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Vaccinium myrtillus leaves and *Frangula alnus* bark derived extracts as potential antistaphylococcal agents*

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Due to constantly increasing antibiotic resistance of pathogens and participation of the biofilms they make in various types of infections, a development of alternative therapeutic strategies becomes an urgent need. Taking advantage of the biological activity of plant-derived compounds can solve this problem. In this study antimicrobial, including those synergistic with classic antibiotics, and cytotoxic properties of newly-obtained extracts from *Vaccinium myrtillus* leaves (VLE) and *Frangula alnus* bark (FBE) were evaluated. Both tested extracts exhibited relevant antistaphylococcal activity (MIC range 0.75-1.5 mg/mL) accompanied by a relativly low cytotoxic effect on mammalian cells (BI>1). Phytochemical analysis of the extracts tested showed a high total content of phenolic compounds with the predominance of hydroxycinnamic acids in VLE and hydroxybenzoic acids and flavanols in FBE. Widely described in the literature antimicrobial properties of phenolics were probably connected with the biological activity of the extracts tested. We also report that the presence of VLE or FBE at sub-MIC concentrations enhances biocidal potential of vancomycin and linezolid. Therefore, we are considering a possibility of an alternative therapy for local infections caused by *S. aureus* by combining classic antibiotics with plant-derived extracts.

Key words: phytocompounds, antimicrobial activity, synergism with antibiotics, biocompatibility index

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INTRODUCTION

Traditional therapy against bacterial infections has become ineffective since the number of strains resistant to antibiotics and participation of microbial biofilm in the development of infectious lesions is constantly increasing. Research into new treatment options effective against both, planktonic and biofilm forms of microbial life, involves looking for the substances with various types of activity. These could be compounds which not only have direct antimicrobial properties but also exhibit synergistic effect with classic pharmacological agents (enhance or restore their activity), restrict the expression of microbial virulence factors, prevent microbial adhesion and aggregation or activate host immune defense mechanisms designed to combat infections (Budzyńska *et al.*, 2011; Cushnie & Lamb, 2011; Gauwerky *et al.*, 2009; Kurlenda & Grinholc, 2012; Kuźma *et al.*, 2012). Nature may be a good source of such substances. Health-promoting and antimicrobial properties of natural substances have been used in folk medicine for thousands of years, even despite the lack of knowledge of their mechanisms of action. Many plant extracts or other formulations prepared as infusions, decoctions, or poultices are used even until today in modern phytomedicine. They often possess multidirectional biological properties such as antioxidant, anti-inflammatory, anti-allergic, anti-tumor and antimicrobial activities (Alviano & Alviano, 2009; Budzyńska *et al.*, 2011; Fraga *et al.*, 2010; Różalski *et al.*, 2013; Rösch *et al.*, 2003; Tegos *et al.*, 2002). Plant-derived products exhibiting antimicrobial activity usually fall into a class of phytoalexins — secondary metabolites synthesized to prevent insect, fungal and microbial infections and to repair tissue damage. Among them, polyphenols (with the largest and the best known group of flavonoids) and essential oils are the most promising. Important advantages of most of the natural products include their wide-spectrum of activity and effectiveness irrespective of the drug susceptibility of target microorganisms, demonstrating at the same time an extremely low risk of giving rise to resistance mechanisms (Bakkali *et al.*, 2008; Budzyńska *et al.*, 2011; Chaieb *et al.*, 2011; Cushnie & Lamb, 2005; 2011; Fankam *et al.*, 2011; Martos *et al.*, 2013).

The aim of our study was to test antimicrobial activity, including that synergistic with classic antibiotics, of newly-prepared plant extracts from *Vaccinium myrtillus* leaves (VLE) and *Frangula alnus* bark (FBE). In addition, commercially available polyphenol-type phytocompounds such as gallic acid, carvacrol, and oleanolic acid were examined. According to phytochemical analysis, these compounds are (among others) ingredients of the tested extracts. We considered a possibility of using phytocompounds in supporting treatment of local skin infections caused by staphylococci. To our knowledge, both plant-derived extracts have not yet found practical and clinically approved application in this area. Due to the literature data on the negative effects of some components of

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Abbreviations: BI, biocompatiblity index; CFU, colony forming units; DMSO, dimethyl sulfoxide; Et-OH, ethanol; FBE, *Frangula alnus* bark extract; FCS, fetal calf serum; HPLC, high-performance liquid chromatography; MBC, minimum bactericidal concentration; MHA, Müeller-Hinton Agar; MHB, Müeller-Hinton Broth; MIC, minimum inhibitory concentration; VLE, *Vaccinium myrtillus* leaves extract

plant extracts on eukaryotic cell viability and functions, cytotoxicity tests on mouse fibroblasts line L929 were also performed for each phytocompound.

MATERIALS AND METHODS

Plant materials, extraction and isolation of phytocompounds. Bark of Alder Buckthorn (*Frangula alnus*) was in a form of commercial samples supplied by KAWON-HURT Nowak Sp.j. (Gostyń, Poland). Leaves of European blueberry (*Vaccinium myrtillus*) were collected in August 2010 in the forest near Łódź (central Poland). The plant material was air-dried at 60°C for 4 h, ground to fine powder and stored in a refrigerator. The dry leaves (10 g) or bark (20 g) were extracted with acetone-water (70:30, v/v) at a solid to liquid ratio 1:10 (w/v), at room temperature for 30 min and then centrifuged at 2500 × *g* for 15 min. The pellets were re-extracted twice with 70% aqueous acetone for 15 min and the supernatants were combined. After removal of acetone with a vacuum rotary evaporator (Rotavapor RII, Büchi, Switzerland) at <40°C, the extracts were subjected to liquid-liquid partition with chloroform (1:1, v/v) three- and eight-times, for blueberry leaves and Alder Buckthorn bark respectively. The water fractions were concentrated under vacuum at <40°C and the remaining extracts were lyophilized (Alpha 1–2 LD plus, Christ) to yield 1.597 g of blueberry leaves extract (VLE) and 2.920 g of Alder Buckthorn bark extract (FBE). The VLE and FBE extracts were assessed for their biological activities and chemical profile. Dried extracts were reconstituted at 5 mg/mL in water before chemical analysis.

Phytochemical analysis. Quantification of the phenolic profile by HPLC method. HPLC-PDA analysis of different groups of phenolic compounds was performed by using analytical reversed-phase HPLC system (Waters) with an autosampler 2707 and binary HPLC pump 1525 coupled to a 996 photodiode array detector (2998), controlled by the Waters Breeze 2 software (Waters, Milford, MA). Separation was performed on a SYMMETRY C18 column (250 mm × 4.6 mm, 5 μm, Waters). The binary mobile phase according to Dyrby *et al.* (2001) consisted of water and formic acid in the ratio of 90:10 (v/v), respectively (solvent A), water, acetonitrile and formic acid in the ratio of 49:50:10 (v/v/v), respectively (solvent B). Separation of phenolics was performed using the following gradient program with a flow rate of 1 mL/min: 0 min, 88% A + 12 % B; 26 min, 70% A + 30% B; 40–43 min, 0% A + 100% B; 43–50 min, 88% A + 12% B. On the basis of spectral identification and maximum of UV-Vis absorption, the phenolics obtained were qualified into four subclasses: flavanols and hydroxybenzoic acids (detection at 280 nm; expressed as mg of gallic acid equivalents/g of extract), hydroxycinnamic acids (detection at 320 nm; expressed as mg of chlorogenic acid equivalents/g of extract), flavonols (detection at 360 nm; expressed as mg of rutin equivalents/g of extract), and anthocyanins (detection at 520 nm; expressed as mg of cyanidin 3-glucoside equivalents/g of extract).

Determination of total flavanols. Total flavanol content in extracts was determined by the vanillin assay method described by Swain and Hillis (1959). The following reaction mixtures a: 0.1–2 mL sample + 4 mL 1% vanillin in 70% H_2SO_4, b: 0.1–2 mL sample + 4 mL 70% H_2SO_4, c: 2 mL water + 4 mL 1% vanillin in 70% H_2SO_4 and d: 2 mL water + 4 mL 70% H_2SO_4 were prepared and incubated for 10 min in a cold water bath. The mixtures were measured at 500 nm using UV-Vis spectrophotometer (Unicam, England). Absorbance was calculated as: $A = (A_a–A_b–A_c)$. (+)-Catechin was employed as a calibration standard and results were expressed as mg of (+)-catechin equivalents/g of extract.

Determination of total proanthocyanidins. The proanthocyanidins were determined after acid depolymerization to the corresponding anthocyanidins as described by Rösch *et al.* (2003). The extracts were dissolved in a mixture containing concentrated hydrochloric acid and *n*-butanol (1:9, v/v). Then the samples were heated for 90 min in a boiling water bath. After the solution was cooled to room temperature, the absorbance was read at 550 nm. The content of proanthocyanidins (mg of cyanidin equivalents/g of extract) was calculated by the molar extinction coefficient of cyanidin ($\varepsilon = 17360$ L mol^{-1} cm^{-1} and molar mass 287 g mol^{-1}).

Determination of total hydrolyzable tannins. The content of hydrolyzable tannins was estimated with a high-performance liquid chromatography (HPLC) method after acidic hydrolysis of gallotannins into methyl gallate and acidic hydrolysis of ellagitannins into ellagic acid (Hartzfeld *et al.*, 2002). The methyl gallate and ellagic acid were analyzed directly by HPLC as described above, and quantified as mg of methyl gallate or ellagic acid equivalents per 1 g of the extract, respectively.

Commercial phytocompounds. Gallic acid, carvacrol, and oleanolic acid were purchased from Sigma-Aldrich (Germany). Gallic acid and carvacrol were initially dissolved in 96% ethanol (Et-OH; Sigma-Aldrich, Germany), oleanolic acid in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Germany), then diluted to tested concentrations in such a way that the final concentration of Et-OH or DMSO did not to exceed 5% or 2% for bacteria, respectively, and 0.5% for eukaryotic cells, which was considered to be safe (not affecting the viability of microbial or eukaryotic cells).

Evaluation of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of phytocompounds. Reference strain of *Staphylococcus aureus* ATCC 29213 and clinical *S. aureus* H9 strain were used. Bacteria were grown for 24 h at 37°C on Müeller-Hinton Agar — MHA (BTL, Poland) and microbial suspensions (5×10^5 CFU/mL) were prepared in Müeller-Hinton Broth — MHB (BTL, Poland) based on nephelometric measurements of the suspension at OD_{535}, corresponding to a specified density of staphylococcal suspension. MIC values were determined by a microdilution broth assay according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (2009). Stock solutions of plant-derived extracts and commercial phytocompounds were prepared in 100% DMSO or 96% Et-OH. The final highest DMSO or Et-OH concentrations were 1.25% and 4.8%, respectively, which did not affect bacterial growth. The concentration ranges of tested phytocompounds (in a two-fold dilution system) were 0.375–6.0 mg/mL for plant-derived extracts and 0.0078–0.5 mg/mL for commercial phytocompounds, with an extension to the concentration of 0.75 mg/mL for oleanolic acid. Bacterial suspensions (100 μL) were mixed 1:1 with serial dilutions of the phytocompounds and incubated at 37°C for 18 h. The highest dilution showing no turbidity was recorded as MIC. Since the color of the extracts at higher concentrations made the reading of turbidity difficult, bacterial growth on MHA (10 μL from each well after vigorous stirring; linear culture incubated for the subsequent 18 h at 37°C) was tested concurrently. The concentrations of the compounds bactericidal to ≥99.9% of the inoculum (MBC) were determined using the same method. In each case,

the experiments were carried out in duplicate on two different preparations.

Determination of plant-derived products synergy with the antibiotics by microdilution method. Serial two-fold dilutions of antibiotics, starting from 2× MIC (final concentration) for each *S. aureus* strain, were prepared in 96-well culture plates (NUNC, Denmark), 50 μL/well in MHB. Then, 50 μL of tested phytocompounds at final concentrations ½ or ¼ MIC, and 100 μL of bacterial suspensions in MHB (5×10^5 CFU/mL) were added to the wells. For the controls of antibiotic activity, 50 μL of MHB was added to the wells instead of plant-derived products. Plates were incubated for 18 h at 37°C. The optical density was measured at 600 nm using a multi-reader Victor2 (Wallac, Finland). Since the color of some plant-derived solutions interfered with optical measurements, 50 μL aliquots of each sample were spotted onto MHA plates. After 18 h incubation at 37°C, staphylococcal growth intensity was evaluated to confirm MIC values. All experiments were prepared in duplicate. The MIC values of antibiotics alone were compared to those obtained in the presence of phytocompounds to assess the occurrence of a synergistic effect.

Evaluation of phytocompound cytotoxicity towards eukaryotic cells. An immortalized cell line: mouse fibroblasts L929 (recommended for a cytotoxicity test in accordance with EN ISO 10993-5) cultured in RPMI-1640 medium (Sigma, Germany) containing 10% fetal calf serum (FCS; Cytogen, Poland) and 2 mM/mL L-glutamine (Sigma, Germany) were used. The monolayer of a 24-h old culture of L929 in 96-wells microplates (cell inoculum: 100 μL/well from 1×10^6 cell/mL) was exposed to various concentrations of plant products for 30 min. (minimal exhibition in accordance with the guidelines for cytotoxicity testing) and 24 h at 37°C, with 5%CO_2. The concentration ranges of the phytocompounds used in the test (prepared as a two-fold dilution system) were 7.8–2000 μg/mL for the extracts and 3.9–500 μg/mL for commercial phytochemicals. In parallel, the controls of the lowest cytotoxicity (cells treated with the culture medium) and the highest cytotoxicity (cells treated with 2% Triton X-100; Merck, Germany) were prepared. Then, analysis of phytocompounds cytotoxicity was performed on the basis of activity of lactate dehydrogenase (LDH) released from the damaged/dead cells (LDH Cytotoxicity Kit, Clontech, USA; as recommended by the manufacturer). This study was designed to determine the cytotoxicity index — IC_{50}, understood as the concentration of a phytocompound causing a cytotoxic effect in 50% of the population of tested cells. Microbiological screening (MBC evaluation), and IC_{50} were used to calculate the value of BI (biocompatiblity index) based on the formula: BI = IC_{50}/MBC. If BI ≥ 1.0 an extract/commercial phytocompound express the best basic biological activity parameters, understood as high antimicrobial activity accompanied by low cytotoxicity towards eukaryotic cells.

RESULTS AND DISCUSSION

Vaccinium myrtillus (Bilberry) has a long history of medicinal use and its berries have been extensively studied in the recent years. They are known for exceptionally high contents of anthocyanins with powerful antioxidant capacity and many flavonoids. Bilberries possess beneficial health effects, like protective role in cardiovascular diseases and cancer, also exhibit antimicrobial (including antiviral, antibacterial, antifungal), and antiallergenic activity (Hokkanen *et al.*, 2009; Trumbeckaite *et al.*, 2013; Yamaura *et al.*, 2011). Less attention has been paid to the chemical composition of leaves and stems as they are not widely used. However, according to our HPLC analysis (Table 1), it seems that *V. myrtillus* leaves contain as high amounts of phenolic compounds as berries. Most of the detected phenols were hydroxycinnamic acids as was calculated based on the chlorogenic acid content. VLE also contained quite a high amount of hydroxybenzoic acids and flavanols expressed as gallic acid equivalents (Table 1). *Frangula alnus* (*Rhamnus frangula*), called Alder Buckthorn is a shrub commonly present across Poland. It occurs in a variety of forest communities – from dry coniferous forests, through the deciduous and mixed forests to swampy places. *F. alnus* bark (*Cortex frangulae*) is regarded as herbal material, serving as an ingredient in many herbal mixtures for the preparation of decoction. It stimulates laxative action and bowel peristalsis. Therefore, it is used in chronic constipation. *F. alnus* bark contains various biologically active substances, like anthraquinones (e.g. frangulin, also known as rhamnoxantin), flavonoids, tannins and saponins (Kharlamova *et al.*, 2009; Manojlovic *et al.*, 2005). As shown in Table 1, HPLC analysis of FBE indicated high total content of phenols with majority of hydroxybenzoic acids and flavanols, expressed as gallic acid equivalents. In contrast to *V. myrtillus* extract, extract from *F. alnus* contained both, condensed tannins (proanthocyanidins) and hydrolyzable tannins (ellagitannins).

Table 1. Phenolic composition (mg/g) of plant-derived extracts.

	V. myrtillus leaves (VLE)	*F. alnus* bark (FBE)
Total phenolics[1]	399.94±15.31	254.75±34.90
Hydroxybenzoic acids + flavanols[2]	75.56±2.16	242.73±33.18
Hydroxycinnamic acids[3]	295.49±12.10	7.45±1.53
Flavonol derivatives[4]	28.89±1.04	4.57±0.19
Total flavanols[5]	25.92±1.84	56.93±5.32
Total proanthocyanidins[6]	25.45±1.18	0
Hydrolyzable tannins[7]	1.54±0.08	0

Values are means ± S.D., $n \geq 3$. [1]sum of different phenolic groups determined by HPLC method; [2]determined by HPLC method at 280 nm as gallic acid equivalents; [3]determined by HPLC method at 320 nm as chlorogenic acid equivalents; [4]determined by HPLC at 360 nm as quercetin equivalents; [5]determined by vanillin reagent as (+)catechin equivalents; [6]determined after acid depolymerization as cyanidin equivalents; [7]determined by HPLC at 280 nm after acid hydrolysis as ellagic acid equivalents

Both plant-derived extracts tested have not yet found a practical and clinically approved application in the treatment of bacterial infections. Generally, a little research concerns the antimicrobial activity of *Cortex frangulae*. It was shown, that *F. alnus* extract possesses anti-fungal properties with the rate of growth inhibition between 25% and 68% for *Penicillium verrucosum* and *Mucor mucedo*, respectively (Manojlovic *et al.*, 2005). Whereas anthraquinones and synthesized frangula-emodine derivatives used at micromolar concentrations are able to inhibit HIV-1 polymerase and RNase H functions, showing anti-viral properties (Kharlamova *et al.*, 2009). However, the detected high content of hydroxybenzoic acids and flavanols as gallic acid equivalents

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the phytocompounds for *S. aureus* ATCC 29213 reference strain and *S. aureus* H9 clinical strain, determined by a microdilution broth assay accompanied by assessment of bacterial growth on solid media.

	S. aureus ATCC 29213		*S. aureus* H9	
[μg/mL]	MIC	MBC	MIC	MBC
Plant extracts:				
VLE (*V. myrtillus*)	1500	1500	750	750
FBE (*F. alnus*)	750	1500	750	750
Commercial phytochemicals:				
Gallic acid	125	125	62,5	62.5
Carvacrol	250	500	250	500
Oleanolic acid	>750	>750	>750	>750

(mainly in FBE) may suggest a strong antibacterial activity. Gallic acid is a type of phenolic acid, known as 3,4,5-trihydroxybenzoic acid, found in sumac, witch hazel, tea leaves, oak bark, and many other plants. It possesses a relevant antioxidant activity used to protect human cells against oxidative damage, and shows cytotoxicity against cancer cells (Appeldoorn *et al.*, 2005). Gallic acid and its dimeric derivative ellagic acid, are found in a free form or as part of gallo- and ellagitannins. Salts and esters of gallic acid called 'gallates' are best known in the context of antimicrobial activity. Examples of gallic acid derivatives are flavan-3-ols present in green tea: epicatechin gallate (ECG), epigallocatechin gallate (EGCG) or gallocatechin gallate (GCG) (Al-Zahrani, 2012). Our study confirmed the literature data on the antimicrobial activity of gallic acid, which proved to be the strongest compound acting against staphylococci among all tested commercial phytochemicals (Table 2). Oleanolic acid exhibited much lower activity against *S. aureus*. Because of poor solubility of oleanolic acid and the need to prepare its primary solution in 100% DMSO (biocidal for all microorganisms), there was no possibility to test oleanolic acid at a concentration higher than 750 μg/mL. Both tested plant extracts also showed strong antistaphylococcal activity with MIC range (depending on the strain) 750–1500 μg/mL (Table 2). It is worth noting that *S. aureus* H9 clinical isolate selected for this experiment is a member of an important group of "alert" human pathogens — MRSA (methicillin resistant *S. aureus*). Therefore, we have shown a significant direct biocidal activity of the extracts from *V. myrtillus* leaves and *F. alnus* bark against Gram-positive bacteria, including the multidrug-resistant *S. aureus* strain. Antimicrobial properties of plant products are determined on the basis of a susceptibility test by demonstrating MIC in the range of 100 to 1000 μg/ml (Tegos *et al.,* 2002). However, plant-derived extracts tested contained a mixture of various phenolic derivatives and other waste substances. Thus, the obtained values of MIC/MBC equal to 750 μg/mL should be considered very promising.

Table 3. Analysis of phytocompound cytotoxicity towards mouse fibroblasts line L929 — determination of the cytotoxicity index (IC50) using LDH Cytotoxicity Kit and calculation of the biocompatiblity index (BI).

	Time [h]	IC_{50} ± SD* [μg/mL]	BI** (a)	BI** (b)
Plant extracts:				
VLE (*V. myrtillus*)	0.5	1144.5 ± 18.0	0.763	1.526
	24	2041.1 ± 587.1	1.361	2.721
FBE (*F. alnus*)	0.5	1877.8 ± 617.9	1.252	2.504
	24	1357.1 ± 168.8	0.905	1.809
Phytochemicals:				
Gallic acid	0.5	135.3 ± 32.6	1.082	2.164
	24	326.1 ± 98.9	2.608	5.217
Carvacrol	0.5	425.7 ± 393.4	0.851	0.851
	24	115.1 ± 72.5	0.230	0.230
Oleanolic acid	0.5	1128.7 ± 355.5	1.505	1.505
	24	201.5 ± 9.3	0.269	0.269

* IC_{50} is mean value from two independent experiments each performed in duplicate ± standard deviation (SD);** BI was calculated as: BI = IC_{50}/MBC, based on MBC of phytochemicals determined for *S. aureus* ATCC 29213 (a) or *S. aureus* H9 (b) strain and mean value of IC_{50} for mouse fibroblasts.

In the light of the knowledge of the ever-increasing resistance of pathogens towards classic antibiotics and the participation of their aggregates/biofilms in the development of many pathological changes, it seems necessary to design new approaches for the therapy efficient against the microorganisms and their communities. Such study may include a search for substances which have not only a direct biocidal effect, but also exhibit synergistic activity with available pharmacological agents, restrict the expression of microbial virulence factors, prevent microbial adhesion and aggregation or activate host immune defense mechanisms designed to combat infections (Budzyńska *et al.*, 2011; Cushnie and Lamb, 2011; Gauwerky *et al.*, 2009; Kurlenda and Grinholc, 2012; Kuźma *et al.*, 2012). Several studies have suggested that phytocompounds, besides their direct biostatic/biocidal activity, can also enhance effects of other biocides (antibiotics) by increasing permeability of microbial cell walls for antibiotic penetration or blocking specific mechanisms of drug resistance. They can deactivate enzymes that degrade antibiotics, impair MDR efflux pumps, or inhibit synthesis of selected envelope proteins of resistant bacteria (Aqil *et al.*, 2005; Cushnie & Lamb, 2011; Gibbons, 2008; Nguyen & Graber, 2010; Palombo, 2011; Rafii & Shahverdi, 2007). Some phytocompounds exhibit direct microbicidal effects comparable to those of antibiotics. For example, it has been found that an extract from *Myrtus communis* leaves inhibits, similarly to the widely used pharmacological complex trimethoprim-sulfamethoxazole, growth of over 99% of the studied clinical isolates of *S. aureus* (Gholamhoseinian *et al.*, 2009). Essential oils of *Salvia officinalis* (sage), *Salvia triloba* (sage shrub), *Origanum minutiflorum* (wild oregano), *Origanum onites* (oregano), *Thymbra spicata* (black thyme), *Satureja cuneifolia* (wild savory) and their components such as carvacrol efficiently inhibited growth of *S. aureus* (Alviano and Alviano, 2009). Moreover, it was demonstrated that some of these compounds are also active against the biofilm form of microorganisms, often interfering at the stage of their initial adhesion and aggregation (Chaieb *et al.,* 2011). In our previous study we showed that plant-derived products, such as diterpenoids salvipisone and aethiopinone from *Salvia sclarea* hairy roots, taxodione-derivative isolated from *Salvia austriaca* roots, essential oils of *Lavendula angustifolia*, *Melaleuca alternifolia*, *Melissa officinalis* and their major constituents: linalool, linalyl acetate, α-terpineol, terpinen-4-ol, preparations obtained from *Humulus lupulus* L. display promissing activity against *Staphylococcus* biofilm. These products influenced

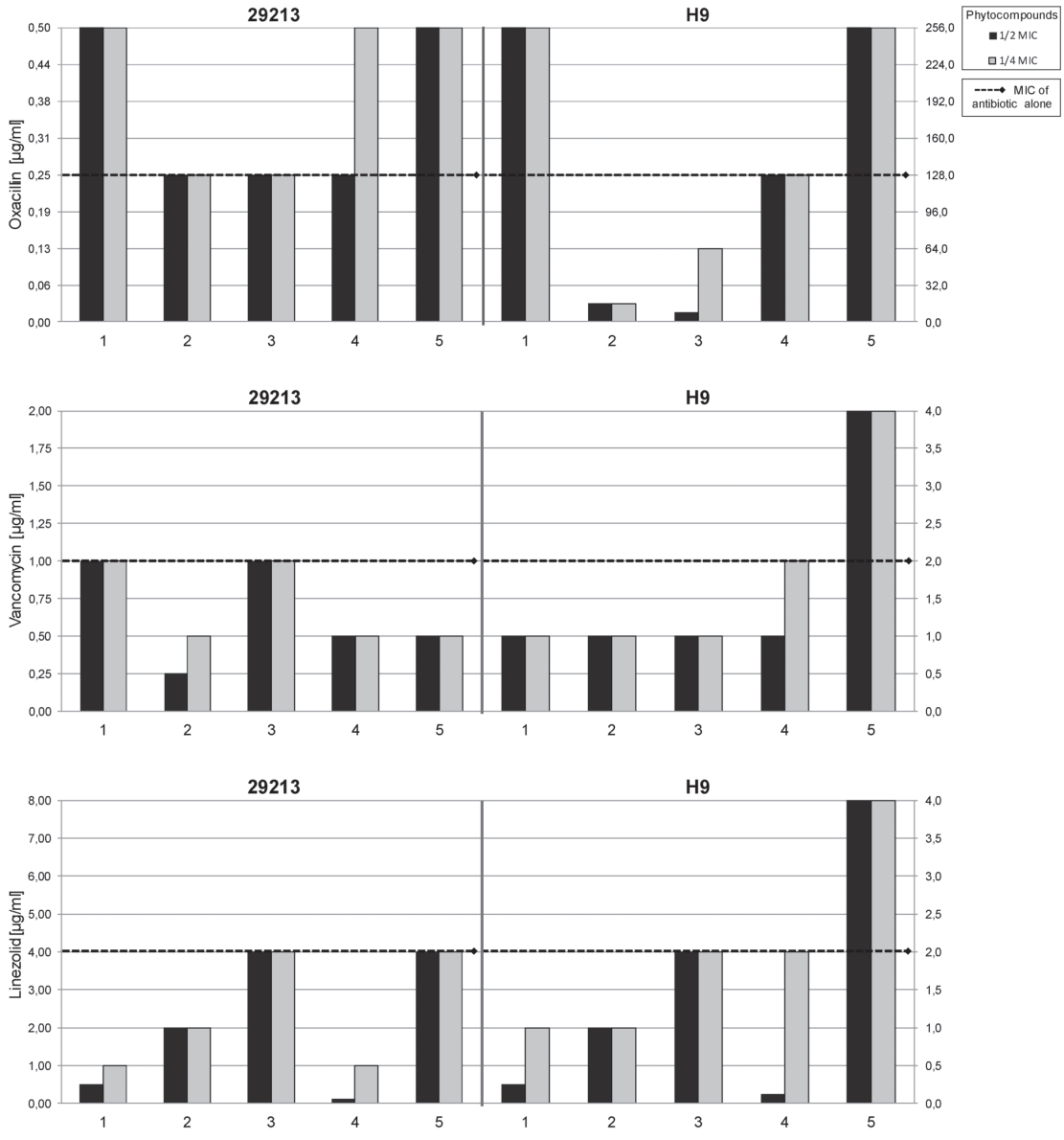

Figure 1. Synergistic effect of phytocompounds (1 — gallic acid; 2 — carvacrol; 3 — oleanolic acid; 4 — VLE; 5 — FBE) used at ½ and ¼ MIC with oxacillin (A), vancomycin (B) or linezolid (C) against *S. aureus* ATCC 29213 and *S. aureus* H9 evaluated by microdilution method.

both, staphylococcal adhesion and biofilm development, as well as were capable of partial destruction and eradication of mature 24-h-old biofilms (Budzyńska *et al.*, 2011; Kuźma *et al.*, 2012; Różalski *et al.*, 2013; Walencka *et al.*, 2007).

The current paradigm in the discovery of novel promising antimicrobial therapeutics postulates their targeting of pathogen virulence factors/abilities rather than direct biocidal activity, and their synergistic effect with classic drugs (Budzyńska *et al.*, 2011; Cushnie & Lamb, 2001; Fankam *et al.*, 2011; Gauwerky *et al.*, 2009; Kurlenda & Grinholc, 2012; Nguyen & Graber, 2010; Rafii & Shahverdi, 2007). In our research we used vancomycin and linezolid (antibiotics recommended for treatment of staphylococcal infections) to study possible synergy of the tested phytocompounds with these drugs. We also included oxacillin to examine the influence of plant-derived preparations on the status of *S. aureus* drug resistance - one of important staphylococcal virulence factors. Here, we report on the possibility of enhancing antimicrobial activities of classic antibiotics in the presence of commercial pytocompounds or plant-derived extracts used at ½ and ¼ MIC. When VLE and FBE were added into the medium together with the antibiotics, the MICs of vancomycin (glycopeptide) and linezolid (oxazolidinone) but not oxacillin (beta-lactam) were decreased (Fig. 1). The obtained results seem to be very important especially since the use of vancomycin and linezolid has recently increased. Both antibiotics are mainly administered to patients during hospitalization that often develop *S. aureus* (including MRSA) infections. The best example is life-threatening hospital-aquired pneumonia — a type of infection frequently aquired in intensive care units and mostly caused by these pathogens (Kalil *et al.*, 2013). *V. myrtillus* leaves extract demonstrated the best synergistic activity together with linezolid, whose MIC was reduced 16-32x depending on the tested strain. It partially corresponded with the result obtained for gallic acid — the phytocompound being the representative of the polyphenols group, which was described as the most prominent

component of the plant-derived extracts tested. MIC of linezolid against both *S. aureus* strains was reduced 8x in the presence of gallic acid at ½ MIC (Fig. 1). Linezolid acts as an inhibitor of protein synthesis by blocking the 23S ribosomal subunit (Kalil *et al.*, 2013). Since many plant products have been demonstrated to affect microbial cell wall and membrane integrity, it can be suggested that the synergy observed in this study is due to the facilitation of antibiotic penetration by phytocompounds. The results obtained for carvacrol seem to confirm this hypothesis. Based on IC_{50} (Table 3), carvacrol expressed a higher cytotoxic effect on eukaryotic cells, and probably also on bacterial cells. At the same time, it was the best enhancer of antimicrobial activity of all antibiotics tested. MIC values of oxacillin, vancomycin and linezolid were decreeased 8×, 2–4× and 2×, respectively, by adding carvacrol at sub-MIC (Fig. 1). Similar enhancement of antibiotic activity was observed by Rafii and Shahverdi (2007) using nitrofurantoin combined with essential oils against enterobacteria. On the other hand, the opposite effect of sub-MIC concentration of Tea Tree oil (*Melaleuca alternifolia*) reducing bacterial susceptibility to antibiotics was also demonstrated. The authors explained this phenomenon as microbial adaptation due to membrane changes inhibiting antibiotic penetration (Bakkali *et al.*, 2008; McMahon *et al.*, 2007). In our study such a mode of action was showed by gallic acid or FBE used together with oxacillin against both *S. aureus* strains (Fig. 1). Thus, the final effect of the phytocompound action on cell membranes can lead to enhanced bacterial susceptibility or resistance. It depends on many factors, including phytocompound concentration, dissolving, rate of diffusion, salt forming, even microbial inoculum and a chosen technique. Therefore, a detailed study of a specific phytocompound, antibiotic and group of microorganisms should be performed every time.

A great number of plant extract constituents are typical lypophiles. They are able to cross the cell wall and cytoplasmic membrane, often disrupting their structure. Biological activity of many pytocompounds is based on cell membrane permeabilization. Such a process occuring in microorganisms leads to a loss of ions, leakage of macromolecules, reduction of membrane potential and fluidity, decrease in the ATP pool, and finally to osmotic lysis and microbial death (Alviano & Alviano, 2009; Bakkali *et al.*, 2008; Cushnie & Lamb, 2005; 2011; Martos *et al.*, 2013; McMahon *et al.*, 2007). Antimicrobial activity of phytocompounds may also derive from their pro-oxidative properties. Accumulation of reactive oxygen substances (ROS) inside cells causes oxigenation of macromolecules followed by metabolic disturbance and cell death (Alviano & Alviano, 2009; Gholamhoseinian *et al.*, 2009). Some studies pointed to flavonoids as compounds which are able to inhibit DNA synthesis (e.g. epigallocatechin, quercetin, rutin) or energy metabolism (e.g. retrochalacones, lonchocarpol A), causing a reduction in macromolecule synthesis (RNA, DNA, cell wall and proteins). It must, therefore, be assumed that phytochemicals may target different structures and physiological processes in bacterial cells (Cushnie & Lamb, 2005; 2011; Gibbons, 2008). Similar cytotoxic effect leading to induction of apoptosis and necrosis can be also observed in eukaryotic cells. Cytotoxicity of many plant-derived compounds used at microbicidal concentrations is a major limitation in their application as antimicrobials in humans (Alviano & Alviano, 2009; Bakkali *et al.*, 2008). Therefore, it is necessary to test cytotoxicity of the preparations destined for human usage (Müller & Kramer, 2008). In this study we used an *in vitro* method with mouse fibroblasts line L929 to estimate the concentration of phytocompounds causing cytotoxic effect in half a population of the cells tested (IC_{50}) during 0.5 and 24 hours of co-incubation. As we expected, plant-derived extracts exhibited much lower cytotoxicity than comercial phytochemicals (Table 3). Based on IC_{50} and MBC against *S. aureus* strains, the biocompatibility index (BI) was calculated. As it has been shown in Table 3, the BIs of plant extracts were >1 for almost all conditions, which indicated these phytocompounds as possessing relevant antimicrobial activity accompanied by a relatively low cytotoxic effect against eukaryotic cells. A similar result was obtained for gallic acid (regardless of the exposure time of L929 cells) and oleanolic acid affecting cells during 0.5 h.

Recently, a growing concern is focused on the impasse of antibiotic therapy, resulting from increasing resistance of microorganisms to the available chemotherapeutic agents, as well as from participation of their biofilms in the development of pathological changes. Thus, there is an ongoing need to investigate new therapeutic strategies and innovative approaches for eradicating multidrug-resistant microorganisms and their biofilms. The use of plant extracts (alone or synergistically with conventional antibiotics) for this purpose is possible, but requires detailed research. The proposed study on an assessment of chemical composition of the newly-prepared plant extracts (from *V. myrtillus* leaves and *F. alnus* bark) and their biological activity, including antimicrobial, synergistic with other biocides and cytotoxic effect on mammalian cells gives a theoretical basis for future applications of these phytocompounds. We believe that the described results provide a reliable scientific background in this area and may serve as an explanation for the proposed alternative therapy of skin and soft tissue infections caused by a very dangerous human pathogen — *S. aureus*.

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