

IN VITRO INTERACTION BETWEEN *Agrimonia eupatoria* L. EXTRACTS AND ANTIBIOTIC

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ABSTRACT. Synergistic activity between water, acetone, ethanol and diethyl ether extract of *Agrimonia eupatoria* L. and commonly used antibiotic (ampicillin) were evaluated. *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were used. Interaction between plant extracts and antibiotic were tested by checkerboard method and expressed as fractional inhibitory concentration (FIC) index showed indifferent, additive and synergistic effects. Synergism was observed against *E. coli* for every combination of agents. FICI values were ranged from 0.03 to 0.29. Inhibitory concentration (IC₅₀) was evaluated for every combination of tested extracts and antibiotic and the best combinations for every tested bacteria were combination of diethyl ether extract + ampicillin and combination of acetone extract + ampicillin.

Key words: *Agrimonia eupatoria*, plant extract, herb-drug interaction

INTRODUCTION

Agrimonia eupatoria L. (family Rosaceae), is widespread throughout Europe, Asia, Africa, North America. Its habitat is on slopes, rocky areas, in arid forests, by roadsides, on dry grasslands. It is a perennial herbaceous plant with upright, hairy stem with few branches. The leaves are leathery, plumose and the lower ones frequently form a rosette. The flowers are arranged in thick, spiky bunches and the fruit grows downwards (JOSIFOVIĆ *eds.*, 1972).

A. eupatoria is traditionally used in folk medicine to treat various inflammatory diseases. It is well-known for its usage as a raw material for the extraction of medicinal ingredients or production of drugs in the pharmaceutical industry. According to the previous studies, *A. eupatoria* is very rich in secondary metabolites and it was detected that it contained: tannin, flavonoids, phenolic acids, triterpenoids (SENDA and ZIEBA, 1972; BILLA *et al.*, 1993a; BILLA *et al.*, 1993b; FENG *et al.*, 2013; GRANICA *et al.*, 2013). It is known that plant synthesizes secondary metabolites that exhibit antimicrobial activity (DUGLER and GONUZ, 2004; CWIKLA *et al.*, 2010; MURUZOVIĆ *et al.*, 2016). According to our previous

study, *A. eupatoria* extracts showed antimicrobial activity (MURUZOVIĆ *et al.*, 2016), but there are no data on synergy between extracts of this plant and antibiotics.

Since the discovery of antibiotics and their uses as chemotherapeutic agents, there was a belief that this would lead to the eradication of infectious diseases. However, diseases and disease agents that were once thought to have been controlled by antibiotics are returning in new forms resistant to antibiotic therapies. 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 organisms multiply and spread to other geographic locations as well as to other microbes by transfer of resistance genes (LEVY and MARSHALL, 2004).

The bacterial resistance is great problem in modern medicine, and this problem has lead to screening of plants extracts as a source of bioactive compounds. There have been many studies about synergistic interaction between plant extracts or pure isolated compounds with commonly used antibiotics against resistance bacteria (ESIMONE *et al.*, 2006; HORIUCHI *et al.*, 2007; STEFANOVIC *et al.*, 2011; OLAJUYIGBE and AFOLAYAN, 2012; STEFANOVIC *et al.*, 2012).

Considering that *A. eupatoria* has been insufficiently studied, the aim of this study was to establish synergy between water, acetone, ethanol and diethyl ether extracts and commonly used antibiotic (ampicillin). Another aim was to determine the inhibitory concentration (IC₅₀) values. Interactions between extracts of this plant and antibiotic have not been investigated yet.

MATERIALS AND METHODS

Chemicals

Ampicillin was obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Organic solvents (ethanol, diethyl ether and acetone) were purchased from Zorka Pharma (Šabac, Serbia). Dimethyl sulfoxide (DMSO) was purchased from Acros Organics (New Jersey, USA). Resazurin was obtained from Alfa Aesar GmbH & Co. (Karlsruhe, Germany). Nutrient liquid media, a Mueller–Hinton broth was purchased from Torlak (Belgrade, Serbia).

Plant material

Agrimonia eupatoria aerial parts in the flowering stage were collected on Mt. Bukulja (Serbia) during the summer of 2012. Identification and classification of the plant material was performed at the Faculty of Science, University of Kragujevac. The voucher samples were deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. The collected plant materials were air-dried in darkness at ambient temperature.

Preparation of plant extracts

The dried, ground plant material was separately extracted by maceration with diethyl ether, ethanol, acetone and water. Briefly, 30g of the plant material was soaked with 150 ml of the solvent. The plant material was macerated three times at room temperature using fresh solvent every 24 hours. After every 24 hours, the samples were filtered through filter paper (Whatman No.1) and the filtrates were collected and evaporated to dryness using a rotary evaporator (IKA, Germany) at 40°C. The extracts were kept in sterile sample tubes and stored at -20°C.

Determination of antimicrobial activity

Test microorganisms

The following G⁻ species of human-pathogenic bacteria were tested: *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. All microorganisms were clinical isolates from the Institute of Public Health, Kragujevac and stored in microbiological collection at the Microbiology Laboratory (Faculty of Science, University of Kragujevac).

Suspension preparation

Bacterial suspensions were prepared by the direct colony method (ANDREWS, 2005). The turbidity of initial suspension was adjusted using 0.5 McFarland densitometer (Bio San, Latvia). Initial bacterial suspensions contain about 10⁸ colony forming units (CFU)/ml. 1:100 dilutions of initial suspension were additionally prepared into sterile 0.85% saline.

Estimation of synergy between plant extracts and antibiotic

Synergy between the water extract/ampicillin, diethyl ether extract/ampicillin, acetone extract/ampicillin and ethanol extract/ampicillin was studied by the checkerboard assay method (SATISH *eds.*, 2005).

A series of twofold dilutions of ampicillin were constructed. From the first to twelfth well in one column the 50 µl extracts solution was diluted 2-fold in Mueller-Hinton broth in order to obtain the final concentration, starting from MIC, which were previously determined for every tested extract. Twofold dilutions of the antibiotic (50 µl) were then added, from the first to the twelfth well in column, starting from MIC, which was also determined. Briefly, in the first well of the one column was the strongest combination of the extract and antibiotics (MIC combinations), and at the last well of the column were the lowest concentrations of extract and antibiotics. The plate was inoculated with 10 µl of the prepared bacterial suspension 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 growth control, solvent control and sterility control.

Each test included growth control consisting of the medium with the solvent (10% DMSO) and medium with bacterial suspension as well as sterility control. In our experiments, it was observed that 10% DMSO did not inhibit the growth of microorganisms. All tests were performed in duplicates.

In vitro interactions between antimicrobial agents were determined and quantified by calculating the fractional inhibitory concentration (FIC) index using the following formula:

$$\text{FIC index} = (\text{MIC of drug A in combination} / \text{MIC of drug A alone}) + (\text{MIC of drug B in combination} / \text{MIC of drug B alone}).$$

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 individual agents
- additive if their joint effect is equal to the sum of effects of 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.

In order to find IC₅₀ value (concentration of extract + concentration of antibiotic) for each bacteria, it was used ELISA plate reader (RT-2100C, Rayto, Shenzhen, China) at wavelength of 600 nm. All obtained values for absorbance at 600 nm for analyzed samples were reduced for absorption of sterile medium with extracts to avoid absorption of extracts at 600 nm. Inhibitory concentration (IC₅₀) was defined as the combination of the lowest concentration of extract and concentration of antibiotic that showed 50% inhibition on the growth of tested bacteria. IC₅₀ was calculated graphic, using Microsoft Excel (Redmond, Washington, DC, USA).

RESULTS

Antibacterial activity and combining effects of extracts and antibiotic

The results of antibacterial activity of water, diethyl ether, acetone and ethanol extracts from *A. eupatoria* as well as activity of ampicillin (antibiotic) against 4 species of bacteria are presented in Table 1. (MURUZOVIĆ *et al.*, 2016).

Table 1. Antibacterial activity of the extracts of *Agrimonia eupatoria* L. (MURUZOVIĆ *et al.*, 2016)

Species	Water extract	Diethyl ether extract	Acetone extract	Ethanol extract	Ampicillin
	MIC ¹				
<i>E. coli</i>	> 20	> 20	10	20	2.1
<i>P. mirabilis</i>	5	> 20	2.5	2.5	> 128
<i>K. pneumoniae</i>	> 20	> 20	10	20	> 128
<i>P. aeruginosa</i>	10	20	0.625	1.25	> 128

¹ Minimum inhibitory concentration (MIC) values given as mg/ml for plant extract and µg/ml for antibiotic

We chose these G⁻ strains of bacteria because they showed resistance or low effect on one or both of the tested agents, so we wanted to determine whether the synergistic effect is better. *E. coli* showed resistance to water and diethyl ether extracts, *P. mirabilis* on diethyl ether extract and ampicillin, while *K. pneumoniae* showed resistance to water and diethyl ether extracts and ampicillin. *P. aeruginosa* showed resistance on ampicillin (MURUZOVIĆ *et al.*, 2016).

In this work, possible joint activity of *A. eupatoria* extracts and ampicillin (antibiotic) was evaluated. The results of the checkerboard combination assays are presented in Table 2.

Table 2. Interaction between extracts of *A. eupatoria* and ampicillin.

Species	Water extract + Ampicillin	FIC index	Diethyl ether extract + Ampicillin	FIC index	Acetone extract + Ampicillin	FIC index	Ethanol extract + Ampicillin	FIC index
	MIC*		MIC		MIC		MIC	
<i>E. coli</i>	0.0097+0.06	0.29 (S)	0.0097+0.12	0.06 (S)	0.0049+0.06	0.29 (S)	0.0049+0.06	0.03 (S)
<i>P. mirabilis</i>	2.5+64	1 (A)	10+64	1 (A)	1.25+64	1 (A)	0.625+64	0.5 (A)
<i>K. pneumoniae</i>	>10+>128	n.d.	>10+>128	n.d.	>10+>128	n.d.	>10+>128	n.d.
<i>P. aeruginosa</i>	10+128	2 (I)	10+64	1 (A)	0.3125+64	1 (A)	0.625+64	1 (A)

*MIC values of the most effective combinations given as mg/ml for plant extract and µg/ml for antibiotic; n.d.- not determined

FIC indices were calculated and the concentrations at which the highest level of activity was exhibited are listed in Table 2. For every combination of agents, synergism was recorded in relation only for *E. coli*.

The strength of synergistic effects between the water extract/ampicillin, diethyl ether extract/ampicillin, acetone extract/ampicillin and ethanol extract/ampicillin are indicated by the FICI values, which ranged from 0.03 to 0.29 for *E. coli*. Inhibition of bacterial growth was recorded at lower concentrations compared to the individually tested values for *E. coli* and *P. mirabilis*.

Combinations of the water extract and ampicillin gave the following kinds of effects against 4 human pathogenic bacteria (Table 2): synergistic against *E. coli*, additive against *P. mirabilis* and indifference against *P. aeruginosa*, while interactions between ethanol extract and ampicillin; diethyl ether extract and ampicillin and acetone extract and ampicillin were: synergistic against *E. coli*, additive against *P. mirabilis* and *P. aeruginosa*. *K. pneumoniae*, resistant to ampicillin, showed no effect on tested MIC combinations of agents. No antagonistic effect against tested bacteria was observed for any tested combination.

In order to find IC₅₀ value for each tested bacteria, it was used ELISA plate reader. The results of IC₅₀ value were shown in Table 3.

Table 3. IC₅₀ values of the most effective combination between *A. eupatoria* extracts and ampicillin.

Species	Water extract + Ampicillin	Diethyl ether extract + Ampicillin	Acetone extract + Ampicillin	Ethanol extract + Ampicillin
	IC ₅₀ *			
<i>E. coli</i>	n.d.	n.d.	n.d.	n.d.
<i>P. mirabilis</i>	0.06+5.08	15.46+96	0.04+0.07	0.35+0.33
<i>K. pneumoniae</i>	1.8+11	0.4+3.40	0.4+4.80	n.d.
<i>P. aeruginosa</i>	4.04+79.05	5.8+32.55	0.13+59.10	0.93+61

*IC₅₀ values of the most effective combinations given as mg/ml for plant extract and µg/ml for antibiotic measured at 600 nm; n.d.- not determined

Since the results for *E. coli* showed synergism on very low concentration in all tested combinations, IC₅₀ was not determinate. The best IC₅₀ value for *P. mirabilis* gave the combination of acetone extract (0.04 mg/ml) + ampicillin (0.07 µg/ml), for *K. pneumoniae* combination of diethyl ether extract (0.4 mg/ml) + ampicillin (3.4 µg/ml) and for *P. aeruginosa* the best was combination of diethyl ether extract (5.8 mg/ml) + ampicillin (32.55 µg/ml) and combination of acetone extract (0.13 mg/ml) + ampicillin (59.10 µg/ml). To the best of the authors' knowledge, the synergism between *A. eupatoria* extracts and ampicillin has not been investigated before.

DISCUSSION

Antibiotic resistance of bacteria is a fast-emerging global crisis. Understanding of the resistance mechanisms is paramount for design and development of new therapeutic strategies (KUMAR and SCHWEIZER, 2005). The resistance of the G⁻ bacteria could be attributed to its cell wall structure, because G⁻ bacteria have an effective permeability barrier, comprised of a thin lipopolysaccharide exterior membrane, which could restrict the penetration of the active compounds from plant extracts (ELOFF, 1988).

SHIOTA *et al.* (2004) showed that one of the effective approaches to overcome bacterial resistance is restoration of antibiotic activity through the synergistic action of antibacterial materials from natural and synthesized agents. In our research, synergism was recorded in relation only for *E. coli*, for every combination of tested agents. No antagonistic effect against tested bacteria was observed for any tested combination.

Synergistic interactions are a result of a combined effect of active compounds from plant extracts and antibiotic. It seems that both active compounds, directly or indirectly attach the same site on bacterial cell. Some authors suggest that phytochemicals 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 (SHIOTA *et al.*, 2004; SIBANDA and OKOH, 2007).

Understanding of mechanisms of synergy is fundamental to development of pharmacological agents against bacterial infection. This way of synergistic interaction, against resistant microorganisms may lead to new ways of treating infectious diseases and probably this represents a potential area for further future investigations. Combination therapy may be helpful and useful for patients with serious infections caused by drug resistant pathogens.

CONCLUSION

The results of this work indicate the potential antibacterial efficacy of acetone, water, ethanol and diethyl ether extract of *A. eupatoria* in combination with ampicillin against some G⁻ bacteria which showed low sensibility or to the tested antibiotic or to the tested extracts. The detection of synergy between the extracts and ampicillin demonstrates the potential of this plant as a source of compounds which modify the antibiotic resistance.

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