Chapter 2

# Synergistic Activity of Antibiotics and Bioactive Plant Extracts: A Study Against Gram-Positive and Gram-Negative Bacteria

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**Abstract**

The alarming growth of the number of antibiotic resistant bacteria and difficulties in treatment of infections have initiated a search for new antibacterial compounds and develop new alternative strategies in combating bacterial infections. Plant-derived compounds could exhibit a direct antibacterial activity and/or an indirect activity as antibiotic resistance modifying compounds, which, combined with antibiotics, increase their effectiveness. This ability of plant active substances reflects in modification or blocking of resistance mechanism so that bacterium becomes sensitive to antibiotic or the antibiotic acts when in lower concentrations. The systematic screening of plant-derived bioactive compounds, including those which can synergistically act with antibiotics, as resistance modifying agents represents a potential approach to overcome bacterial resistance. Therefore, the goals of this chapter are (i) an update of literature review on synergism between plant extracts and antibiotics, (ii) presentation of experimental results of synergistic activity of selected plant extracts and antibiotics and (iii) concluding remarks.

**Keywords:** antibacterial activity, synergism, antibiotic, plant extract, mode of action

## 1. Introduction

From the beginning of the antibiotic era, it was noticed that bacteria had the potential to develop resistance to antibiotics. Those early treatment failures with antibiotics did not represent a significant clinical problem because other classes of agents, with different cellular targets, were available [1]. But, in time, the number of antibiotic resistant bacteria has increased and antibiotic resistance has become a global public health threat [2].

The remarkable ability of bacteria to adapt to adverse environmental conditions makes them capable of surviving at clinically relevant concentrations of existing antibiotics resulting in the selection of resistant strains. The misuse and overuse of antibiotics are accelerating this process. An antibiotic, as a selective agent, induces genetic changes of bacteria, contributing to development, selection and spreading of resistant strains [3]. This process of acquired resistance is supported by rapid mutation and horizontal transfer of resistance genes. Resistance genes (via plasmids, transposons) may be transferred between individuals of the same or related bacterial species, between members of commensal or pathogenic microbiota and between different environmental habitats, thus spreading the resistance. Even more, there is evidence that some clinically relevant resistance genes have environmental origin [4]. The final score is the list of multi-drug, health-threatening resistant bacteria: methicillin-resistant *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci, vancomycin-resistant *Enterococcus* species (VRE), extended-spectrum β-lactamases producing Enterobacteriaceae, multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, at present.

The alarming growth of the number of antibiotic resistant bacteria and difficulties in treatment of infections have initiated a search for new antibacterial compounds and develop new alternative strategies in combating bacterial infections. Medicinal plants, with their long history of use in folk medicine for the treatment of infectious diseases, have become a promising new source of antibacterial agents. Plant-derived compounds could exhibit a direct antibacterial activity and/or an indirect activity as antibiotic resistance modifying compounds, which, combined with antibiotics, increase their effectiveness [5]. The systematic screening of plant-derived bioactive compounds, including those which can synergistically act with antibiotics, as resistance modifying agents represents a potential approach to overcome bacterial resistance. Therefore, the goals of this chapter are: (i) an update of literature review on synergism between plant extracts and antibiotics, (ii) presentation of experimental results of synergistic activity of selected plant extracts and antibiotics and (iii) concluding remarks.

## 2. Plant-derived antibacterial compounds

Plants produce a whole series of different compounds, which are not of particular significance for primary metabolism, but represent an adaptive ability of a plant to adverse abiotic and biotic environmental conditions. They have a remarkable effect to other plants, microorganisms and animals from their immediate or wider environment. All these organic compounds are defined as biologically active substances and generally represent secondary metabolites, given the fact that they occur as an intermediate or end products of secondary plant metabolism. Apart from determining unique plant characteristics (color, scent, flavor), these compounds also complete the functioning of plant organism, showing both biological and pharmacological activities of a plant [6]. They represent a structurally diverse group of compounds, classified in three major groups: phenolic compounds (simple phenols, phenolic acids, flavonoids, quinones, tannins and coumarins), terpenes and alkaloids. These compounds can be isolated from plant material as a solvent extract, an essential oil or a supercritical extract. Crude extracts represent complex mixtures of compounds (of both secondary

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and primary metabolites), belonging to different biosynthetic and chemical classes that share some general mutual characteristics, such as polarity and/or volatility [7]. Plant extracts have long been known to possess broad antimicrobial activity and were frequently studied and reviewed [7–13]. Their marked antibacterial activity, classification as GRAS (generally recognized as safe) substances and low risk of bacterial resistance development have made them as suitable source for development of novel antibacterial agents.

### 2.1. Mechanisms of action of plant-derived antibacterial compounds

The antibacterial efficiency of plant compounds depends on several factors: (i) characteristics of target microorganism (the type, genus, species, strain), (ii) characteristics of plant material (botanical source, composition of the bioactive compounds as well as time of harvesting, stage of development or method of extraction) and (iii) chemical properties (hydrophilicity, lipophilicity, concentration, pH value). It is widely accepted that plant extracts, because of complex nature, possess multiple mechanisms of action. Plant extracts and their main components may exhibit activity by: (i) inhibiting bacterial growth or viability, (ii) targeting bacterial virulence factors or (iii) potentiating effectiveness of antibiotics as resistance modifying agents.

The inhibition of bacterial growth occurs through several mechanisms: disruption of membrane function and structure (including the efflux system), interruption of DNA/RNA synthesis and function, interference with intermediary metabolism and induction of coagulation of cytoplasmic constituents [7, 8, 14, 15].

Phenolic compounds, initially, affect cell membrane, as high correlation between toxicity and hydrophobicity of different phenolic compounds, changing the permeability and causing the leakage of cellular content or interfere with membrane proteins resulting in structure disrupting [7, 8, 14–16]. Besides the effect on cellular membrane, flavonoids, also, inhibit nucleic acid synthesis (caused by topoisomerase inhibition) and energy metabolism (caused by NADH-cytochrome c reductase or ATP synthase inhibition) as well as interrupt cell wall and cell membrane synthesis [17]. Quinones have a potential to form irreversible complex with nucleophilic amino acids in proteins. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides and membrane-bound enzymes [8]. Tannins are subdivided into two groups: hydrolysable (gallotannins and ellagitannins) and condensed (proanthocyanidins) tannins. Proanthocyanidins possess several mode of action such as destabilization of cell membrane, inhibition of extracellular microbial enzymes, direct actions on microbial metabolism or deprivation of the substrates required for microbial growth [14]. The activity of gallotannins is attributable to their strong affinity for iron, and it is also related to the inactivation of membrane-bound proteins [18]. Coumarins cause a reduction in cell respiration [8]. Terpenes, compounds built up from isoprene subunits, because of lipophilic nature cause cell membrane disruption [19]. Alkaloids, one of the earliest isolated bioactive compounds from plants, possess ability to intercalate with DNA, interrupt activity of enzymes (esterase, DNA-, RNA-polymerase) or cell respiration [19].

Above that plant compounds have impact on growth and viability of bacteria, several research papers have discussed the effects of these compounds in modulating various aspects of bacterial virulence. It was observed that plant extracts inhibit bacterial biofilm formation, motility,

attachment and cell communication [20–23]. Biofilm formation is additional virulence factor that helps in the persistence of pathogens, and bacteria within a biofilm are more resistant to host defense and to antibiotics what make difficulties in eradication of infections. Moreover, plant extracts and their main compounds are able to suppress bacterial toxin production by reducing the expression of major virulence genes. Upadhyay et al. [24] have summarized the published results and showed that selected plant extracts inhibit the production of cholera toxin by *Vibrio cholerae*, reduce the production of *Staphylococcus aureus* α-hemolysin, enterotoxins and toxic shock syndrome toxin 1, reduce the production of verotoxin and inactivate Shiga toxins.

Finally, numerous investigations have shown that plant extracts in combination with antibiotics increase their activity and decrease the doses of antibiotics and their side effects. These positive interactions are considered as a potential strategy to combat bacterial resistance. The following sections will focus on synergistic activities of plant extracts and antibiotics.

### 2.2. Synergistic antibacterial activity of plant extracts and antibiotics

Synergistic interaction between two agents, in which one agent enhances the effect of the other and together they act more efficiently than as individual agents, motivated many scientists to examine and assess the significance of synergistic acting of plant-derived compounds and traditional antibiotics [25, 26]. It is well known that plant extracts possess antibacterial properties but, also, the ability to enhance the activity of an antibiotic in combination with it. That ability of plant active substances reflects in modification or blocking of resistance mechanism so that bacterium becomes sensitive to antibiotic or the antibiotic acts when in lower concentrations. Such an approach, besides reducing the effective dose of antibiotics on one side, also reduces the side effects of antibiotics as medicine on the other.

Numerous *in vitro* researches have confirmed synergistic effects of plant extracts and antibiotics with a significant reduction of minimum inhibitory concentration (MIC) in antibiotics. Scientists have tested various types of extracts of numerous plants in combination with different antibiotics. These were primarily antibiotics from the group of inhibitors of cell wall synthesis and protein synthesis. The tests included both Gram-positive and Gram-negative bacteria.

The ethanol extract of *Punica granatum* rind showed very good synergistic activity with ciprofloxacin resulting in upto 34-fold reduction of MIC and re-sensitization of *Klebsiella pneumoniae* resistant strain [27]. The antibacterial and modulatory potential of the ethanol extracts obtained from leaves and bark of *Azadirachta indica* in combination with aminoglycosides and carbapenems against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* was tested. The association between the ethanol bark extract and amikacin against *P. aeruginosa* PA 24 strain showed synergistic effect. Synergistic effect was also combined with ethanol bark extract and imipenem, amikacin or gentamicin against *E. coli* strains and with imipenem against *S. aureus* strains [28]. Results of combination assay between grape pomace extract and antibiotics showed that the extract combined with representatives of different classes of antibiotics as β-lactam, quinolone, fluoroquinolone, tetracycline and chloramphenicol acted in synergy in all *S. aureus* and *E. coli* strains tested with fractional inhibitory concentration index (FICI) values varying from 0.031 to 0.155. The MIC of antibiotics was reduced 4- to 75-fold.

The most abundant phenolic compounds identified in the extract were quercetin, gallic acid, protocatechuic acid and luteolin. It was also shown that combinations of grape pomace extract with antibiotics are not toxic for the HeLa cell line at concentrations in which the synergistic effect was observed [29]. Different interactions (synergistic, additive and indifference) were observed between *Thymbra spicata* L. extracts and certain antibiotics, including ampicillin, cefotaxime, amikacin and ciprofloxacin. The FICI ranged from 0.02 to 1.5 for *S. aureus* and 0.25 to 2 for *K. pneumoniae* strains. The best synergistic capacity appeared with cefotaxime against *S. aureus* strains, and the activity of cefotaxime was increased from 8- to 128-fold [30]. The hydroalcoholic extracts obtained from the leaves of *Psidium guajava* L. and *Psidium brownianum* Mart ex DC synergistically acted with gentamicin, amikacin and ciprofloxacin against *E. coli*, *P. aeruginosa* and *S. aureus* [31]. The antibiotic potentiating property of *Vangueria madagascariensis* (fruit and leaf extracts) against clinical isolates of *Acinetobacter* spp. was observed. The extracts were found to potentiate the activity of chloramphenicol and ciprofloxacin in a ratio of 50% extract: 30% antibiotic [32]. Thakur et al. [33, 34] analyzed the synergistic antibacterial potential of hydroethanolic extracts of the stem bark of *Berberis aristata* and *Camellia sinensis* with third-line antibiotics against carbapenem-resistant *E. coli*. The analysis of *Berberis aristata*/antibiotics combinations revealed synergistic behavior (FICI < 1) with colistin, tigecycline and amoxicillin/clavulanate potassium, whereas antagonism (FICI > 1) was seen with ertapenem and meropenem [33]. *Camellia sinensis*/antibiotics combinations showed synergism with tigecycline, ertapenem, meropenem, colistin and amoxicillin/clavulanate potassium [34]. Active substances of water extract of tea (*Camellia sinensis*) modified the resistance of methicillin-resistant *S. aureus* (MRSA) as well as the resistance of β-lactamases-producing *S. aureus.* The extract diluted 40- to 100-fold reduced MIC of methicillin from ≥256 to ≤0.12 μg/ml, whereas the extract diluted 40-fold reduced MIC of penicillin up to ≤0.12 μg/ml [35].

Different scientific papers reported results of synergistic antibacterial activity of various plant extracts in the presence of different antibiotics, such as oxacillin, tetracycline, nalidixic acid, ofloxacin, chloramphenicol, gentamicin, erythromycin, penicillin, ampicillin, kanamycin and ciprofloxacin. *Piper betle* L. extracts/antibiotics combination indicated additive and synergistic effects. The greatest synergy was observed against *P. aeruginosa* (FICI 0.09) in the 70% acetone extract—30% chloramphenicol combination. Synergy was also observed against *S. aureus*, *Propionibacterium acnes*, *S. epidermidis* and *Streptococcus pyogenes* [36]. The extracts from *Beilschmedia acuta* leaves and bark and those from the leaves of *Newbouldia laevis* and *Polyscias fulva*, at their concentration of 1/2 MIC and 1/5 MIC, were enhanced activity of tetracycline, chloramphenicol, ampicillin, kanamycin and ciprofloxacin against multi-drug resistant bacteria [37]. In the case of *Juglans regia* extract, 10-fold reduction in MICs was observed against *S. aureus* when it used in combination with oxacillin. In this combination, oxacillin was able to inhibit MRSA strains at concentration of 0.312 μg/ml, MIC in combination was 64-fold lower than the MIC of oxacillin alone and indicated a reversion of methicillin resistance [38]. *Indigofera suffruticosa*, a popular plant used to treat infections, was investigated as modulator of antibiotic effectiveness against *S. aureus*. Acetone extract and erythromycin showed synergistic effects (55.56%; FICI values ranged from 0.3 to 0.5), additive effects (0.6 ≤ FICI ≤0.8) in three and an indifferent effect in only one (ratio of 1:9, drug: extract; FICI = 1.7). For the chloroform extract and erythromycin combinations, both synergistic (0.2 ≤ FICI ≤ 0.4) and additive (0.7 ≤ FICI ≤ 0.9) effects were equally found in four ratios and only one ratio gave a non-interaction (1:9, drug:

extract; FICI = 1.7). No synergistic effect was seen with ether extracts, but eight ratios resulted in additive effects (0.6 ≤ FICI ≤0.9) and one ratio an indifferent (3:7, drug: extract; FICI = 1.2) [39]. Ethanol extract of *Hyptis martiusii*, with concentration of 32 μg/ml, reduced the effective concentrations of antibiotics (amikacin, gentamicin, kanamycin, neomycin and tobramycin) over 100-fold, where the tested concentrations of antibiotics ranged from 8 to 256 μg/ml, and they were reduced upto ≤1 μg/ml [40]. Sub-inhibitory concentrations of water extract of *Catha edulis* (5 mg/ml) enhanced the activity of tetracycline two- to fourfold, against resistant strains of periodontal bacteria (*Streptococcus sanguis* TH-13, *S. oralis* SH-2 and *Fusobacterium nucleatum*) [41]. Nostro et al. [42] showed that combinations of propolis and *Zingiber officinale* with clarithromycin intensified controlling of *Helicobacter pylori*. Sibanda and Okoh [43] detected the synergism of acetone extract of *Garcinia kola* nuts with amoxicillin, ciprofloxacin, tetracycline and chloramphenicol, whereas the ethanol extracts from *Aegopodium podagraria* L. and *Torilis anthriscus* in combination with streptomycin and chloramphenicol exhibit synergistic and additive effects [44, 45]. Synergistic effect was also established between ciprofloxacin and chloroform extract of *Jatropha elliptica* at the level of concentration of 1/8 MIC of antibiotics. It was found that the extract contained active substances inhibiting NorA efflux mechanism [46].

In addition to the synergistic effects observed for the plant extracts, *in vitro* studies reported the capacity of pure compounds to potentiate the activity of antibiotics. The carnosic acid, the main bioactive compound of *Rosmarinus officinalis* extracts, was capable of acting synergistically with gentamicin against *S. aureus* clinical isolates. In addition, the carnosol, γ-lactone derivative of carnosic acid, isolated from a crude extract from *Salvia officinalis* L. reduced the MICs of aminoglycosides in vancomycin-resistant enterococci. Carnosic acid (8 μg/ml) or carnosol (16 μg/ml) reduced the MICs of several aminoglycosides in vancomycin-resistant *Enterococcus faecium* and *E. faecalis* round 8- to 128-fold [47]. Combinations of tetracycline or β-lactam antibiotics with baicalein (5,6,7-trihydroxyflavone) exhibit synergistic effects against MRSA [48]. Moreover, it has been reported that epigallocatechin gallate is synergistically active in combination with β-lactams, tetracycline, oxytetracycline [49, 50]. The geranylated flavanones from *Paulownia tomentosa* fruits showed a promising synergistic potential with antibiotics [51]. Curcumin, a flavonoid isolated from the rhizome of a plant, *Curcuma longa* L., markedly reduced the MICs of the antibiotics oxacillin, ampicillin, ciprofloxacin and norfloxacin used against MRSA. The combined activity of curcumin and antibiotics resulted in a 2- to 128-fold reduction in MIC values [52]. Allicin, antibacterial compound from garlic (*Allium sativum*), potentiated the action of cefazolin (4- to 128-fold) and oxacillin (32- to 64-fold), against *Staphylococcus* sp. and cefoperazone (8- to 16-fold) against *P. aeruginosa* [53].

### 2.3. Mechanisms of synergistic antibacterial activity of plant extracts and antibiotics

When a number of scientific researches have confirmed the synergistic activity of plant extracts and antibiotics certainly, the next step was to investigate the mechanisms of the synergistic action. It is believed that active compounds from plants modify and inhibit the mechanisms of acquired resistance in bacterial cell and thus exhibit a synergistic effect with antibiotics [54, 55]. The mechanism of synergistic action is explained by: (i) modification of active sites on bacterial cell, (ii) inhibition of enzymes, which catalyze degradation or modification of antibiotics, (iii) increase of membrane permeability and (iv) inhibition of efflux pumps (**Figure 1**).

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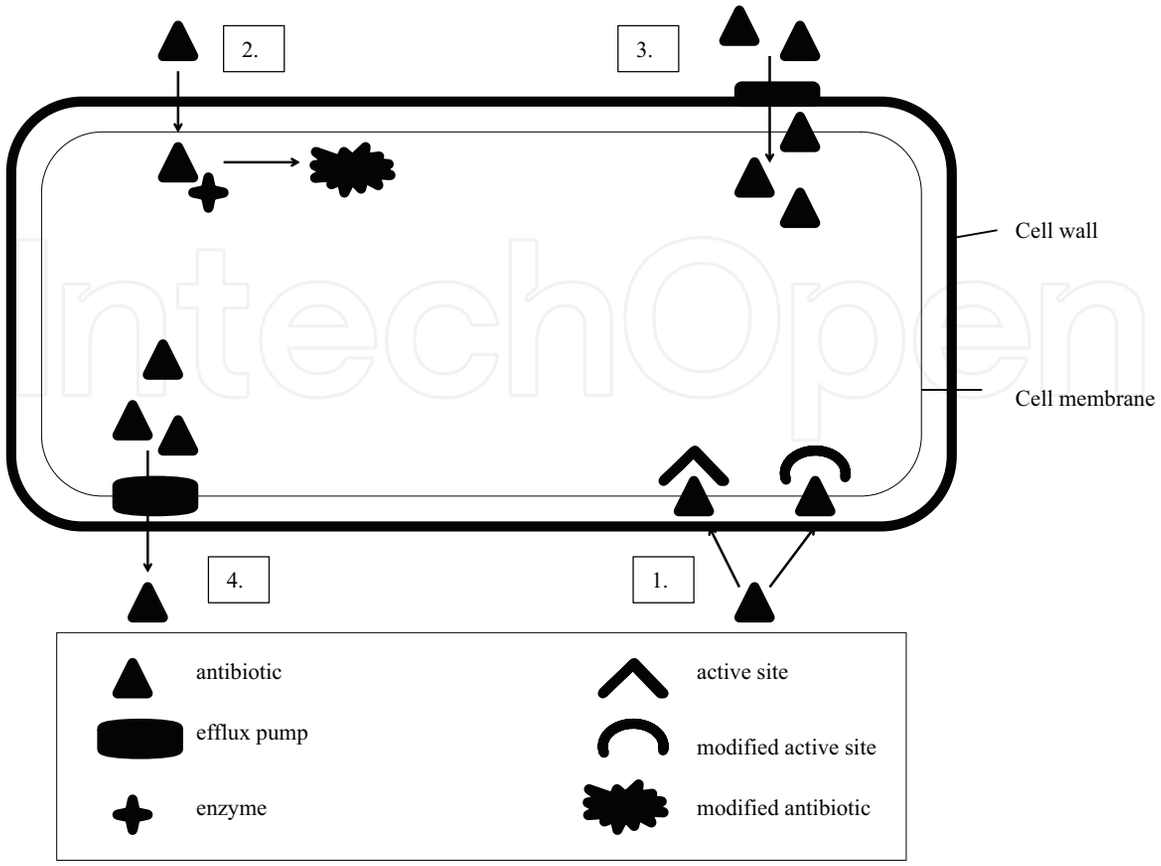


**Figure 1.** Mechanisms of synergistic antibacterial activity: (1) modification of the active site, (2) enzymatic degradation of antibiotic, (3) increase of membrane permeability and (4) inhibition of efflux pump.

### *2.3.1. Modification of active sites on bacterial cell*

Modification of active sites is a common mechanism of resistance and may occur for diverse classes of antibiotics. Resistance to β-lactam antibiotics is especially present in Gram-positive bacteria. β-lactam antibiotics inhibit metabolism of peptidoglycan binding themselves to penicillin-binding proteins (PBPs), which catalyze cross linking of peptidoglycan in mesh structure. Without the mesh structure, cell wall becomes mechanically weak, which undermines integrity of the bacteria cell. Resistance occurs due to reduced affinity of PBPs for antibiotics or due to reduced production of these proteins. All the factors modifying structure, activity or synthesis of PBPs influence the reduction of resistance. Numerous scientific papers confirmed the change in resistance to β-lactam antibiotics through synergistic acting of antibiotics and plant secondary metabolites. Flavonoid baicalin isolated from *Scutellaria amoena* [56], polyphenol corilagin from *Arctostaphylos uva-ursi* [57], tellimagrandin and rugosin B from wild rose (*Rosa canina*) [58] and epigallocatechin gallate from green tea (*Camellia sinensis*) [59] significantly reduce MIC of β-lactam antibiotics, especially in case of MRSA. Methicillin resistance is due to the expression of an additional penicillin-binding protein (PBP2a), regulated by *mecA* gene, which has a low β-lactams-binding affinity, and it enables cell wall synthesis. Methicillin inhibits the transpeptidational activities of other PBPs, but PBP2a remains active ensuring the cross-linking of the glycan chains in peptidoglycan. PBP2a is not able to

completely compensate for the other PBPs because cells grown in the presence of methicillin exhibit a marked reduction in the degree of cross-linking. However, the limited degree of cross-linking is enough to ensure survival of the cell [60].

### *2.3.2. Enzymatic degradation or modification of antibiotics*

Bacterial cell consists of various enzymatic systems, which inactivate antibiotics. It occurs through the processes of hydrolysis, replacement of active groups (acetylation, phosphorylation glycosylation and adenylation) and processes of oxidation-reduction [61]. One of the well-examined groups of enzymes is the group of β-lactamases. β-lactamases (penicillins and cephalosporins) are the enzymes from the group of lyases, which destroy the amino link of β-lactam ring turning it into inactive form. On grounds of scientific studies, it has been showed that active plant compounds may inhibit these enzymes preventing degradation of antibiotics. For example, it is well known that epigallocatechin gallate from green tea in synergistic interactions with antibiotics increases the effect of antibiotics by inhibiting β-lactamases [62, 63]. *Stephania suberosa* extracts possess multiple mode of action, inhibit β-lactamases activity and increase cell membrane permeability against ampicillin-resistant *S. aureus* [64].

### *2.3.3. Increase of membrane permeability*

Cell wall is a first barrier, which antibiotics and other compounds must overcome to achieve their targets and demonstrate their inhibitory activity. In Gram-positive bacteria, the cell wall is composed of several layers of peptidoglycan, which are mostly permeable to different compounds, while in Gram-negative bacteria, the cell wall is complex. It is constructed of a single layer of peptidoglycan and a layer of lipoproteins and lipopolysaccharides known as outer membrane. The outer membrane is significant barrier for many compounds including antibiotics because of several reasons: (i) polysaccharides restrict or completely prevent penetration of antibiotics with high molecular weight, (ii) a lipid layer limits penetration of hydrophilic molecules and (iii) porins enable transport of hydrophilic molecules. Entrance to the periplasmic space might occur via diffusion through porins or through the lipid bilayer by solubilization. After crossing the outer membrane, compounds can be taken out from the periplasmic space by the efflux pumps or inactivated by enzymes, before effectively reaching cell membrane. The cell membrane is another barrier, which restricts influx of compounds in cytoplasm. Phenolic compounds and terpenes change function and structure of membrane. They affect membrane permeability mainly due to perturbation of the lipid bilayer causing decrease in lipid density in the bilayer. Reduced density of the lipids results in a permeable membrane [65]. Probably, the increased membrane permeability resulted in increased level of the antibiotics inside bacterial cells and their better interaction with intracellular targets. Hemaiswarya et al. [66] noticed synergistic interactions of eugenol from plant *Eugenia aromatic* with 10 different hydrophobic and hydrophilic antibiotics in case of five Gram-negative bacteria. Synergism occurred due to ability of eugenol to increase the permeability of cell membrane, and the concentration of 1 mM caused up to 50% of damage of cell membrane.

#### *2.3.4. Inhibition of efflux pumps*

One of the mechanisms of resistance is also utilized for developing efflux pumps by bacteria to expel antibiotics from cells. Efflux pumps work through ATP hydrolysis or on grounds of difference in concentration of ions. Numerous plant-derived compounds with significant activity as inhibitors of efflux pumps were discovered. Primarily, these compounds are active against Gram-positive bacteria [67]. For examples, carnosic acid and carnosol, isolated from chloroform extract of *Rosmarinus officinale*, acted as inhibitors of efflux pumps. A 10 μg/ml of carnosic acid and carnosol increased by the activity of tetracycline two- to fourfold in case of a *S. aureus* strain that had Tet (K) pump. Active compounds inactivated efflux pump and prevented expelling of tetracycline from the cell. Carnosic acid also increased the activity of erythromycin eightfold in case of a *S. aureus* strain that had Msr (A) pump by inhibiting its activity [68]. The same authors tested the active substance from herb *Lycopus europaeus* in combination with tetracycline and erythromycin and detected doubled intensity of antibiotics activity in case of *S. aureus* strains that had Tet (K) and Msr (A) pumps [69]. Baicalein isolated from the leaves of *Thymus vulgaris* demonstrated synergy with ciprofloxacin against MRSA strains and with gentamicin against vancomycin-resistant enterococci, apparently by the inhibition of the NorA efflux pump [70]. Shahverdi et al. [71] discovered that cinnamaldehyde, from *Cinnamomum zeylanicum* bark essential oil, reduced clindamycin resistance in *Clostridium difficile* inhibiting CdeA efflux pump system, the first multidrug efflux transporter, which is identified in *C. difficile*. Recently, *Punica granatum* extract inhibits efflux pump of multidrug resistant *K. pneumoniae* [27].

## 3. *In vitro* testing of antibacterial synergistic activity of selected plant extracts and antibiotics

In this study, different combinations of selected plant extracts and commonly used antibiotics were tested, emphasizing the potential role of phytocompounds in increasing the effectiveness of antibiotics. The experiment involved ethanol, ethyl acetate and acetone extracts from five plant species: *Cychorium intybus* L. (Asteraceae), *Salvia officinalis* L., *Clinopodium vulgare* L. (Lamiaceae), *Cytisus nigricans* L. and *Dorycnium pentaphyllum* Vill. (Fabaceae). The plant species were selected on the basis of several factors: (i) use in traditional medicine, (ii) phytochemical composition, (iii) known *in vitro* antibacterial activity and (iv) insufficient data on synergistic activity.

### 3.1. Materials and methods

#### *3.1.1. Plant material*

The aerial parts of *C. vulgare, D. pentaphyllum* and *C. nigricans* were collected from the different regions of Serbia, while *S. officinalis* (leaves) and *M. officinalis* (leaves) were supplied from the commercial source. Identification and classification of the plant material were performed at the Faculty of Science, University of Kragujevac. The voucher specimens are deposited at

the Herbarium of the Faculty of Science, University of Kragujevac. The collected plant materials were air-dried under shade at room temperature and then ground into small pieces, which were stored into paper bags at room temperature.

### *3.1.2. Preparation of samples for testing*

Dried, ground plant material was extracted by static maceration with ethanol, ethyl acetate and acetone for 3 days at room temperature. Every 24 h, 30 g of plant material was soaked with 150 ml of solvent (3 × 150 ml). After filtration, the extracts were concentrated using a rotary evaporator at 40°C to obtained dry extracts without trace of solvent (duration of solvent evaporation was 20 min for acetone extract, 30 min for ethyl acetate extract and 45 min for ethanol extract). The crude plant extracts were stored at −20°C. Before the testing, the crude extracts were dissolved in dimethyl sulfoxide (DMSO) and then diluted into nutrient liquid medium to achieve a concentration of 10% DMSO. The concentrations used in the experiments were based on the dry weight of the extracts.

Four antibiotics, amoxicillin, cephalexin, gentamicin and chloramphenicol, were used. Stock solutions of antibiotics were prepared in Mueller-Hinton broth. Each extract was combined with two antibiotics of different modes of action (cephalexin/gentamicin, amoxicillin/chloramphenicol).

### *3.1.3. Microorganisms*

The following bacteria were used: *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and clinical isolate of *S. aureus* (PMFKg-B30), *Bacillus subtilis* (PMFKg-B2), *K. pneumoniae* (PMFKg-B26), *E. coli* (PMFKg-B32), *P. aeruginosa* (PMFKg-B28) and *P. mirabilis* (PMFKg-B29). All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. Bacteria were stored in microbiological collection at −70°C (Laboratory of Microbiology, Faculty of Science, University of Kragujevac).

Bacterial suspension was prepared from overnight cultures by the direct colony method. Colonies were taken directly from the plate and suspended into 5 ml of sterile 0.85% saline. The turbidity of initial suspension was adjusted comparing with 0.5 Mc Farland standard. When adjusted to the turbidity of a 0.5 Mc Farland standard, a suspension of bacteria contains about $10^8$ colony forming units (CFUs)/ml. Ten-fold dilutions of initial suspension were additionally prepared into sterile 0.85% saline to achieve $10^6$ CFU/ml.

### *3.1.4. Combination assay*

Prior to performing the synergy test, the minimum inhibitory concentrations (MICs) of plant extracts and antibiotics were determined using microdilution plate method with resazurin in Mueller-Hinton broth [72]. Briefly, 96-well microtiter plates were prepared by dispensing 100 μl of Mueller-Hinton broth into each well. A 100 μl from the stock solution of tested compound was added into the first row of the plates. Then, twofold serial dilutions were performed by transferring 100 μl of solution from one row to another, using a multichannel pipette. The obtained concentration range was from 0.156 to 20 mg/ml for plant extracts and from 0.12 to 1000 μg/ml for antibiotics. Ten microlitres of each $10^6$ CFU/ml bacterial

suspension was added to appropriate wells. Finally, 10 μl of resazurin solution was added. Resazurin is an oxidation-reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated microtiter plates were incubated at 37°C for 24 h. MIC was defined as the lowest concentration of the tested compounds that prevented resazurin color change from blue to pink.

The combined activity of plant extracts and antibiotics was evaluated by checkerboard method [73]. The testing was performed in 96-well microtiter plates using a 6-by-6 well configuration. Twofold dilutions of each antibacterial compounds were prepared. First, 100 μl of Mueller-Hinton broth was added into 36 wells of a 96-well microtiter plate. Then, 50 μl of each dilutions of extract was added horizontally into six rows, and 50 μl of each dilutions of antibiotic was added vertically into six columns. The final volume was 200 μl. The final concentration range corresponded to 1/32 MIC – MIC. Each well contained unique combination of plant extract and antibiotic concentration. Ten microlitres of each $10^6$ CFU/ml bacterial suspension and 10 μl of resazurin solution were added. The microtiter plates were incubated for 24 h at 37°C. The combination of the compounds in which resazurin color change did not appear (growth inhibition) is taken as effective MIC for the combination. Each test included growth control and sterility control.

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

$$\text{FICI} = \frac{\text{MICa in combination}}{\text{MICa}} + \frac{\text{MICb in combination}}{\text{MICb}} \quad (1)$$

where MICa is MIC of plant extract and MICb is MIC of antibiotics.

Interpretation of the FICI was as follows: FICI ≤ 0.5 synergy; FICI > 0.5–1 additivity; FICI > 1–4 indifference and FICI > 4 antagonism. The action of antibacterial agents was considered to be:

- Synergistic, if their joint effect was stronger than the sum of effects of the individual agents
- Additive, if their joint effect was equal to the sum of effects of the individual agents
- Indifferent, if their joint effect was equal to the effect of either individual agent
- Antagonistic, if their joint effect was weaker than the sum of effects of the individual agents or weaker than the effect of either individual agent [73].

The mean FICI of all combination was used to categorize results as synergy, additivity, indifference and antagonism.

*3.1.5. Statistical analysis*

Statistical evaluation of the data was performed by Student's *t*-test using the SPSS statistical software package, version 20 for Windows. The results were considered to be statistically significant at $p < 0.05$. The results of antibacterial activity of plant extracts were statistically analyzed.

## 3.2. Results and discussion

### *3.2.1. Antibacterial activity*

The antibacterial activity of tested plant extracts was previously evaluated, and results were reported in [68]. Intensity of antibacterial activity depended on the species of bacteria, plant species and the type of extract. The MIC values were in range from 0.019 to >20 mg/ml (**Table 1**). In general, the significant antibacterial activity was obtained with acetone extract from *S. officinalis* and ethyl acetate and acetone extract from *C. intybus.* Other tested extracts exhibited moderate activity. Statistically significant difference in activity between extracts of *C. intybus*, *S. officinalis* and *C. vulgare* was noticed (**Table 1**). Antibacterial activity of ethyl acetate ($p = 0.001$) and acetone extract ($p = 0.002$) of *C. intybus* was statistically higher than the activity of ethanol extract. Acetone extract was the most active ($p = 0.018$). Moreover, the activity of acetone extract of *S. officinalis* was higher than activity of ethanol ($p = 0.004$) and ethyl acetate extract ($p = 0.001$). Ethyl acetate ($p = 0.015$) and acetone extract ($p = 0.018$) of *C. vulgare* acted better than ethanol extract. Between ethyl acetate and acetone extract, no statistically significant difference in activity was noted ($p = 0.756$). There is no statistically significant difference in action between extracts of *C. nigicans* and *D. pentaphyllum* ($p < 0.05$) (**Table 1**).

The tested bacterial strains showed different level of sensitivity to the antibiotics (**Table 2**). The resistance profile of bacteria was determined according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [74]. In relation to chloramphenicol and amoxicillin, all clinical isolates were resistant. Gentamicin and cephalexin were active against clinical isolates of *B. subtilis*, *S. aureus* and *E. coli.* Isolates of *K. pneumoniae, P. aeruginosa* and *P. mirabilis* were resistant to all tested antibiotics.

### *3.2.2. Synergistic activity of plant extracts and antibiotics*

*In vitro* testing of combined activity of ethanol, ethyl acetate and acetone extracts of five plant species (*C. intybus*, *S. officinalis, C. vulgare, C. nigricans* and *D. pentaphyllum*) and four antibiotics (cephalexin, amoxicillin, gentamicin and chloramphenicol) showed three types of interaction: synergism, additivity and indifference in relation to tested strains of bacteria. In general, according to obtained results, the following remarks could be made:

- *S. officinalis*, *C. vulgare* and *C. nigricans* acted synergistically with tested antibiotics, while *C. intybus* and *D. pentaphyllum* showed additive effect.
- The activity of tested antibiotics was increased up to 32-fold depending on the kind of extract and type of bacteria.
- The concentrations of tested extracts, corresponding to values ranging from 1/4 MIC to 1/32 MIC, increased the activity of antibiotics.
- The synergism was observed almost in case of all tested bacteria, with exception of *E. coli* for which there was no synergism observed in any of the combinations considered. For other bacteria, this ratio was shown in the following decreasing order: *P. mirabilis* > *K. pneumoniae* > *B. subtilis* > *P. aeruginosa* ATCC 2785 > *S. aureus* > *S. aureus* ATCC 25923 >

| Plant species | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| | | MIC (mg/ml) | | | | | | | | |
| *Cichorium intybus* | E[a] | 5 | 10 | 10 | 10 | 20 | 20 | 20 | 5 | 2.5 |
| | Et[b] | 2.18 | 2.18 | 2.18 | 8.75 | 8.75 | 2.18 | 2.18 | 2.18 | 1.09 |
| | Ac[c] | 2.5 | 2.5 | 2.5 | 5 | 5 | 2.5 | 2.5 | 2.5 | 2.5 |
| *Salvia officinalis* | E[a] | 5 | 5 | 5 | >20 | 10 | >20 | >20 | >20 | 2.5 |
| | Et[a] | 10 | 20 | 20 | >20 | >20 | >20 | >20 | >20 | 2.5 |
| | Ac[b] | 0.03 | 0.15 | 0.31 | 20 | 1.25 | 20 | 0.31 | 0.156 | 0.019 |
| *Clinopodium vulgare* | E[a] | 1.25 | >20 | >20 | >20 | 20 | >20 | 20 | 2.5 | 20 |
| | Et[b] | 0.625 | 20 | 10 | 10 | 10 | 10 | 10 | 2.5 | 10 |
| | Ac[b] | 1.25 | 20 | 10 | 10 | 10 | 20 | 10 | 0.625 | 10 |
| *Cytisus nigricans* | E[a] | 2.5 | 20 | 20 | 10 | 10 | 20 | 20 | 5 | 1.25 |
| | Et[a] | 5 | 20 | 20 | 20 | 20 | 20 | 20 | 5 | 5 |
| | Ac[a] | 2.5 | 20 | 20 | 10 | 20 | >20 | 20 | 2.5 | 10 |
| *Dorycnium pentaphyllum* | E[a] | 5 | 10 | 20 | 10 | 5 | 20 | 20 | 2.5 | 10 |
| | Et[a] | 1.25 | 20 | 20 | 10 | 10 | >20 | 20 | 1.25 | 20 |
| | Ac[a] | 1.25 | 20 | 20 | 5 | 5 | 20 | 10 | 1.25 | 10 |

1. *B. subtilis*; 2. *K. pneumoniae*; 3. *S. aureus*; 4. *P. aeruginosa*; 5. *P. mirabilis*; 6. *E. coli*; 7. *E. coli* ATCC 25922; 8. *S. aureus* ATCC 25923; 9. *P. aeruginosa* ATCC 27853; E: ethanol extract; Et: ethyl acetate extract; Ac: acetone extract; Superscript with different letters are significantly different at $p < 0.05$, for every plant species separately.

**Table 1.** Antibacterial activity of tested plant extracts.


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*P. aeruginosa* > *E. coli* ATCC 25922. Extracts of *Salvia officinalis*, together with amoxicillin and chloramphenicol, have synergistically acted to most bacteria.

- The ethanol and ethyl acetate extracts of *S. officinalis* and *C. nigricans* intensified the activity of amoxicillin, gentamicin, cephalexin and chloramphenicol reducing the effective concentration by 32-fold against Gram-positive bacteria *B. subtilis* and *S. aureus* and Gram-negative bacteria *K. pneumoniae* and *P. mirabilis.*
- The extracts of *C. vulgare* in combination with cephalexin and gentamicin showed synergistic effect of less intensity. The most active was the combination of acetone extract and gentamicin against *B. subtilis* in which case the MIC of antibiotics was decreased by 16-fold.
- This was the first observation of synergistic effect of *C. vulgare* and *C. nigricans* and additive effect of *C. intybus* and *D. pentaphyllum* with tested antibiotics.

*3.2.2.1. Interaction between S. officinalis extracts and antibiotics*

The results of combined acting of ethanol, ethyl acetate and acetone extract of *S. officinalis* and antibiotics (chloramphenicol and amoxicillin) expressed in FICI are indicated in **Table 3**. Synergistic, additive and indifferent effects were observed. FICI was ranged in intervals from 0.30 to 1.37.

The extracts showed a better synergistic capacity with amoxicillin than with chloramphenicol (**Table 4**). In reference with initial MIC values, activity of amoxicillin was increased by 4- to 32-fold depending on the species of bacteria. Of nine tested bacteria, amoxicillin acting with ethanol and acetone extract showed synergism against eight bacteria and in case of ethyl acetate extract against two bacteria. Only in case of *E. coli*, there was no synergistic, but only indifferent effect observed. On grounds of FICI values, it may be noticed that intensity of synergistic effect was different and that extracts of *S. officinalis* significantly increased the activity of amoxicillin (FICI 0.31–0.35) (**Table 4**).

| Bacteria | CEF | AMO | GEN | CHL |
|---|---|---|---|---|
| | MIC (μg/ml) | | | |
| *B. subtilis* | 12.5 | 31 | 3.125 | 250 |
| *K. pneumoniae* | 500 | 250 | 6.25 | 250 |
| *S. aureus* | 1.56 | 500 | 0.39 | 500 |
| *E. coli* | 1.56 | >1000 | 1.56 | >1000 |
| *P. aeruginosa* | >1000 | >1000 | >1000 | >1000 |
| *P. mirabilis* | >1000 | >1000 | >1000 | >1000 |
| *E. coli* ATCC 25922 | 6.25 | 6.25 | 0.39 | 250 |
| *S. aureus* ATCC 25923 | 6.25 | 1.9 | 0.19 | 125 |
| *P. aeruginosa* ATCC 27853 | >1000 | 0.12 | 0.098 | 250 |

CEF: cephalexin; AMO: amoxicillin; GEN: gentamicin; CHL: chloramphenicol.

**Table 2.** Antibacterial activity of tested antibiotics.

| Bacteria | Ethanol extract | | Ethyl acetate extract | | Acetone extract | |
|---|---|---|---|---|---|---|
| | CHL | AMO | CHL | AMO | CHL | AMO |
| *B. subtilis* | 0.44 (S) | 0.32 (S) | 0.40 (S) | 0.35 (S) | 1.37 (I) | 0.50 (S) |
| *K. pneumoniae* | 0.44 (S) | 0.32 (S) | 0.35 (S) | 1.37 (I) | 1.37 (I) | 0.35 (S) |
| *S. aureus* | 0.44 (S) | 0.39 (S) | 0.49 (S) | 1.37 (I) | 1.37 (I) | 0.35 (S) |
| *E. coli* | 1.37 (I) | 1.25 (I) | 1.37 (I) | 1.37 (I) | 1.37 (I) | 1.37 (I) |
| *P. aeruginosa* | 1.37 (I) | 0.5 (S) | 1.37 (I) | 1.37 (I) | 1.37 (I) | 1.37 (I) |
| *P. mirabilis* | 1.37 (I) | 0.37 (S) | 1.37 (I) | 0.53 (A) | 1.37 (I) | 0.49 (S) |
| *E. coli* ATCC 25922 | 0.61 (A) | 1.18 (I) | 0.61 (A) | 0.42 (S) | 1.37 (I) | 0.38 (S) |
| *S. aureus* ATCC 25923 | 0.61 (A) | 0.37 (S) | 1.37 (I) | 1.37 (I) | 1.37 (I) | 0.35 (S) |
| *P. aeruginosa* ATCC 27853 | 0.30 (S) | 0.31 (S) | 0.56 (A) | 1.37 (I) | 0.61 (A) | 0.35 (S) |

CHL: chloramphenicol; AMO: amoxicillin.

**Table 3.** Interaction between *S. officinalis* extract and antibiotics expressed as FICI.

Chloramphenicol showed synergism with ethanol and ethyl acetate extract (**Table 4**). In case of combination of chloramphenicol/ethanol extract, synergism was observed against four bacteria, while in case of combination of chloramphenicol/ethyl acetate extract, synergism was observed against three bacteria. These combinations reduced the MICs of chloramphenicol even 32-fold in case of mention strains of bacteria. In case of other bacteria, as

| Bacteria | Amoxicillin | | | Chloramphenicol | |
|---|---|---|---|---|---|
| | Ethanol extract | Ethyl acetate extract | Acetone extract | Ethanol extract | Ethyl acetate extract |
| | MIC* | | | | |
| *B. subtilis* | $1/16_E + 1/8_A$ | $1/16_E + 1/32_A$ | $1/4_E + 1/4_A$ | $1/4_E + 1/8_A$ | $1/8_E + 1/32_A$ |
| *K. pneumoniae* | $1/8_E + 1/16_A$ | / | $1/32_E + 1/16_A$ | $1/4_E + 1/8_A$ | $1/16_E + 1/32_A$ |
| *S. aureus* | $1/4_E + 1/16_A$ | / | $1/32_E + 1/16_A$ | $1/4_E + 1/8_A$ | $1/4_E + 1/32_A$ |
| *P. aeruginosa* | $1/4_E + 1/4_A$ | / | / | $1/32_E + 1/8_A$ | / |
| *P. mirabilis* | $1/32_E + 1/4_A$ | / | $1/32_E + 1/4_A$ | / | / |
| *E. coli* ATCC 25922 | / | $1/8_E + 1/16_A$ | $1/8_E + 1/16_A$ | / | / |
| *S. aureus* ATCC 25923 | $1/8_E + 1/8_A$ | / | $1/32_E + 1/16_A$ | / | / |
| *P. aeruginosa* ATCC 27853 | $1/8_E + 1/16_A$ | / | $1/32_E + 1/16_A$ | / | / |

"/" no synergism; E – extract; A – antibiotic.
*The most active combination.

**Table 4.** Synergism between *S. officinalis* extracts and antibiotics.

well as in combination of chloramphenicol/acetone extract, additive and indifferent effects were observed. Horiuchi et al. [47] observed that acetone extract of *S. officinalis* and isolated components, carnosol and carnosic acid, increased the activity of aminoglycosides against vancomycin-resistant enterococci. These herbal components reduced the MIC of antibiotics by 8- to 128-fold depending on the type of bacteria.

*3.2.2.2. Interaction between C. vulgare extracts and antibiotics*

The results of combined acting of ethanol, ethyl acetate and acetone extract of *C. vulgare* and antibiotics (cephalexin and gentamicin) expressed in FICI are presented in **Table 5**. Synergistic and indifferent effects were observed. FICI was ranged in intervals from 0.39 to 1.67. The ethanol extract exhibited the best synergistic capacity with antibiotics.

For most tested bacteria, interactions of extracts and antibiotics were indifferent. Synergism was observed against four bacteria: *B. subtilis*, *K. pneumoniae*, *P. aeruginosa* and *P. mirabilis*. There was a synergistic acting of gentamicin with all three extracts in relation to *B. subtilis,* while cephalexin acted synergistically with ethanol and ethyl acetate extract (**Table 6**). In these combinations, FICI was 0.39 and 0.44. As for *K. pneumoniae,* the strain that was resistant to cephalexin, the combination of cephalexin with ethanol and acetone extracts showed a synergism. The concentrations of extracts of 1/4 MIC intensified the activity of cephalexin, and MIC was decreased fourfold.

The ethanol extract showed with both tested antibiotics a synergistic activity against *P. aeruginosa* and *P. mirabilis.* The test results were interesting because the strains showed resistance to cephalexin and gentamicin. In synergistic combinations, it was observed that sub-inhibitory concentrations of extracts (1/4 MIC and 1/8 MIC) modified activity of antibiotics by reducing effective concentrations of antibiotics up to 16-fold.

| Bacteria | Ethanol extract | | Ethyl acetate extract | | Acetone extract | |
|---|---|---|---|---|---|---|
| | GEN | CEF | GEN | CEF | GEN | CEF |
| *B. subtilis* | 0.44 (S) | 0.44 (S) | 0.44 (S) | 0.44 (S) | 0.39 (S) | 1.29 (I) |
| *K. pneumoniae* | 1.33 (I) | 0.50 (S) | 1.33 (I) | 1.67 (I) | 1.33 (I) | 0.50 (S) |
| *S. aureus* | 1.04 (I) | 1.23 (I) | 1.24 (I) | 1.23 (I) | 1.19 (I) | 1.07 (I) |
| *E. coli* | 1.56 (I) | 1.67 (I) | 1.67 (I) | 1.67 (I) | 1.33 (I) | 1.67 (I) |
| *P. aeruginosa* | 0.50 (S) | 0.50 (S) | 1.33 (I) | 1.33 (I) | 1.33 (I) | 1.33 (I) |
| *P. mirabilis* | 0.44 (S) | 0.50 (S) | 1.34 (I) | 1.39 (I) | 1.34 (I) | 1.39 (I) |
| *E. coli* ATCC 25922 | 1.56 (I) | 1.45 (I) | 1.56 (I) | 1.33 (I) | 1.35 (I) | 1.33 (I) |
| *S. aureus* ATCC 25923 | 1.24 (I) | 1.34 (I) | 1.12 (I) | 1.19 (I) | 1.24 (I) | 1.19 (I) |
| *P. aeruginosa* ATCC 27853 | 1.19 (I) | 1.24 (I) | 1.14 (I) | 1.24 (I) | 1.19 (I) | 1.24 (I) |

GEN: gentamicin; CEF: cephalexin.

**Table 5.** Interaction between *C. vulgare* extracts and antibiotics expressed as FICI.

| Bacteria | Gentamicin | | | Cephalexin | | |
|---|---|---|---|---|---|---|
| | Ethanol extract | Ethyl acetate extract | Acetone extract | Ethanol extract | Ethyl acetate extract | Acetone extract |
| | MIC* | | | | | |
| *B. subtilis* | $1/8_E + 1/4_A$ | $1/4_E + 1/8_A$ | $1/4_E + 1/16_A$ | $1/8_E + 1/4_A$ | $1/4_E + 1/8_A$ | / |
| *K. pneumoniae* | / | / | / | $1/4_E + 1/4_A$ | / | $1/4_E + 1/4_A$ |
| *P. aeruginosa* | $1/4_E + 1/4_A$ | / | / | $1/4_E + 1/4_A$ | / | / |
| *P. mirabilis* | $1/4_E + 1/8_A$ | / | / | $1/4_E + 1/4_A$ | / | / |

"/" no synergism; E – extract; A – antibiotic.
*The most active combination.

**Table 6.** Synergism between *C. vulgare* extracts and antibiotics.

### *3.2.2.3. Interaction between C. nigricans extracts and antibiotics*

The results of combined acting of ethanol, ethyl acetate and acetone extract of *C. nigricans* and antibiotics (cephalexin and gentamicin) expressed in FICI are presented in **Table 7**. Synergistic and indifferent effects were observed. FICI was ranged in intervals from 0.30 to 1.56. The ethanol extract exhibited the best synergistic capacity with antibiotics.

The ethanol extract synergistically acted with both antibiotics against *B. subtilis*, *K. pneumoniae* and *P. mirabilis* (**Table 8**). The strain of *Proteus mirabilis* was resistant to gentamicin and cephalexin, but in combination with ethanol and ethyl acetate extract, the MIC of antibiotics was reduced by 32-fold.

| Bacteria | Ethanol extract | | Ethyl acetate extract | | Acetone extract | |
|---|---|---|---|---|---|---|
| | GEN | CEF | GEN | GEN | CEF | GEN |
| *B. subtilis* | 0.38 (S) | 0.38 (S) | 1.29 (I) | 1.29 (I) | 1.29 (I) | 1.29 (I) |
| *K. pneumoniae* | 0.40 (S) | 0.37 (S) | 1.47 (I) | 1.33 (I) | 1.56 (I) | 1.33 (I) |
| *S. aureus* | 1.2 (I) | 1.07 (I) | 1.27 (I) | 1.23 (I) | 1.29 (I) | 1.23 (I) |
| *E. coli* | 1.35 (I) | 1.44 (I) | 1.47 (I) | 1.33 (I) | 1.56 (I) | 1.33 (I) |
| *P. aeruginosa* | 1.33 (I) | 1.33 (I) | 1.33 (I) | 1.33 (I) | 1.33 (I) | 1.33 (I) |
| *P. mirabilis* | 0.30 (S) | 0.37 (S) | 0.30 (S) | 1.07 (I) | 0.40 (S) | 0.40 (S) |
| *E. coli* ATCC 25922 | 1.41 (I) | 1.56 (I) | 1.41 (I) | 1.56 (I) | 1.41 (I) | 1.41 (I) |
| *S. aureus* ATCC 25923 | 1.12 (I) | 1.24 (I) | 1.32 (I) | 1.12 (I) | 1.42 (I) | 1.24 (I) |
| *P. aeruginosa* ATCC 27853 | 0.44 (S) | 1.24 (I) | 1.12 (I) | 1.20 (I) | 1.12 (I) | 1.24 (I) |

GEN: gentamicin; CEF: cephalexin.

**Table 7.** Interaction between *C. nigricans* extracts and antibiotics expressed as FICI.

| Bacteria | Gentamicin | | | Cephalexin | |
|---|---|---|---|---|---|
| | Ethanol extract | Ethyl acetate extract | Acetone extract | Ethanol extract | Acetone extract |
| | MIC* | | | | |
| *B. subtilis* | $1/8_E + 1/8_A$ | / | / | $1/8_E + 1/8_A$ | / |
| *K. pneumoniae* | $1/4_E + 1/16_A$ | / | / | $1/32_E + 1/4_A$ | / |
| *P. mirabilis* | $1/8_E + 1/32_A$ | $1/8_E + 1/32_A$ | $1/16_E + 1/4_A$ | $1/4_E + 1/32_A$ | $1/4_E + 1/16_A$ |
| *P. aeruginosa* ATCC 27853 | $1/4_E + 1/8_A$ | / | / | / | / |

"/" no synergism; E – extract; A – antibiotic.
*The most active combination.

**Table 8.** Synergism between *C. nigricans* extracts and antibiotics.

### *3.2.2.4. Interaction between C. intybus extracts and antibiotics*

The results of combined acting of ethanol, ethyl acetate and acetone extract of *C. intybus* and antibiotics (amoxicillin and chloramphenicol) expressed in FICI are presented in **Table 9**. Additive and indifferent effects were observed. FICI was ranged in intervals from 0.56 to 1.37. The results demonstrated that extracts increased activity of amoxicillin better than activity of chloramphenicol. The most active combinations were with ethanol extract in relation to *B. subtilis* and *P. mirabilis*. For these combinations, additive effects were observed, and MICs of antibiotics decreased twofold. On the other side, Ahmad and Aquil [75] noticed synergism between ethanol extract and tetracycline, chloramphenicol and ciprofloxacin.

| Bacteria | Ethanol extract | | Ethyl acetate extract | | Acetone extract | |
|---|---|---|---|---|---|---|
| | CHL | AMO | CHL | AMO | CHL | AMO |
| *B. subtilis* | 0.56 (A) | 0.61 (A) | 1.37 (I) | 1.37 (I) | 0.91 (A) | 0.86 (A) |
| *K. pneumoniae* | 0.68 (A) | 0.68 (A) | 1.37 (I) | 0.84 (A) | 0.91 (A) | 0.68 (A) |
| *S. aureus* | 1.29 (I) | 0.7 (A) | 1.37 (I) | 0.62 (A) | 0.74 (A) | 0.59 (A) |
| *E. coli* | 1.37 (I) | 1.37 (I) | 1.37 (I) | 0.7 (A) | 1.37 (I) | 0.7 (A) |
| *P. aeruginosa* | 1.37 (I) | 1.37 (I) | 1.37 (I) | 1.37 (I) | 1.37 (I) | 1.37 (I) |
| *P. mirabilis* | 1.37 (I) | 0.56 (A) | 1.37 (I) | 0.7 (A) | 1.37 (I) | 0.67 (A) |
| *E. coli* ATCC 25922 | 1.37 (I) | 1.37 (I) | 1.37 (I) | 1.37 (I) | 1.37 (I) | 0.87 (A) |
| *S. aureus* ATCC 25923 | 1.37 (I) | 0.7 (A) | 1.37 (I) | 1.37 (I) | 1.37 (I) | 1.37 (I) |
| *P. aeruginosa* ATCC 27853 | 1.37 (I) | 1.37 (I) | 1.37 (I) | 1.37 (I) | 1.37 (I) | 1.37 (I) |

CHL: chloramphenicol; AMO: amoxicillin.

**Table 9.** Interaction between ***C. intybus*** extracts and antibiotics expressed as FICI.

| Bacteria | Ethanol extract | | Ethyl acetate extract | | Acetone extract | |
|---|---|---|---|---|---|---|
| | GEN | CEF | GEN | CEF | GEN | CEF |
| *B. subtilis* | 1.13 (I) | 1.29 (I) | 1.031 (I) | 1.24 (I) | 1.29 (I) | 0.56 (A) |
| *K. pneumoniae* | 1.30 (I) | 1.33 (I) | 0.75 (A) | 1.37 (I) | 1.24 (I) | 1.30 (I) |
| *S. aureus* | 1.29 (I) | 1.23 (I) | 0.56 (A) | 1.12 (I) | 1.07 (I) | 1.18 (I) |
| *E. coli* | 1.56 (I) | 1.75 (I) | 2.00 (I) | 1.67 (I) | 1.67 (I) | 1.33 (I) |
| *P. aeruginosa* | 1.33 (I) | 1.33 (I) | 1.031 (I) | 1.34 (I) | 1.37 (I) | 1.37 (I) |
| *P. mirabilis* | 1.17 (I) | 1.07 (I) | 0.84 (A) | 1.37 (I) | 0.68 (A) | 0.91 (A) |
| *E. coli* ATCC 25922 | 1.18 (I) | 1.30 (I) | 0.63 (A) | 1.33 (I) | 1.33 (I) | 1.35 (I) |
| *S. aureus* ATCC 25923 | 1.07 (I) | 1.12 (I) | 0.75 (A) | 1.33 (I) | 1.19 (I) | 1.24 (I) |
| *P. aeruginosa* | 1.12 (I) | 1.20 (I) | 1.25 (I) | 1.33 (I) | 1.37 (I) | 1.19 (I) |
| ATCC 27853 | | | | | | |

GEN: gentamicin; CEF: cephalexin.

**Table 10.** Interaction between *D. pentaphyllum* extracts and antibiotics expressed as FICI.

#### *3.2.2.5. Interaction between D. pentaphyllum extracts and antibiotics*

The results of combined acting of ethanol, ethyl acetate and acetone extract of *D. pentaphyllum* and antibiotics (cephalexin and gentamicin) expressed in FICI are presented in **Table 10**. Additive and indifferent effects were observed. FICI was ranged from 0.56 to 2.0. The ethanol extract indifferently acted with antibiotics. Additive effect was noticed in combination with ethyl acetate and acetone extract. In these combinations, MIC values of antibiotics were decreased two times in presence of 1/4 MIC of extracts.

## 4. Concluding remarks

The problem of bacterial resistance is growing, and the outlook for the use of antibacterial drugs in the future is still uncertain. Even though pharmacological industries have produced a number of new antibiotics in the last few decades, resistance to these drugs by bacteria has increased. Plants are valuable sources of new and biologically active molecules possessing antibacterial properties. This activity can be attributable both to direct action against bacteria or as synergistic activity with antibiotics. The *in vitro* synergistic activity of plant active compounds against multidrug-resistant bacteria has been widely shown by the numerous scientific studies. This progress in synergy research enhances the possibility of designing new antibacterial agents of plant origin for the treatment of infections. However, the mechanisms underlying these synergy effects are still poorly explored. Only with exact knowledge of these mechanisms, it will be possible to develop a new generation of standardized, effective preparations. Furthermore, *in vivo* testing of activity, toxicity and bioavailability will determine

their actual relevance for treatment of human infection diseases. Finally, an excellent database of active compounds is formed, and future studies on bioavailability, pharmacodynamics and mechanism of action will contribute in the development of new antibacterial agents.

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