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NATURAL DRUGS

SYNERGISTIC ANTIBACTERIAL ACTIVITY OF *SALVIA OFFICINALIS* AND *CICHORIUM INTYBUS* EXTRACTS AND ANTIBIOTICS

OLGICA D. STEFANOVIĆ*, DRAGANA D. STANOJEVIĆ and LJILJANA R. ČOMIĆ

Laboratory of Microbiology, Department of Biology and Ecology,
Faculty of Science, University of Kragujevac, Radoja Domanovica 12, 34 000 Kragujevac, Serbia

Abstract: Synergistic activity of *Salvia officinalis* and *Cichorium intybus* extracts and commonly used antibiotics, amoxicillin and chloramphenicol, were evaluated. Interactions between plant extracts and antibiotics were tested by checkerboard method and interpreted as FIC index. *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and clinical isolates *Staphylococcus aureus*, *Bacillus subtilis*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Escherichia coli* and *Proteus mirabilis* were used. *Salvia officinalis* showed better synergistic capacity than *Cichorium intybus*. Synergistic interactions were observed between amoxicillin and acetone or ethyl acetate extract of *Salvia officinalis* and between chloramphenicol and ethyl acetate extract of *Salvia officinalis*. In the presence of sub-inhibitory concentration (1/4 MIC to 1/32 MIC) of sage extracts, the MIC values of antibiotics were decreased by 2- to 10-fold. Synergism was observed against all test bacteria, except *Escherichia coli*. The combinations of acetone and ethyl acetate extract from *Cichorium intybus* and antibiotics resulted in additive and indifferent effects against tested bacteria.

Keywords: antibacterial activity, herb-drug interaction, plant extracts

The development of resistance in bacteria is one of the mechanisms of natural adaptation to the presence of an antimicrobial agent that inhibits susceptible organisms and selects the resistant ones. Under continued selection pressure, the selected resistant bacteria multiply and spread to other geographic locations as well as to other bacteria by transfer of resistance genes (1). Selection of resistant strains occurs so rapid for some bacteria that clinical usefulness of the antibiotics is lost within a 5 year period (2).

The emergence and spread of microbes that are resistant to cheap and effective first-choice antibiotics has become a common occurrence. Faced with this problem, there is need to develop alternative approaches in addition to the search for new antimicrobial compounds (3).

Plants have traditionally provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being (4). In recent years, there have been many studies about the beneficial role of bioactive plant extracts and pure isolated compounds in increasing the *in vitro* efficacy of commonly used antibiotics against variety of microorganisms. A number of studies have reported the use of plant extracts in combination with antibiotics, with significant reduction in the minimum inhibitory concentrations of the antibiotics. Betoni et al. (5) tested synergistic interactions between methanol extracts of 8 Brazilian medicinal plants and 13 antibiotics against 15 strains of *Staphylococcus aureus* (5). Estimone et al. (6) investigated the herb-drug interaction between tea extract and penicillin G against *Staphylococcus aureus* and showed that the interactions were mainly additive (6). Nostro et al. (7) demonstrated that combinations of propolis extract + clarithromycin and *Zingiber officinale* + clarithromycin have the potential to help control *Helicobacter pylori* – associated with gastroduodenal disease (7). Sibanda and Okoh (8) showed potentials of synergy between acetone extracts of *Garcinia kola* seeds and amoxicillin, ciprofloxacin, tetracycline and chloramphenicol against pathogenic microorganisms. This ability of plant extracts to act synergistically with antibiotics could be a new approach to solve the problem of bacterial resistance and less susceptible bacteria (9–11).

Salvia officinalis L. (*Lamiaceae*) and *Cichorium intybus* L. (*Asteraceae)* are well-known medic-

* Corresponding author: e-mail: e-mail: olgicas@gmail.com

inal plants. *S. officinalis* (sage) is one of the oldest and still the most popular medicinal plants. Besides many others therapeutical properties, *S. officinalis* has broad spectrum antibacterial activity (12–14). Tannin-based astringent activities of sage reduced growth of plaques, inhibit gingival inflammation and have beneficial effects on carries prophylaxis (15). Recently, antimicrobial activity of *S. officinalis* was showed against vancomycin-resistant enterococci (16).

Antibacterial activity of *Cichorium intybus* (chicory) is also known. Inhibitory effects of chicory on growth of various Gram-positive and Gram-negative pathogenic bacteria, as antibiotic susceptible and resistant strains, were evaluated (17–19). The extracts from *C. intybus* were screened for anti-*Helicobacter* activity (20) as well as against multidrug resistant *Salmonella typhi* (21).

Regarding the fact that *S. officinalis* and *C. intybus* extracts are well known antibacterial agents, the aim of this work was to establish *in vitro* synergy between ethyl acetate and acetone extracts of these medicinal plants and commonly used antibiotics (chloramphenicol and amoxicillin) emphasizing the potential role of phytochemicals in increasing the effectiveness of antibiotics.

EXPERIMENTAL

Preparation of samples for testing

S. officinalis (leaf) and *C. intybus* (root) were obtained commercially. Dried, ground plant material (30 g for each extract) was extracted with ethyl acetate and acetone in Soxhlet apparatus. The extracts were concentrated to dryness using a rotary evaporator at 40°C. The obtained extracts were dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) and then diluted into Mueller-Hinton broth (Torlak, Belgrade) to achieve a concentration of 10% DMSO. Solutions of chloramphenicol (Panpharma, France) and amoxicillin (Panfarma, Belgrade) were obtained by dissolving in a Mueller-Hinton broth.

Tested microorganisms

The following bacteria were used: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and clinical isolates of *Staphylococcus aureus* (PMFKg-B30), *Bacillus subtilis* (PMFKg-B2), *Enterobacter cloacae* (PMFKg-B22), *Klebsiella pneumoniae* (PMFKg-B26), *Escherichia coli* (PMFKg-B32) and *Proteus mirabilis* (PMFKg-B29). All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. Bacteria were stored in microbiological collection at the Laboratory of Microbiology (Faculty of Science, University of Kragujevac).

Antibacterial assay

Before the synergism assay was evaluated, the minimal inhibitory concentrations (MICs) of ethyl acetate extracts, acetone extracts and antibiotics were determined by tube dilution method (22). Briefly, 1 mL of the stock solution of tested compound was mixed with 1 mL of Mueller-Hinton broth and then twofold serial dilutions were prepared. The concentration range was from 40 mg/mL to 0. 019 mg/mL. Each dilution was inoculated with 50 μL of diluted inoculum. Overnight cultures of bacterial species were used for inoculum preparation. Bacterial suspensions were prepared by direct colony suspension method and adjusted to obtain the turbidity of 0.5 McFarland standard (approximately 10^8 colony-forming units (CFU)/mL) and then diluted in 1:100 ratio. The final concentration of the bacteria in the test tubes was 5×10^5 CFU/mL. The test tubes were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration at which visible growth was inhibited.

Each test included two growth controls consisting of the medium with the solvent (10% DMSO) and medium with bacterial suspension as well as sterility control. All tests were performed in duplicate.

The synergistic interactions were evaluated by checkerboard method (23). Briefly, a series of twofold dilutions of stock solutions of amoxicillin or chloramphenicol and a series of twofold dilutions of stock solutions of either acetone or ethyl acetate extracts from *S. officinalis* or *C. intybus* were prepared. Plant extract (0.25 mL, final concentration from MIC to 1/32 MIC) and 0. 25 mL of antibiotic (final concentration from MIC to 1/32 MIC) were added into 0.5 mL of Mueller-Hinton broth to achieve a final volume of 1 mL. Because the final volume (1 mL) is four times as great as the volume of each antimicrobial (0.25 mL), the antimicrobial concentrations used in the stock solutions are four times greater than the desired final concentrations. Each test tube contained unique combination of plant extract/antibiotic concentration. The tested tubes were inoculated with 50 μL of the inocula prepared as those used for MIC assay and incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of antimicrobial agents in combination at which visible bacterial growth was inhibited. Each test included two growth controls consisting of

Table 1. Antibacterial activity and type of interaction between extracts of *Salvia officinalis* and antibiotics.

Species	MIC_{Ch}	MIC_{A}	MIC_{Ac}	Acetone extract + chloramph.	Acetone extract + amoxicillin	MIC_{Et}	Et. acetate extract + chloramph	Et. acetate extract + amoxicillin
Escherichia coli ATCC 25922	0.25	0.0625	0.3125	1.37* (I)	0.38 (S)	40	0.615 (A)	0.42 (S)
Staph. aureus ATCC 25923	0.125	0.0019	0.156	1.37 (I)	0.35 (S)	40	1.37 (I)	1.37 (I)
P. aeruginosa ATCC 27853	0.25	0.00012	0.019	0.615 (A)	0.35 (S)	2.5	0.56 (A)	1.37 (I)
Bacillus subtilis	0.25	0.031	0.039	1.37 (I)	0.50 (S)	10	0.40 (S)	0.35 (S)
Enterobacter cloacae	0.25	2	0.156	0.615 (A)	0.35 (S)	20	0.49 (S)	1.37 (I)
Klebsiella pneumoniae	0.25	0.25	0.156	1.37 (I)	0.35 (S)	20	0.35 (S)	1.37 (I)
Staphylococcus aureus	0.5	0.5	0.3125	1.37 (I)	0.35 (S)	20	0.49 (S)	1.37 (I)
Escherichia coli	35	2	20	1.37 (I)	1.37 (I)	40	1.37 (I)	1.37 (I)
Proteus mirabilis	17.5	4	1.25	1.37 (I)	0.49 (S)	40	1.37 (I)	0.53 (A)

* mean FIC index; MIC_{Ch} – MIC values for chloramphenicol (mg/mL); MIC_{A} – MIC values for amoxicillin (mg/mL); MIC_{Ac} – MIC values for acetone extract (mg/mL); MIC_{Et} – MIC values for ethyl acetate extract (mg/mL); I – indifferent effect; A – additive effect; S – synergistic effect.

the medium with the solvent (10% DMSO) and medium with bacterial suspension as well as sterility control.

In vitro interactions between antimicrobial agents were determined and quantified by calculating the fractional inhibitory concentration (FIC) index using the following formula: FIC index = (MIC of plant extract in combination/MIC of plant extract alone) + (MIC of antibiotic in combination/MIC of antibiotic alone). Interpretation of the FIC index (FICI) was as follows: FICI ≤ 0.5 – synergy; FICI > 0.5–1 – additive; FICI > 1–4 –indifference and FICI > 4 – antagonism. The action of antimicrobial agents is considered to be:

– synergistic if their joint effect is stronger than the sum of effects of the individual agents;

– additive if their joint effect is equal to the sum of effects of the individual agents;

– indifferent if their joint effect is equal to the effect of either individual agent;

– antagonistic if their joint effect is weaker than the sum of effects of the individual agents or weaker than the effect of either individual agent (23).

When more than one combination resulted in a change in the MIC value of the extract or antibiotic, the FIC index was expressed as the average of the FIC values.

RESULTS

The results of *in vitro* testing of antibacterial activity of ethyl acetate and acetone extracts of *S. officinalis* and *C. intybus* are shown in Tables 1 and 3. The solvent (10% DMSO) did not inhibit the growth of tested bacteria. The extracts showed selective antibacterial properties and the activity depended both on the species of bacteria and on the type of extract. In general, the most active extract was acetone extract from *S. officinalis* with MIC values between 0.019 mg/mL and 1.25 mg/mL, except for *Escherichia coli* (MIC was 20 mg/mL). The MIC values of *C. intybus* acetone and ethyl acetate extracts were in the range from 1.09 mg/mL to 8.75 mg/mL. The highest MIC values were shown by ethyl acetate extract of *S. officinalis*, from 2.5 mg/mL to 40 mg/mL.

The MIC values for chloramphenicol were lower than 1 mg/mL, except for *Proteus mirabilis* and *Escherichia coli* 17.5 mg/mL and 35 mg/mL, respectively. The MIC values for amoxicillin were between 0.00012 mg/mL and 4 mg/mL.

The joint activity of *S. officinalis* extracts and antibiotics produced synergistic, additive and indifferent effects (Table 1). The extracts showed better synergistic capacity with amoxicillin than with chloramphenicol. The acetone extract/amoxicillin com-

Table 2. Synergistic interactions between extracts from *Salvia officinalis* and antibiotics.

Species	Amoxicillin				Chloramphenicol	
	Acetone extract		Ethyl acetate extract		Ethyl acetate extract	
	FICI	MIC*	FICI	MIC	FICI	MIC
Escherichia coli ATCC 25922	0.38	$1/8_E + 1/16_A$	0.42	$1/8_E + 1/16_A$		
Staph. aureus ATCC 25923	0.35	$1/32_E + 1/16_A$				
P. aeruginosa ATCC 27853	0.35	$1/32_E + 1/16_A$				
Bacillus subtilis	0.50	$1/4_E + 1/4_A$	0.35	$1/16_E + 1/32_A$	0.40	$1/8_E + 1/32_A$
Enterobacter cloacae	0.35	$1/32_E + 1/16_A$	0.49	$1/4_E + 1/32_A$		
Klebsiella pneumoniae	0.35	$1/32_E + 1/16_A$	0.35	$1/16_E + 1/32_A$		
Staphylococcus aureus	0.35	$1/32_E + 1/16_A$	0.49	$1/4_E + 1/32_A$		
Proteus mirabilis	0.49	$1/32_E + 1/4_A$				

* The most active combination ($1/8_E + 1/16_A$ means 1/8 conc. of MIC of extract + 1/16 conc.of MIC of antibiotic).

Table 3. Antibacterial activity and type of interaction between extracts of *Cichorium intybus* and antibiotics.

Species	MIC_{Ch}	MIC_{A}	MIC_{Ac}	Acetone extract + chloramph.	Acetone extract + amoxicillin	MIC_{Et}	Et. acetate extract + chloramph.	Et. acetate extract + amoxicillin
Escherichia coli ATCC 25922	0.25	0.0625	2.5	1.37* (I)	0.87 (A)	2.18	1.37 (I)	1.37 (I)
Staph. aureus ATCC 25923	0.125	0.0019	2.5	1.37 (I)	1.37 (I)	2.18	1.37 (I)	1.37 (I)
P. aeruginosa ATCC 27853	0.25	0.00012	1.25	1.37 (I)	1.37 (I)	1.09	1.37 (I)	1.37 (I)
Bacillus subtilis	0.25	0.031	2.5	0.91 (A)	0.86 (A)	2.18	1.37 (I)	1.37 (I)
Enterobacter cloacae	0.25	2	5	0.7 (A)	0.68 (A)	2.18	0.87 (A)	0.7 (A)
Klebsiella pneumoniae	0.25	0.25	2.5	0.91 (A)	0.68 (A)	2.18	1.37 (I)	0.84 (A)
Staphylococcus aureus	0.5	0.5	2.5	0.74 (A)	0.59 (A)	2.18	1.37 (I)	0.62 (A)
Escherichia coli	35	2	2.5	1.37 (I)	0.7 (A)	2.18	1.37 (I)	0.7 (A)
Proteus mirabilis	17.5	4	5	1.37 (I)	0.67 (A)	8.75	1.37 (I)	0.7 (A)

* mean FIC index; MIC_{Ch} – MIC values for chloramphenicol (mg/mL); MIC_{A} – MIC values for amoxicillin (mg/mL); MIC_{Ac} – MIC values for acetone extract (mg/mL); MIC_{Et} – MIC values for ethyl acetate extract (mg/mL); I – indifferent effect; A – additive effect; S – synergistic effect.

bination showed synergy for 88.9% (8 of 9 strains) and ethyl acetate extract/amoxicillin combination for 22.2% (2 of 9 strains) of tested bacteria (Table 2). The activity of amoxicillin was increased from 2- to 10-fold depending on bacteria. The mean FIC indices for combinations of amoxicillin with acetone or ethyl acetate extract were from 0.35 to 1.37. The chloramphenicol only with ethyl acetate extract showed synergism for 44.4% (4 of 9 strains) of tested bacteria (Table 2). Synergism was observed against *Bacillus subtilis*, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. The activity of chloramphenicol was increased 10-fold in relation to these strains. For the other strains, as well as chloramphenicol/acetone extract combinations, additive and indifferent effects were demonstrated. The mean FIC indices were in range from 0.35 to 1.37.

Comparing to *S. officinalis*, extracts of *C. intybus* did not show synergistic effect with antibiotics (Table 3). Additive and indifferent effects were observed. The mean FIC indices were from 0.59 to 1.37. Amoxicillin with acetone extract or ethyl acetate extract showed additive effect for 77.8% (7 of 9 strains) and 55.6% (5 of 9 strains) of the tested bacteria, respectively. Chloramphenicol with acetone extract or ethyl acetate extract showed additive effect for 44.4% (4 of 9 strains) and 11.1% (1 of 9 strains) of the tested bacteria, respectively.

DISCUSSION

This study has shown that acetone and ethyl acetate extracts of *S. officinalis* and *C. intybus* in combinations with antibiotics inhibited the growth of tested bacteria at a lower concentration than when the single drugs were tested separately. This effect was synergistic or additive for the most of the tested strains. The combinations between acetone extract of *S. officinalis* and amoxicillin showed the most potent synergy. It was found that the presence of sub-inhibitory concentrations (between 1/4 MIC to 1/32 MIC) of the acetone extract modulated the activity of amoxicillin by reducing the concentration of antibiotic needed to inhibit the growth of bacteria. These findings indicate the potential of *S. officinalis* as a source of antibiotic resistance modifying compounds. The synergy was observed against all test bacteria, except *Escherichia coli*. Horiuchi et al. (24) also reported synergistic activity between acetone extract from *S. officinalis* and aminoglycosides against vancomycin-resistant enterococci and isolated an effective compound, carnosol. The ethyl acetate extract of *S. officinalis* demonstrated weak antibacterial activity (MIC = 2.5–40 mg/mL) but besides that, it showed ability to improve activity of antibiotics against tested bacteria (synergistic and additive effects). This is interesting to note that the synergistic capacity of plant extracts could be investigated independently of their antimicrobial activity. In this study, according to FIC indices, the ethyl acetate and acetone extracts from *C. intybus* showed additive and indifferent effects. Aqil and Ahmad noticed synergism between ethanolic extract from *C. intybus* and tetracycline, chloramphenicol and ciprofloxacin, but it was tested by disk-diffusion method and synergistic effect was observed on the basis of enlargement of inhibition zone (17).

Synergistic and additive interactions are a result of a combined effect of active compounds from extracts and antibiotics. Plant extracts contain a great number of different compounds (phenols, flavonoids, tannins, coumarins, alkaloids, terpenoids) which have an impact on growth and metabolism of microorganisms (25). The chemical composition of *S. officinalis* leaf extract is very complex. Many polyphenols (diterpene, triterpene, tannins, flavones) have been isolated (13, 16, 24, 26–28). The root extract of *C. intybus* is rich in inulin, alkaloids, sesquiterpene lactones, flavonoids, saponins (18, 29, 30). It seems that both active compounds from extracts and antibiotics directly or indirectly attach to the same site on bacterial cell. Mechanism of synergy is still insufficiently researched. Some authors suggest that phytocompounds disturb cell wall or increase permeability of the cytoplasmic membrane and thereby facilitate the influx of antibiotics, produce efflux pump inhibitors or inhibit penicillin-binding proteins (3, 24). The combinations of antibiotics with plant extracts could be a significant basis for development of new approach in resistance modifying agents because the use of extracts shows a low risk of increasing bacterial resistance to their action. Actually, the extracts contain mixtures of different bioactive compounds, which make microbial adaptability very difficult comparing to single-constituent antibiotics. Moreover, using plant products with antibiotics could decrease undesirable side effects of antibiotics (31, 32). However, the understanding of mechanism of synergy is fundamental to development of pharmacological agents against bacterial infection.

In conclusion, the antibacterial activity of plant extracts from *S. officinalis* and *C. intybus* were confirmed and synergistic activity of extracts of *S. officinalis* and tested antibitics could suggest an alternative manner to overcome a problem of bacterial infections. Further research is necessary to identify

active compounds and research mechanism of interaction with antibiotics.

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