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Communication

Leonurus cardiaca L. herb — a derived extract and an ursolic acid as the factors affecting the adhesion capacity of *Staphylococcus aureus* in the context of infective *endocarditis**

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The objective was an assessment of the impact of *Leonurus cardiaca* L. extract (*LCE*) and ursolic acid (*UA*) on the adhesive properties of *Staphylococus aureus* NCTC 8325 strain, expressing virulence factors important in the pathogenesis of infective *endocarditis*. The adhesion and biofilm formation of bacteria cultured in the presence of subinhibitory concentrations of *LCE* or *UA* on the abiotic surface or covered with fibrinogen, fibronectin or collagen, were evaluated. Inhibitory effects of *LCE* and *UA* on staphylococcal adherence to both types of surface were demonstrated. This, in the case of *UA*, resulted in a significant reduction of biofilm formation.

Key words: ECM, *Leonurus cardiaca*, ursolic acid, *Staphylococcus aureus*

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INTRODUCTION

Infective *endocarditis* (IE) is often a fatal disease, which mostly develops as a complication of heart surgery or anatomical disorders, mainly in the valves. Occasionally, but in last few years with significantly growing frequency, it may occur in individuals without clear risk factors. The native valve staphylococcal IE is usually caused by *Staphylococcus aureus*. In contrast, the prosthetic valve infection is more often caused by a coagulase-negative staphylococci (*S. epidermidis*). Both bacteria species possess numerous surface-bound and extracellular proteins recognizing extracellular matrix components (ECM), which are exposed on the inflamed heart tissue. The resulting "vegetations" are composed of the bacterial cells forming biofilm, fibrin, platelet clots, leukocytes and erythrocytes. It has been found that plasma-derived protein "bridges", between staphylococcal receptors and thrombocytes, are formed from fibrinogen, fibrin, thrombospondin and von Willebrand factor (Hauck & Ohlsen, 2006). Staphylococci bind also avidly to endothelial cells through adhesin-receptor interactions (SpA, von Willebrand-factor binding protein, FbpA and ClfA, ClfB). Two other *S. aureus* products (secretable) interact with hemostasis system, but at two different stages. Coagulase- prothrombin binding results in the formation of active "staphylothrombin" and fibrin generation, whereas staphylokinase, which binds plasminogen and facilitates plasmin activity is a thrombolytic agent. On the other hand, targets of α-toxin include lymphocytes, macrophages, epithelium, endothelium, and erythrocytes. This toxin has also been proposed to play a role in biofilm formation by increasing the bacterial cell-to-cell interactions (Anderson *et al*., 2012; McAdow *et al*., 2012; Zhang *et al*., 2011).

Because of the risk of serious sequelae, patients with IE undergo compulsory treatment with antibiotics, unfortunately, due to the drug-resistance and the tendency of bacteria to grow in a biofilm, such treatment is often ineffective (Widmer *et al*., 2006). Since the most common cause of death of IE patients is an appearance of clots derived from vegetations, they are routinely administered antiplatelet/anticoagulation drugs. Taking into account that resistance to some of these drugs is also an emerging clinical entity, it would be interesting to determine similar properties of naturally occurring substances (e.g. plant extracts or their components), which in future could be used as the alternatives to the currently known chemotherapeutics (Fraga *et al*., 2010). The results of our unpublished preliminary studies on the biological activity of several plant extracts rich in polyphenols drew attention to the *Leonurus cardiaca* herb extract and one of its compounds — an ursolic acid. Both products demonstrated interesting antimicrobial, antiplatelet and immunomodulatory activities. Another reason for an interest in *L. cardiaca* herb extract is its known beneficial effect on heart and cardiocirculatory system. It is a part of the preparations, such as *Cardiosan, Cardionervit, Cardiogran* and many others used, for example, to strengthen the heart muscle, possessing biological features which are compared in many respects with those derived from *Valerianae* radix and *Melissae* folium (Wojtyniak *et al*., 2013). However, due to the simultaneous antimicrobial activity of *L. cardiaca* extract / ursolic acid, the main objective of the present study is an assessment of their impact on the expression of *S. aureus* attributes, taking part in the initial steps of pathogenesis of infective *endocarditis*. The study includes evaluation of: i) adhesion of pathogens to an inert plastic surface or to a surface covered with ECM components (fibrinogen, fibronectin, collagen), resembling for example a heart valve prosthesis, ii) biofilm formation in the above circumstances, reproducing roughly the bacterial behavior involved in the formation of vegetations.

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*The preliminary report on the same subject was presented at MIKROBIOT 2013 Workshop, Łódź, Poland

Abbreviations: ECM, extracellular matrix proteins; IE, infective endocarditis; *LCE*, *Leonurus cardiaca* extract; *UA*, ursolic acid

MATERIALS AND METHODS

Bacterial strains to be tested. The study was performed on a model *Staphylococcus aureus* 8325-4 (NCTC 8325) strain expressing virulence factors of the MSCRAMMs/SERAMMs group — α-toxin$^+$, SpA$^+$, Coa$^+$, ClfA$^+$ ClfB$^-$, SAK$^+$. Their expression was earlier proved in our Lab. Briefly, SpA (staphylococcal protein A) was detected using goat anti-SpA FITC-labeled IgG; SAK (staphylokinase) was detected on the basis of the reduction of the chromogenic substrate; α-hemolysin secretion was evaluated as an intensity of hemolysis of the sheep red blood cells and with the Western blot using anti-α-tox mAb; expression of a fibrinogen receptor (ClfA, clumping factor A) was assessed using a semi-quantitative Latex *S. aureus* test; coagulase (Coa) production was evaluated in the rabbit plasma coagulation test (Sadowska, 2010).

Preparation and chemical characterization of the *Leonurus cardiaca* extract (*LCE*). Motherwort (*Leonurus cardiaca* L.) was a commercial sample supplied by KAWON-HURT Nowak sp.j. (Gostyń, Poland). Leaves 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 for 15 min (4000 rpm). The pellets were re-extracted twice with 70% aqueous acetone for 15 min and the supernatants were combined. After the 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) nine times. The water fractions were concentrated in vacuum, and lyophilized (Alpha 1–2 LD plus, Christ) with the yield of 14.47%. Dried extracts were reconstituted at 5 mg/ml of water before analysis. Qualitative and quantitative composition of *LCE* was determined using spectrophotometric methods and HPLC-PDA analysis. On the basis of spectral identification and maximum of UV-Vis absorption, phenolics were qualified in four subclasses: flavanols and hydroxybenzoic acids (expressed as gallic acid equivalents; detection at 280 nm), hydroxycinnamic acids (as chlorogenic acid equivalents; detection at 320 nm), flavonols (as rutin equivalents; detection at 360 nm), and anthocyanins (as cyanidin 3-glucoside equivalents; detection at 520 nm). The results were expressed as mg/g of extract.

Ursolic acid (*UA*) ≥ 90% purity was purchased from Sigma, USA.

Antimicrobial activity of *LCE* and *UA*. MIC (minimal inhibitory concentration) values were specified by a standard microdilution broth assay. Stock solution of *LCE* was prepared in 50% DMSO, while *UA* in 96.0% EtOH (PoCh, Poland). The concentration ranges of the compounds (in the two-fold dilutions system in 96-well microplates) were of: 0.0625–2.0 mg/ml (*UA*) and 0.5–6.0 mg/ml (*LCE*). Subsequently, the bacterial suspension (100 µl) was added (1:1) to the wells. Phytocompounds dilutions without bacteria were prepared as the negative control, while for a positive control bacterial suspensions in Mueller-Hinton broth were added to the wells. The final highest DMSO concentration was 1.25% and EtOH was 4.25%, which did not affect bacterial growth. Plates were incubated at 37°C for 18 h and the lowest concentration showing no turbidity was recorded as MIC. In each case, experiments were carried out in quadruplicate in two separate occasions.

Anti-adhesive and anti-biofilm properties of *LCE* and *UA*. The suspension of *S. aureus* strain (1–5 × 10^7 CFU/ml) was prepared in adhesion/biofilm promoting medium — tryptic soya broth (TSB) supplemented with 0.25% glucose (TSB/Glu). The experiment scheme was as follows: (A) — 96-well microplates with immobilized fibrinogen 2 µg/well (Sigma, USA), collagen I, rat tail (Life Technologies, USA) or fibronectin, 1 µg/well (R&D Systems, Inc.) were used; (B) 96-well polystyrene microplates (Nunc, Roskilde, Denmark) with uncoated wells were used. In all cases 100 µl of *S. aureus* suspension and 100 µl of *LCE* or *UA* at final ½ or ¾ MIC were added and incubated for 2 h at 37°C; (C) anti-biofilm activity of *LCE* and *UA* was tested when the above described experiments (A) and (B) were prolonged to 24 h. In this case only ¾ MIC of *LCE* and *UA* was used. Negative controls in each experiment version (A, B, C) were wells containing only dilutions of phytocompounds in DMSO (½ or ¾ MIC), while positive controls were wells containing bacterial suspensions and TSB/Glu. To evaluate adhesion/biofilm formation of bacteria, Alamar Blue (AB, Trek Diagnostic Systems, Inc. USA) staining protocol was used as recommended by the manufacturer. Briefly, 5 µl of Alamar Blue (AB, BioSource, USA) was added to the wells of 96-well tissue culture polystyrene plate (Nunclon Surface, Nunc, Denmark), containing bacterial cultures after an appropriate time of their exposition on phytochemicals or medium (control). Then, the plates were incubated for 1 h at 37°C (with shaking). Finally, the absorbance was determined at 550 nm and 600 nm using a multifunctional counter (Victor2, Wallac, Finland). The percentage of AB reduction was calculated according to the manufacturer formula. Relative changes in bacterial adhesion were expressed as a percentage of attached cells incubated in the presence of subMIC of phytocompounds ±S.D., compared to the positive control. In each case, experiments were carried out in quadruplicate in two separate occasions.

Statistic analysis of data. Differences in parameters were tested for significance using the Mann-Whitney *U* test and the program Statistica 10.0 (Stat Soft Inc.). The differences with $P<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The phytochemical analysis showed that the total content of polyphenols in *L. cardiaca* L. extract (*LCE*), expressed as a gallic acid equivalent averaged from three measurements, was 115.12±11.5 mg/g. The HPLC analysis demonstrated their amount as 182.75±14.57 mg/g. Hydroxycinnamic acids (caffeic acid, ferulic acid and *p*-coumaric acid) content calculated as chlorogenic acids was 172.01±14.34 mg/g. The minimal inhibitory concentration (MIC) of this extract against *S. aureus* 8325-4 (NCTC 8325) was 6 mg/ml, whereas MIC of ursolic acid (*UA*) was 0.25 mg/ml. In subsequent experiments dedicated to the assessment of adhesion/biofilm formation, ½ and ¾ MIC of these products were used. Why were the subinhibitory concentrations exmined? Because it is known that the beneficial effect of antibiotic or other drug is not only achieved when the concentration is above the minimal inhibitory concentration (MIC) between consecutive doses. It has been shown *in vitro* that, depending on the pharmacokinetic and pharmacodynamic properties, the subMIC of a given product is able to affect important bacterial characteristics (Sadowska, 2010). Due to the specific pathogenesis course of infective *endocarditis*, it can be assumed that achieving the MIC concentration of a product at the inflammation site is unlikely. Moreover, the products tested by us possess

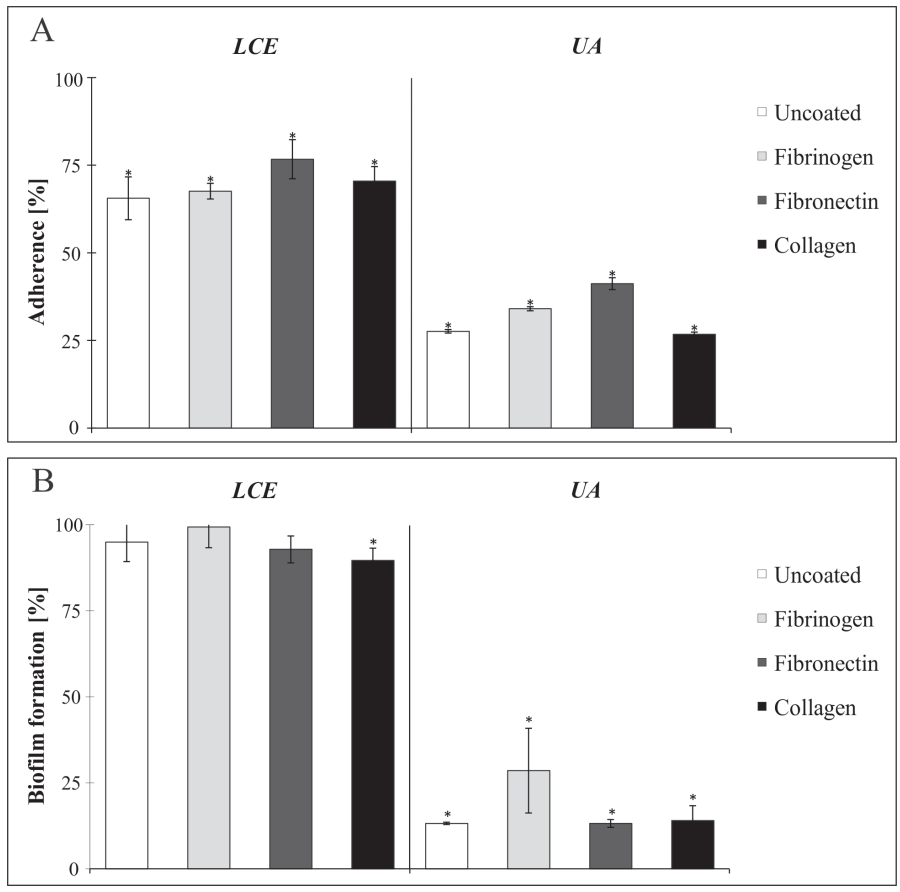

Figure 1. Anti-adherent and anti-biofilm activity of *Leonurus cardiaca*-derived extract and ursolic acid against *S. aureus* NCTC 8325. Bacteria were cultured for 2 or 24 h on microplates without or with immobilized fibrinogen, fibronectin or collagen, in absence or constant presence of the phytocompounds used at their ¾ MIC. Adhesion (**A**) and biofilm formation (**B**) were evaluated by staining with Alamar Blue. Results are presented as the percentage of the biomass viability, compared to the control, accepted as 100%. All presented results are mean from 2 independent experiments performed in quadruplicate ± S.D. *$p<0.05$

(at MIC) unfavorable biocompatibility index, which excludes their use at a higher concentration (not shown).

The microplate Alamar Blue assay (MABA) used in the study showed the inhibitory effects of *LCE* and *UA* on staphylococcal adherence to an inert surface. The percentage of the inhibition of adherence to uncoated wells of polystyrene plates ranged from 14.2% to 72.4%, dependent on the type (*LCE*, *UA*) and concentration of each phytocompound (½ or ¾ MIC). This limitation of bacterial settlement was not caused by the decrease in their viability *per se*, since the growth rate of *S. aureus* suspension culture (tested in parallel after 2 h co-incubation by MABA) was not affected. The specific adherence of bacteria to the microplate wells containing immobilized extracellular matrix proteins (ECM) was also diminished. For example, ursolic acid (*UA*) used in a concentration of ¾ MIC limited bacterial adhesion to collagen-coated wells by 73.2%, to fibronectin-coated wells by 58.8%, and to fibrinogen-coated surface by 65.9%. The *L. cardiac*a extract used at ¾ MIC showed the activity twice as weak as ursolic acid (in the range of 23.2–32.4%), significant only in the case of a collagen coated surface. When *LCE* and *UA* were used at a lower concentration (½ MIC), the obtained inhibitory effect was weaker. The data in Fig. 1A represents the results obtained in experiments where ¾ MICs of the compounds were used.

The exposure of ECM neoepitopes, deposition of platelets and fibrin at the site of inflammation, as well as the expression of the various molecules on capillary endothelial cells, occur in the heart area. These conditions lead to a local inflammatory process and coagulation, and created a perfect surface for the attachment of a pathogen (Chavakis *et al.*, 2005; Edwards & Massey, 2011; McAdow *et al.*, 2011; Kim *et al.*, 2012). Therefore, a limitation of the adhesion by the *L. cardiaca* extract and ursolic acid indicate a new potential application of these compounds. In another study, conducted by us at the same time in a different direction than the one presented here, it was found that both phytocompounds (at concentrations of 0.5 to 50 μg/ml) inhibit the adhesion of platelets to immobilized collagen. Moreover, these products, when co-incubated with human plasma, inhibit (in a dose dependent manner) fibrinogen polymerization (data not yet published). Thus, the *L. cardiaca* extract as well as ursolic acid, in addition to the very interesting anti-staphylococcal activity detected in the present study, not only modulate platelet functions but may also change properties of fibrinogen, which is a key protein in blood coagulation.

Thus, the weakened *S. aureus* adherence caused by the presence of *LCE* and *UA* in the culture medium is a beneficial effect, especially because it resulted in the statistically significant reduction in the metabolic potency of bacterial mass (biofilm) formed during subsequent 24 h incubation (Fig. 1B). If we relate these observations to the real situation *in vivo*, we may suppose and anticipate that vegetations would be smaller/weaker and less prone to detachment, if such a type of products is present in

a given microenvironment. The comparison of the degree of the Alamar Blue reduction by cells in biofilm, which developed in the presence of phytochemicals, showed a decrease in their formation. *LCE* was a weak inhibitor, since it caused only 5% inhibition of biofilm formation on the inert polystyrene surface and approximately 1-10% reduction on an surface coated with ECM proteins, whereas ursolic acid proved to be very effective. At a concentration of ¾ MIC *UA* limited biofilm formation on the surface, both on inert (by 85%) and on coated with ECM proteins (approximately 70-86%). Although the exact mechanism by which the tested phytocompounds affects *S. aureus* cells during the adhesion and biofilm development is at the moment unknown, it can be assumed that it is based on modification of the surface adhesins molecular architecture, their expression and/or influence on the cell-surface hydrophobicity. This remains to be determined in further studies just undertaken in the our Lab. The original plant extract used in the study — *L. cardiaca* is known as a herb used in traditional medicine, being applied in the cases for a variety of illnesses. It contains alkaloid leonurine, which is a mild vasodilator, and therefore, has long been used as a sedative in heart neurosis. Among other biochemical constituents, it also contains bitter iridoid glycosides, di- and triterpenoids, flavonoids, tannins and volatile oils (Wojtyniak *et al.*, 2013). The *L. cardiaca* extract tested in the present study was characterized as presented above and was shown to contain high amounts of known biologically active components. Another product tested in this study — ursolic acid, belongs to the main group of triterpenoids represented by pentacyclic derivatives, which are ubiquitous in the plant kingdom, including *L. cardiaca*. Several biochemical and pharmacological effects of *UA* such as anti-inflammatory, antioxidant, anti-proliferative, anti-cancer, anti-mutagenic, anti-atherosclerotic, anti-hypertensive, anti-leukemic and antiviral properties are reported in a number of experimental systems (Checker & Sainis, 2012). Through the presented results we provide additional valuable information on the biological properties of these phytochemicals, which had not been tested before in the range covered by our research.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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