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EFFECTS OF EXTRACTS FROM MEDICINAL PLANTS ON BIOFILM FORMATION BY *ESCHERICHIA COLI* URINARY TRACT ISOLATES

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ABSTRACT

Bacteria grown as biofilm cause serious trouble in medical practice as a source of both contamination of indwelling medicinal devices, and nosocomial infections. Biofilm microorganisms develop antibiotic resistance more rapidly than plankton. For this reason, novel anti-biofilm strategies address the search of substances that may suppress biofilm growth of pathogens without killing the microorganisms themselves. This report presents the results of a search for plant substances corresponding to these requirements. Three strains of urinary clinical isolates of E. coli, two uropathogenic (UPEC) and one from asymptomatic bacteriuria (ABU), with pre-established biofilm proficiency were compared. Antibiotic resistance of the strains was determined by the disk-diffusion assay. Each of the UPEC strains was resistant to two antibiotics while the ABU strain was multiresistant. The antibacterial and antibiofilm effects of 14 extracts in different organic solvents from four medicinal plants were tested. The dried extracts were dissolved as stocks in ethanol. Disk diffusion assay with different amounts of the extracts showed no antibacterial activity against the selected strains. Biofilm growth was examined by the crystal violet assay after growth for 24 h in M63 medium alone or supplemented with 10 μg/ml from the dried extracts. All of the extracts modulated biofilm growth, and four had significant biofilm suppression effects on the UPEC strains while they stimulated the attached mode of growth of the ABU strain. Meanwhile, the extracts had no significant influence on the growth curves of the UPEC, but could delay the growth of the ABU strain. This implies that they may contain valuable substances for application directed against pathogenic biofilms.

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Introduction

In natural habitats and in their host environment bacteria most often occur attached to surfaces where they form sessile consortia, or biofilms (3). Biofilms that develop on indwelling devices (urinary catheters, pacemakers, voice prostheses, contact lenses, etc.) are of major concern in medical practice (2). The attached mode of growth protects the bacteria from environmental stresses. One serious problem is the much more rapid establishment of antibiotic resistance in biofilm than in plankton (3).

Escherichia coli is among the predominant species isolated from urinary tract infections. The biofilm-forming strains of uropathogenic *E. coli* (UPEC) are often a cause of chronic and recurrent infections (4, 14). UPEC are hard to eradicate by antibiotic therapy, especially in patients with prolonged catheterisation where they develop device-associated biofilms (1, 3, 7, 9, 15). This necessitates the search of novel approaches to biofilm suppression. One prospective source are substances of plant origin, and this is illustrated for instance by the already popular application of cranberry and blueberry juices in cases of urinary tract infections (6, 10). The good therapeutic candidates should suppress biofilm growth while not exerting antibacterial activity so that no selective pressure can result in resistance. Ideally, they should also not influence growth and biofilm formation by the non-pathogenic microflora.

Here we report the results of a screening of 14 different extracts from four medicinal plants for antibacterial and antibiofilm activity against three clinical isolates of *E. coli* – two from urinary-tract infection and one from asymptomatic bacteriuria.

Materials and Methods

Strains, media and cultivation

In this study were included three urinary clinically isolated strains with pre-estimated good biofilm growth capacity (11) from the laboratory collection of the Departnemt of Biochemistry and Microbiology, Plovdiv University. PU-1 was isolated from a woman with pyelonephritis, PU-13 – from a man with cystitis, and PU-19 – from a man with ABU. Estimation of the antibacterial activity of the plant extracts was tested also on reference strains: *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Streptococcus saprophyticus* NCTC 7292.

The strains were stored frozen in Trypticase soy broth (TSB) (Difco) supplemented with 30% glycerol. Before use, samples were inoculated into TSB and incubated overnight at

TABLE 1

Medicinal plants tested in the study

Sample index in 'Results'	Plant species	Initial extract in:	Chemical composition
Rr1	*Rhodiola rosea* (Crassulaceae)	Acetone (total extract)	Oligomeric and polymeric proantocianidines and all components described as components of F1-F3
Rr2		F1*	Phenylpropanoid glycosides
Rr3		F2*	Flavonoids
Rr4		F3*	Flavonoids
Am1	*Arnica montana* (Asteraceae)	Chloroform (total extract)	Sesquiterpene lactones, phenolic acides, flavonoids, triterpenes
Am2		Purified lactone fraction	Sesquiterpene lactones
Pa1	*Petasites album* (Asteraceae)	Methanol (total extract)	All components of hexane, chloroform, butanol entracts described below
Pa2		Hexane	Mono-, sesqui-, tri-terpenoids, nonpolar phenyl ethanoids and propanoids
Pa3		Chloroform	Sesquiterpene alcohols and lactones
Pa4		Buthanol	Glicosides
Ph1	*Petasites hybridus* (Asteraceae)	Methanol (total extract)	All components of hexane, chloroform, butanol entracts described below
Ph2		Hexane	Mono-, sesqui-, tri-terpenoids, nonpolar phenyl ethanoids and propanoids
Ph3		Chloroform	Sesquiterpene alcohols and lactones
Ph4		Buthanol	Glicosides

* F1-F3 – fractions obtained from division of by separation of Rr1 on S LH 20 column

37 °C. Then bacteria were streaked on nutrient agar for single colony separation. Three single colonies were selected, applied separately to slant trypticase soy agar, incubated overnight at 37 °C and kept refrigerated until use but no longer than 30 days. Samples from these were examined in parallel in the biofilm experiments.

Plant extracts

Fourteen different extracts from four medicinal plants were included in the study. Extraction of *Rhodiola rosea* with acetone, *Arnica montana* with chloroform and, *Petasites albus, Petasites hybridus* with methanol gave total extracts (Rr 1, Am 1, Pa 1, and Ph 1), respectively. F1, F2, and F3 were obtained by separation of Rr1 on Sephadex LH-20 column using EtOH for elution. Am1 was prepared by precipitation with $Pb(OAc)_2$, filtration and extraction of the filtrate with $CHCl_3$. Extracts Pa 2 – Pa 4 and Ph 2 – Ph 4 were prepared from Pa 1 and Ph 1, respectively. The methanol extracts were dissolved in 50% aqueous methanol, and successively extracted with hexane, chlorophorm and butanol. The plants, mode of extraction and chemical composition are given in **Table 1**. The extracts were suspended as stocks in ethanol to concentrations of 10 mg/ml and stored frozen until use.

Disk diffusion assay

The classical disk-diffusion assay was applied first to characterize the antibiotic resistance of the urinary strains. This was performed with discs (Bioanalyse-Ankara, Turkey) soaked with the following antibiotics: ampicillin, ampicillin/sulbactam, cefuroxime, cefotaxime, cefotaxime/clavulanic acid, nitrofurantoin, gentamycin, amikacin, nalidixic acid, ciprofloxacin, and trimethophorin/sulfamethoxazole. The disks were applied on Müller-Hinton agar plates (NCIPD, Sofia, Bulgaria). The test was also applied to examine the antibacterial activities of the plant extracts. Initially, discs loaded with 50 µg of the extracts were tested against the set of two Gram-negative and two Gram-positive strains referent for antibiotic activities. In a separate series of experiments, disks were loaded with increasing amounts from each extract: 5, 10, 50, 100, and 200 µg, air dried and applied on the Müller-Hinton agar plated with the tested *E. coli* clinical isolates.

Crystal violet (CV) assay for biofilm growth

E. coli biofilm formation was estimated after cultivation in 100 µl of M63 medium (0.02 M KH_2PO_4, 0.04 M K_2HPO_4, 0.02 M $(NH_4)_2SO_4$, 0.1 mM $MgSO_4$ and 0.04 M glucose) or M63 medium supplemented with 10 µg/ml of the plant extracts. Biofilms were developed on 96-well U-shaped plates for microtitration (Nunc). The wells were inoculated with 10 µl of overnight TSB cultures of the *E. coli* strains (approximately 6

$\times 10^5$ CFU·ml^{-1} as shown by plating) and cultivated for 24 h at 20 ºC. Planktonic cells were removed, the wells were washed 3 times in 0.85% NaCl and the biofilms were stained with 0.1% crystal violet for 10 min. The dye was solubilised with 150 µl 75% ethanol per well and the absorbance was measured on a plate reader at 550 nm. Each variant was applied on 6 wells per sample and repeated with bacteria originating from 3 separate colonies from each strain. The data were processed by variation statistics using the Student-Fisher test.

Effects of the extracts on bacterial growth

To check for the effects of the plant extracts on the bacterial growth course under the nutrient limitation applied in the biofilm trials, plates with 100 µl of M63 – pure or supplemented as above, were cultivated at 37 °C and absorbance was measured hourly at 620 nm wavelength until the stationary phase was reached.

Results and Discussion

We first characterised the antibacterial activities of the plant extracts on a set of Gram-positive and Gram-negative reference strains. The disk diffusion assay showed that the substances were either inactive, or with low activity against the Gram-positive strains only. Bacteriostatic or weak bactericidal activity (inhibition zones c.a. 8-12 mm) against *S. aureus* and *S. saprophyticus* was recorded upon application of 50 µg of Rr4, Pa1, Pa2 and Ph4. As a second task, we applied the disk-diffusion assay to characterise the clinical *E. coli* strains included in the study with regard to their antibiotic sensitivity. The two UPEC strains can be characterised as susceptible to most of the standardly applied antibiotics: PU-1 was inhibited only by ampicillin and ampicillin/sulbactam, and PU-13, by ampicillin and trimethoprim/sulfomethoxazole. Contrary to this, the ABU strain PU-19 was multiresistant and inhibition zones were registered only around disks loaded with cefotaxime/clavulanic acid, nitrofurantoin, and amikacin. Unrelated with the antibiotic-resistance profiles, the disk-diffusion assay characterised the three strains as generally not sensitive to the plant extracts, the only exception being the formation of little inhibition zones around the disks loaded with 50, 100 and 200 µg of Pa2 and applied to PU-19.

Contrary to the lack of effects of the above amounts of the extracts, the addition of only 10 µg/ml during biofilm growth, resulted in a variety of biofilm modulation activities (**Table 2**). Biofilm growth of the UPEC strain PU-1 was generally suppressed by all the supplements, whereas with strain PU-13 the effects ranged from suppression through no statistically significant effect to stimulation. Notably, most of the extracts stimulated biofilm growth in the ABU strain.

The extracts Rr1, Rr2, Am1 and Am2 were biofilm-inhibitory to both UPEC strains, hence promising for practical applications, and were subjected to further analysis. Contrary to UPEC, the four extracts stimulated biofilm growth in the ABU strain PU-19 (**Fig. 1**). This stimulation was the highest with Rr1 where biofilm growth was almost fourfold that of the control.

The extracts had no effect on the plankton growth course in M63 medium of PU-1 and PU-13. However, opposite to biofilm stimulation the four extracts affected the growth of PU-19 (**Fig. 2**). With Rr1, the extract that had the highest biofilm-stimulation effect on this strain, the stationary phase of growth

TABLE 2

Effects (% of control ± standard deviation) of plant extracts on biofilm growth by UPEC clinical isolates

	PU-1	**PU-13**	**PU-19**
control	100	100	100
Rr1	63±26	82±15	367±7
Rr2	55±3	76±8	109±6
Rr3	58±15	104±13	124±19
Rr4	55±6	100±20	143±16
Am1	53±14	84±18	114±10
Am2	70±23	88±11	142±19
Pa1	51±16	125±13	114±20
Pa2	67±11	121±5	109±10
Pa3	45±11	139±14	121±21
Pa4	39±11	138±20	93±1
Ph1	59±8	120±8	146±8
Ph2	72±7	94±12	138±19
Ph3	61±9	96±7	134±5
Ph4	34±4	147±38	107±13

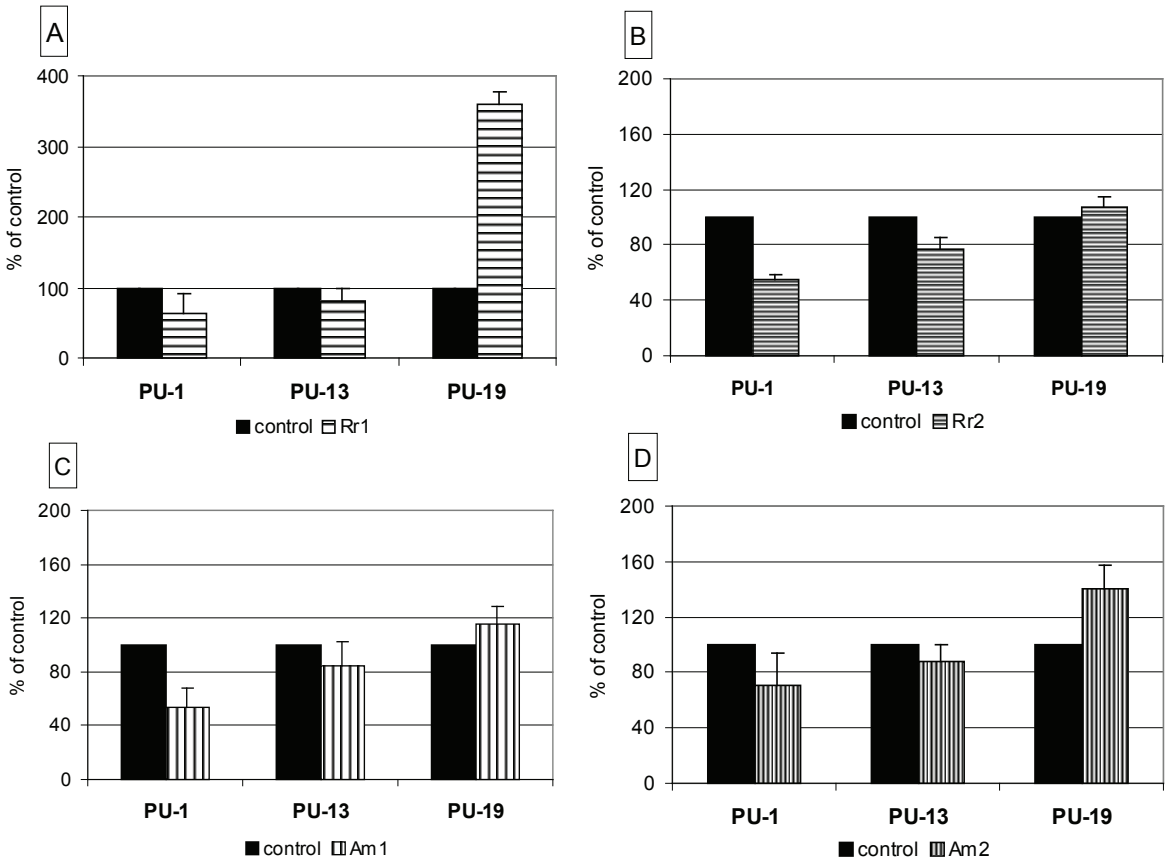

Fig. 1. Effects of the extracts Rr1 (**A**), Rr2 (**B**), Am1 (**C**) and Am2 (**D**) on biofilm formation by the three *E. coli* strains

was recorded at a lower turbidity value than in the control. For the other three extracts, slowing down of bacterial growth was noted in the beginning but the stationary phase was registered at turbidity similar to that of the control.

The chosen plant species are known for their use in non-traditional medicine. The results from the present experiments showed however little potential for antibacterial applications. This was shown with the standard reference strain panel of four Gram-positive and Gram-negative bacteria, where low bacteriostatic or bactericidal effects of Rr4, Pa1, Pa2 and Ph4 were recoded on the Gram-negative strains only. The lack of activity was confirmed for the *E. coli* clinical isolates included in the study.

This is opposed by the wide range of biofilm modulating activities of the plant preparations. The effects varied and were *E. coli* strain-specific. Four of the extracts, Rr1, Rr2, Am1 and Am2 were biofilm suppressing against both UPEC strains, and may be a source of potentially useful substances for medicinal applications. What is more, these substances stimulated instead of suppressing sessile growth in the ABU strain. It is tempting to relate this to antibiotic susceptibility, especially of the multidrug resistance strain *E. coli* PU-19. Since biofilm growth can be accepted as a way of bacteria to overcome unfavourable environmental conditions (8), this strain could be considered as a better survivor than the other two. This suggestion is however not the likely explanation of the results, and is contradicted by the observed suppression effects of Rr1, Rr2, Am1 and Am2 on the growth course of PU-19 in M63 medium. Likely results were earlier observed with spent cultures of several probiotic bacterial species that had antibacterial activity against enteroaggregative *E. coli*, but stimulated instead of suppressing biofilm growth (12). Such results could be explained by switching on of hitherto unknown protective mechanisms in *E. coli*.

Last but not least is the observation of the opposite effects of Rr1, Rr2, Am1 and Am2 on the strains: suppressing the biofilm growth in the UPEC and stimulating it in the ABU. Most of the present-day biofilm research on urinary clinical *E. coli* isolates was concentrated on UPEC strains. ABU strains for which less is known can reside in the urinary tract without causing pathology. Recently, it has been established that ABU strains may be better biofilm formers than UPEC, and can outcompete them in the colonisation of surfaces (5, 13). The here registered opposite effects on pathogenic and

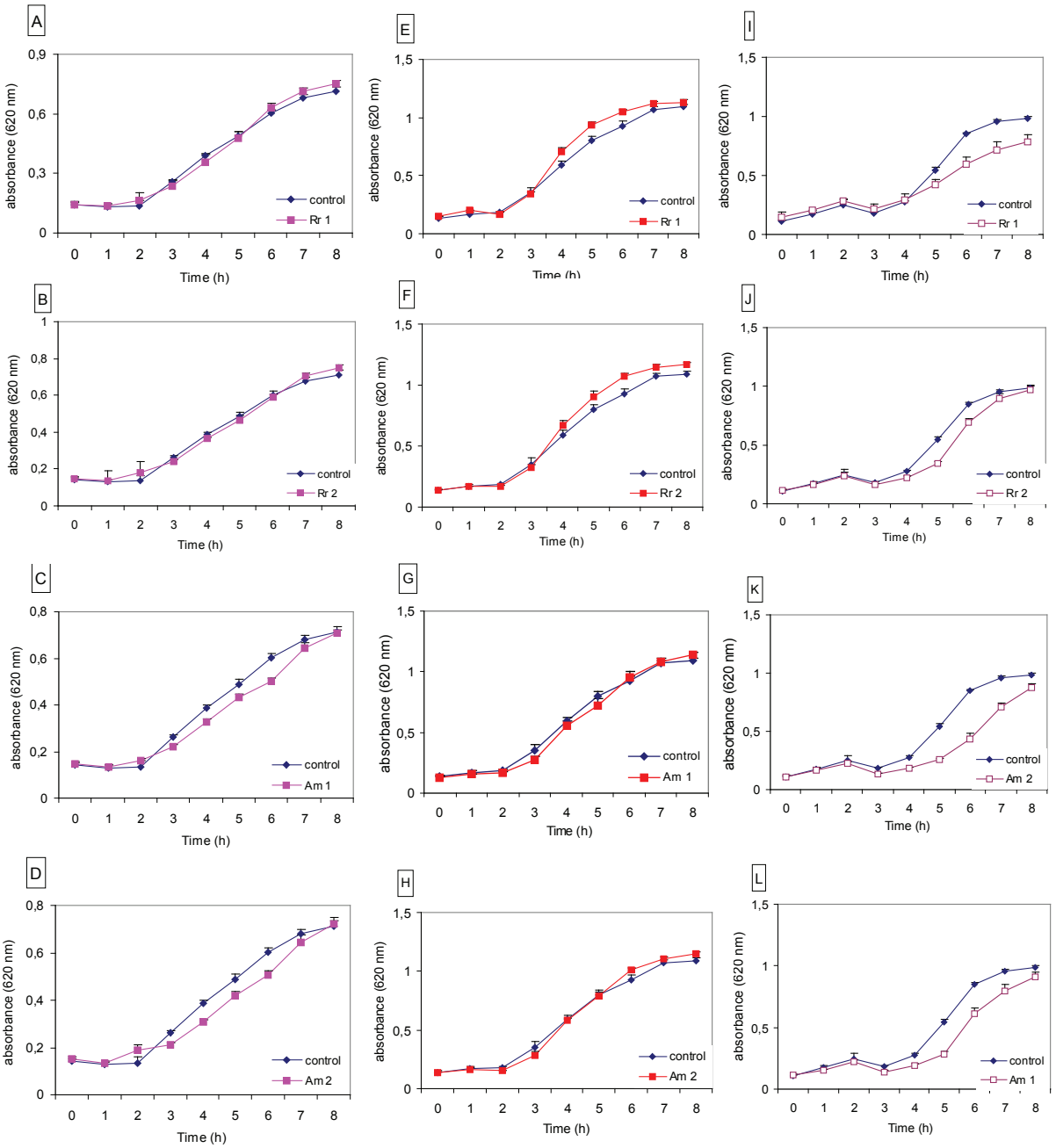

Fig. 2. Effects on the course of growth in M63 medium of *E. coli* PU-1 (**A**, **B**, **C**, **D**), *E. coli* PU-13 (**E**, **F**, **G**, **H**), and *E. coli* PU-19 (**I**, **J**, **K**, **L**) by the extracts Rr1 (**A**, **E**, **I**), Rr2 (**B**, **F**, **J**), Am1 (**C**, **G**, **K**) and Am2 (**D**, **H**, **L**)

non-pathogenic strains by Rr1, Rr2, Am1 and Am2 confirm that they have good potential for antibiofilm applications in medical practice. Further studies are needed to identify the active biofilm-modulating substances.

Conclusions

Fourteen samples from four medicinal plants in different organic solvents were examined for antibacterial and antibiofilm activities. The samples generally lacked antibacterial activity but were characterised by a wide range of biofilm modulating activities. Four of the extracts suppressed biofilm growth in UPEC strains but stimulated it significantly in the ABU strain. Such effect is in accordance with the novel demands for biofilm suppression of pathogens without antibacterial activity so that resistance development can be avoided. The eventual selectivity of the preparations against sessile growth of pathogenic strains only and the nature of the active substances need further examination.

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