infectious diseases [17] . Several studies have shown biologic activities of *S. striata*, including antibacterial [18], anti-inflammatory [19], antioxidant [20], anticancer [21] and healing effects [22].

The aim of this study was to investigate the synergistic effect between SSE and commonly used antibiotics against MRSA and *P. aeruginosa*.

Materials and Methods

Materials and Strains

Nutrient Agar (NA) and Muller Hinton Broth (MHB) culture media were obtained from Merck (Darmstadt, Germany) and used for growing the bacteria and antibacterial activity tests throughout the study.

Standard strains of MRSA (ATCC 33591) and *P. aeruginosa* (ATCC 27853) were obtained from Persian Type Culture Collection in Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. Isolates were maintained in Tryptic Soy Broth (TSB) containing 15% glycerol at -80 ° C until use. Bacterial inocula were prepared from 24 h culture of the organisms grown on nutrient agar (NA) plates. The organisms were harvested, and suspended in normal saline (NS) to produce a MacFarland 0.5 (turbidity equivalent to 10^8 colony forming units (CFU) [23].

Preparation of S. striata Extract

The aerial parts of *S. striata* were collected from the west parts of Iran (Ilam province). The authentication of herb material was performed at the Department of Pharmacognosy, School of Pharmacy, Hamadan University of Medical Sciences. The plant was dried and grounded to a fine powder. The plant hydroalcoholic (ethanol/distilled water 7/3 v/v) extract was prepared by using maceration method [24] .

Preparation of stock and standard solutions

A SSE stock solution of 32mg/ml was prepared by accurately weighing and dissolving the extract in sterile dimethyl sulfoxide (DMSO). Aliquots of the stock solution were brought to 10 ml volume using sterile 0.9% (w/v) normal saline to obtain further dilutions.

Commercial parenteral dosage forms of antibiotics [Co-trimoxazole (CTX), Clindamycin (CLD) Vancomycin (VAN), piperacillin + Tazobctam (PIP-Tazo), Gentamicin (GEN) and Ceftazidime (CEF)] were used for preparing antibiotic solutions. Whole content of one vial or ampule was dissolved and further diluted in normal saline to obtain antibiotic solution with the intended concentration

Determination of Minimum Inhibitory Concentration (MIC)

MIC of *S. striata* extract against MRSA and *P. aeruginosa* were determined on sterile 96 well microdilution plates according to the Clinical and Laboratory Standards Institute (CLSI) Guidelines [25]. SSE solutions in the range of concentrations of 32 - 0.015 mg/ml were prepared through two-fold serial dilution of the stock solution. 100 µl of each solution was mixed with 100 µl of Mueller Hinton Broth (MHB) medium inoculated by bacterial suspension (containing 10^6 CFU/ml) in three wells row of microdilution plate. Four wells without adding the extract were used to show maximum growth for each microorganism and four others containing uninoculated medium were used as negative control to show the aseptic technique.

MIC of the antibiotics against corresponding microorganisms were determined using the same method explained above.

After incubation of the plates at 37° C for 24 h, the lowest concentration at which no growth was observed was determined as MIC [26]. Visual inspection was used to determine any signs of bacterial growth and turbidity. For more accurate determination of MIC, 50 µl of 0.002% w/v sodium resazurin solution was added to the wells and color change was investigated after 1 h incubation at 37 °C. Change of color from blue to purple or red was considered as a sign of bacterial growth [27]. The test was performed in three separate experiments, each one in three replicates. Quantities determined as MIC in at least two experiments were reported as the final MIC.

Determination of Minimum Bactericidal Concentration (MBC)

MBC was determined by transferring 100 µl culture from the wells exhibited no growth on NA plates and incubated at 37° C for 24 h. The lowest concentrations of SSE and antibiotics that show no colony growth on NA were reported as MBC. This test was repeated in three separate experiments. Quantities determined as MBC in at least two tests were reported as the final MBC.

Investigation of Antibacterial Activity of combined S. striata Extract and Antibiotics

The antibacterial activity of combined SSE and antibiotics was investigated using the checkerboard dilution test that is one of the methods used for evaluation of *in vitro* synergy for multiple drugs [28]. This test determines the impact on antibacterial activity of the combination of

antibacterial agents in comparison to their individual activities. Fractional Inhibitory Concentration (FIC) index value was used to evaluate the interaction of the two agents tested. FIC is determined according to the following equation (Eq. 1), where A and B are the MIC for each antibacterial agent when combined in a single well, and MIC_A and MIC_B are the MIC for each agent individually.

FIC Index = FIC_A+FIC_B= $A/MIC_A + B/MIC_B$

FIC Index values are interpreted as follows: FIC Index ≤ 0.5, synergistic, 0.5 ≤ FIC Index ≤ 1, synergistic to additive, 1 ≤ FIC Index ≤ 4, additive, and FIC Index ≥ 4, antagonistic[29].

Checkerboard test was performed for combinations of the SSE with CTX, CLD and VAN against MRSA and with PIP-Tazo, GEN and CEF against *P. aeruginosa*. An 8-by-8 well configuration on sterile 96 well microdilution plates was utilized. Final concentrations of the SSE and antibiotics in the wells ranged from 1/8 × MIC to 4 × MIC. The wells contained MHB medium inoculated with 10^6 CFU/ml of the respective microorganism. Positive and negative controls containing inoculated and uninoculated MHB were set on every plate. After incubation of the plates at 37° C for 24 h, bacterial growth was investigated by visual inspection followed by adding 50 µl resazurin solution to observe the color change. The lowest FIC index of all the non-turbid wells along the turbidity/non-turbidity interface was used for interpretation of the results. This test was performed in triplicate and results observed in at least two replicates were reported.

Statistical Analysis

Microsoft Excel 2016 was used to calculate mean and variance of data.

Results

Antibacterial Activity

The results of the evaluation of the antibacterial activity of SSE showed that this extract has low activity against MRSA (MIC=8 mg/ml) (Figure. 1) and *P. aeruginosa* (MIC= 4 mg/ml, MBC= 8mg/ml) (Figure 2).

The SSE had no bactericidal activity against MRSA at the concentrations of 32-0.015 mg/ml. The MRSA strain was resistant to Clindamycin and Co-trimoxazole. The MIC and MBC of SSE and standard antibiotics are shown in Table.1.

Figure. 1. Determination of MIC for Vancomycin and *Scrophularia striata* against methicillin resistance *Staphylococcus aureus* (ATCC 33591).

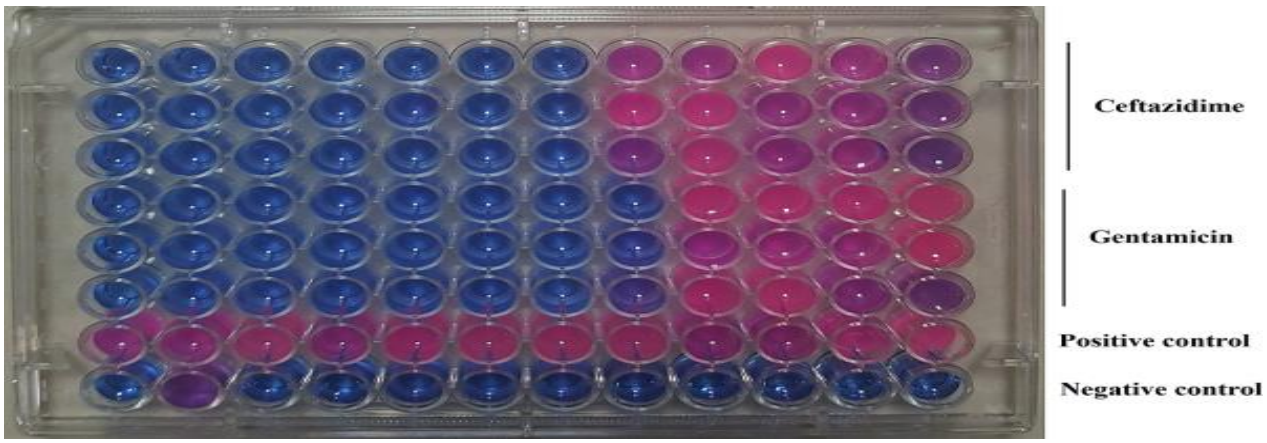

Figure. 2. Determination of MIC for Ceftazidime and Gentamicin against *P. aeruginosa* (ATCC 27853)

Table 1. The antibacterial activity of antibiotics and *S. striata* extract

	MRSA				*P. aeruginosa*			
	SSE	**CTX**	**CLD**	**VAN**	**SSE**	**PIP**	**GEN**	**CEF**
Concentrations	0.015-32 mg/ml	0.25-2000 µg/ml	0.015-256 µg/ml	0.031-64 µg/ml	0.015-32 mg/ml	0.031-64 µg/ml	0.031-64 µg/ml	0.031-64 µg/ml
MIC	8 mg/ml	ND*	ND	2 µg/ml	4 mg/ml	2 µg/ml	0.25 µg/ml	0.062 µg/ml
MBC	ND	ND	ND	ND	8 mg/ml	ND	1 µg/ml	0.5 µg/ml

* Not determined in the concentration ranges

Study of Synergistic Effect between Antibiotics and SSE

The results of interaction between SSE and antibiotics expressed in FICI are shown in Table. 2. The combination of SSE and Vancomycin had synergistic to additive effect against MRSA. The combinations of SSE and Pip + Tazo showed synergism against *P. aeruginosa* and in the case of the combination of SSE and Gentamicin the interaction was synergism to additive. The interaction between SSE and Ceftazidime was additive (Figure 3). The combination of SSE and Pip-Tazo showed the best synergistic capacity *against P. aeruginosa.*

Table 2. The interaction between antibiotics and *S. striata* extract

Bacteria	Antibiotics + SSE	FICI	Interpretation
MRSA	VAN	0.75	synergistic to additive
P. aeruginosa	PIP	0.3	synergism
	GEN	0.75	synergistic to additive
	CEF	1.5	additive

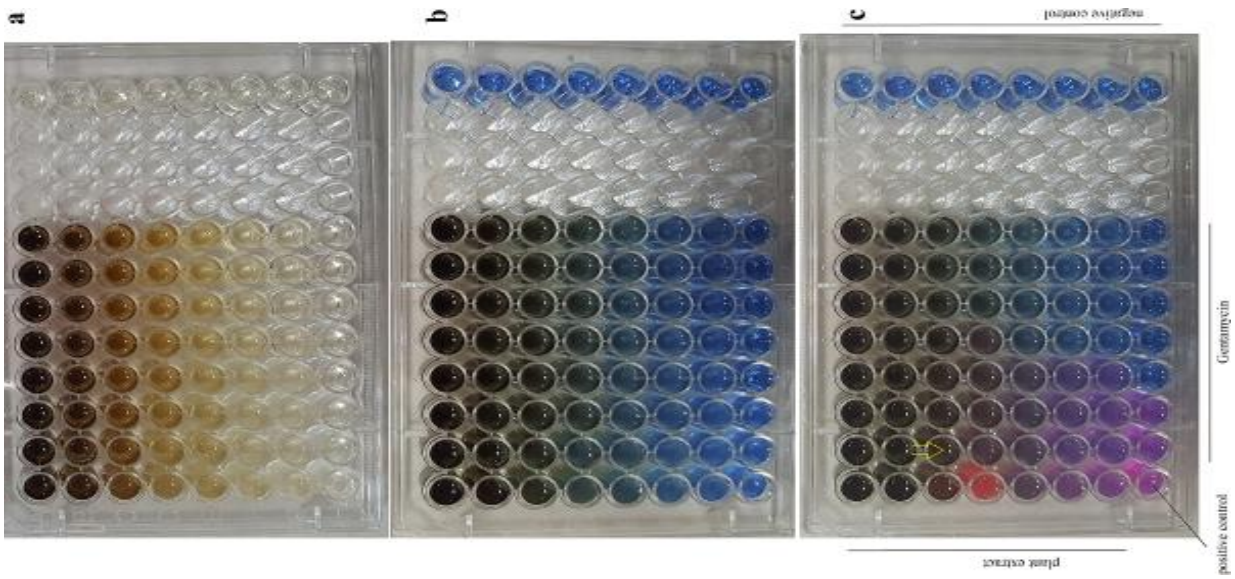

Figure 3. Checkerboard test for determination of combined antibacterial activity of Gentamicin and *Scrophularia. striata* extract against *P. aeruginosa* (ATCC 27853), a) after incubation, b) after adding the resazurin solution and c) after incubation of resazurin

Discussion

As the results of this study showed, the SSE in combination with standard antibiotics has good synergistic and additive effects and has the potential to be used as an adjunct therapy in the treatment of infections caused by resistant microorganisms such as *P. aeruginosa* and MRSA. The mechanism of SSE to enhance the antibacterial activity of antibiotics is unknown. In addition to the direct antibacterial activity of plants, one of the possible mechanisms for the synergistic antibacterial effect of plants extract and antibiotics is the modifying and inhibiting the acquired resistance in bacterial cell and thus enhance the antibiotic antibacterial activity [(13)].

The main compounds that isolated and characterized from SSE were flavonoids such as quercetin, nepitrin and isorhamnetin-3-*O*-rutinoside [(30)]. Flavonoids may affect cellular membrane, inhibit nucleic acid synthesis, and energy metabolism.

Additionally, flavonoids may interrupt cell membrane and cell wall synthesis [31]. According to the results of this study the SSE has better antibacterial activity against *P. aeruginosa* (MIC= 4 mg/ml) comparing to MRSA (MIC= 8 mg/ml) and in combination with Pip- tazobactam has a synergistic effect against *P. aeruginosa*. In addition, SSE enhanced the Gentamicin antibacterial activity against this microorganism. The resistance of *P. aeruginosa* against antibiotics may be intrinsic, acquired or adaptive. This microorganism has a low permeable outer membrane, expresses an efflux pump and produces antibiotic-inactivating enzymes to resist antibiotics, intrinsically. The acquired resistance of this organism may be due to mutation changes or horizontal gene transfer. Previous studies indicated that the phenolic compounds and flavonoids initially change the permeability of cell membrane and this leads to the leaking of cellular content or disrupt the membrane structure by interfering with membrane proteins [32, 33]. Therefore, SSE may increase the entry of antibiotics into bacterial cells by increasing the permeability of bacterial cell membrane.

SSE also enhanced the Vancomycin antibacterial activity against MRSA. Vancomycin is a bactericidal antibiotic that inhibits bacterial cell wall synthesis by binding to D- Ala-D-Ala peptide and following that preventing peptidoglycan cross-Linking by transpeptidation and eventually inhibit the cell wall biosynthesis and bacterial cell death [34]. Vancomycin has been widely used for the treatment of MRSA infections and it has led to the emergence of Vancomycin resistant *S. aureus* [35]. The augmentation of antibacterial activity of Vancomycin by SSE could be a result of SSE antibacterial activity or modifying the mechanism of acquired resistance.

Conclusion

In conclusion, our findings in this study revealed the synergistic and additive activity of SSE combined with standard antibiotics against *P.aeruginosa* and MRSA. Antibiotics resistance is a growing problem and the perspective of antibiotics effectiveness in the future is uncertain. Plants are important sources of biologically active compounds with antibacterial activity. The antibacterial effect of plants can be due to their direct activity against bacteria or their synergistic activity with antibiotics. *S. striata* could be a source of new antibacterial compounds. However, the further studies are needed to explore the mechanism underlying its synergistic effects. In addition, the toxicity, antibacterial activity and bioavailability of the SSE should be studied *in vivo*.

Conflict of Interest

The authors declare there is no conflict of interest.

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