In vitro activity of heather [*Calluna vulgaris* (L.) Hull] extracts on selected urinary tract pathogens

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ABSTRACT

Calluna vulgaris L. Hull (Ericaceae) has been used for treatment of urinary tract infections in traditional medicine. In this study we analyzed *in vitro* antibacterial activity of the plant extracts on different strains of *Escherichia coli*, *Enterococcus faecalis* and *Proteus vulgaris*, as well as the concentrations of total phenols and flavonoids in the extracts. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. The concentrations of total phenols were examined by using Folin-Ciocalteu reagent and ranged between 67.55 to 142.46 mg GAE/g. The concentrations of flavonoids in extracts were determined using spectrophotometric method with aluminum chloride and the values ranged from 42.11 to 63.68 mg RUE/g. The aqueous extract of *C. vulgaris* showed a significant antibacterial activity. The values of MIC were in the range from 2.5 mg/ml to 20 mg/ml for this extract. *Proteus vulgaris* strains were found to be the most sensitive. The results obtained suggest that all tested extracts of *C. vulgaris* inhibit the growth of human pathogens, especially the aqueous extract.

KEY WORDS: heather, phenols, flavonoids, antibacterial activity, drug activity

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INTRODUCTION

Heather, (***Calluna vulgaris*** L. Hull, fam. Ericaceae) is an evergreen shrub. ***C. vulgaris*** can be found in most parts of Europe and Northern America from lowland up to alpine regions. Plant is traditionally used to treat urinary tract infection and inflammatory disorders [1].

C. vulgaris has been used in ethnopharmacology as an antiseptic, antibacterial, cholagogue, diuretic, expectorant, antirheumatic and anti-inflammatory agent [2]. Several studies revealed antioxidant [3-5], antitumor and anti-inflammatory effects of ***C. vulgaris*** extract [6]. Heather leaves represent a very promising source of triterpenoids [7]. Numerous triterpenoids, including ursolic and oleanolic acid, possess antitumor and anti-inflammatory properties [8]. A new property of ursolic acid has been described in an acetone-extract of heather which could help explain the anti-inflammatory characteristics of this plant [9]. Previous research showed the activity ***C. vulgaris*** in the treatment of inflammatory diseases and wounds in the Swedish traditional medicine [10]. A large number of plant species are used in Danish folk medicine for treatment of depression and anxiety. One of the three most active extracts was the aqueous extract of aerial parts of ***C. vulgaris*** for antidepressive treatment [11]. The content of quercetin in ***C. vulgaris*** might explain the reported nerve calming effect of the plant [12].

A review of relevant literature revealed little data on antibacterial activity of different extracts of ***C. vulgaris*** on the most frequent urinary tract pathogens. The aim of this study was to investigate ***in vitro*** antibacterial activity of aqueous, ethanol and ethyl acetate extract from leaves and flowers of this plant. The second aim was to determine a total phenol and flavonoid content in the extracts using spectrophotometric methods.

MATERIALS AND METHODS

Chemicals

Gallic acid, rutin hydrate and aluminium chloride hexahydrate ($AlCl_3$) were purchased from Acros Organics, New Jersey, USA. Organic solvents and sodium hydrogen

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carbonate were purchased from „Zorka pharma" Šabac, Serbia. Chlorogenic acid, Folin-Ciocalteu phenol reagent and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemicals Co, St Louis, MO, USA. Sodium carbonate (Na_2CO_3) was obtained from MP-Hemija, Belgrade, Serbia. Nutrient liquid medium, a Mueller-Hinton broth was purchased from Liofilchem, Italy. An antibiotic, amoxicillin, was purchased from Panfarma, Belgrade, Serbia.

Plant material

During summer of 2010, leaves and flowers of ***C. vulgaris*** were collected from natural populations on Borja Mountain in the region of Teslić municipality in Bosnia and Herzegovina. Plants identified were confirmed and voucher specimen was deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. The collected plant material was air dried in darkness at ambient temperature (20°C). The dried plant material was cut up and stored in paper bags until performing analysis.

Preparation of plant extracts

Prepared plant material (10g) was transferred to dark-colored flasks with 200 ml of solvent (water, ethanol, ethyl acetate) and stored at room temperature. After 24 h, infusions were filtered through Whatman No. 1 filter paper and residue was re-extracted with equal volume of solvents. After 48 hours, the process was repeated. Combined supernatants were evaporated to dryness under vacuum at 40 °C using Rotary evaporator. Aqueous extract of herb was prepared at 80°C. The obtained extracts were kept in sterile sample tubes and stored at –20 °C.

Determination of total phenolic content

The ***C. vulgaris*** extracts were analyzed by spectrophotometry for total phenolics according to Folin-Ciocalteu procedure [13]. The reaction mixture was prepared by mixing 0.2 ml of methanolic solution of extract (1 mg/ml) and 1.5 ml of 110 Folin-Ciocalteu reagent dissolved in water. The mixture was allowed to equilibrate for 5 min and then mixed with 1.5 ml 6% $NaCO_3$ solution. After incubation for 90 minutes at room temperature in darkness, the absorbance of the mixture was read at 725 nm against a blank using spectrophotometer. The blank was prepared with methanol instead of extract solution. The samples were prepared in triplicate and the mean value of absorbance was obtained. The same procedure was repeated for gallic acid, which was used for calibration of standard curve. Total phenol content is reported as gallic acid equivalents by reference to linear equation of the standard curve ($y = 0.008x + 0.0077$, $R^2 = 0.998$). Then the total phenolic content was expressed as gallic acid equivalents in miligrams per gram of extract (mg GAE/g of extract).

Determination of flavonoid concentration

The concentrations of flavonoids were determined using spectrophotometric method with aluminum chloride [14]. The sample contained 1 ml of methanolic solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The mixture was vigorously shaken, and after 10 minutes of incubation at room temperature, the absorbance ***versus*** a prepared blank was read at 430 nm using spectrophotometer. The samples were prepared in triplicate and the mean value of absorbance was obtained. Rutin was used as a standard for calibration of standard curve. The concentrations of flavonoids were calculated from the linear equation of standard curve ($y = 0.021x + 0.040$, $R^2 = 0.999$). Then the concentrations of flavonoids were expressed as miligram of rutin equivalent per gram of extract (mg of RUE/g of extract).

Tested bacterial strains

Antibacterial activity of aqueous, ethanol and ethyl acetate extract from ***C. vulgaris*** was tested against urinary pathogens, extracted from urine samples, including ten different ***Escherichia coli*** strains (Mf-Ec1, Mf-Ec2, Mf-Ec3, Mf-Ec4, Mf-Ec5, Mf-Ec6, Mf-Ec7, Mf-Ec8, Mf-Ec9, Mf-Ec10), ten ***Enterococcus faecalis*** strains (Mf-Ef1, Mf-Ef2, Mf-Ef3, Mf-Ef4, Mf-Ef5, Mf-Ef6, Mf-Ef7, Mf-Ef8, Mf-Ef9, Mf-Ef10) and ten different strains of ***Proteus vulgaris*** (Mf-Pv1, Mf-Pv2, Mf-Pv3, Mf-Pv4, Mf-Pv5, Mf-Pv6, Mf-Pv7, Mf-Pv8, Mf-Pv9, Mf-Pv10).

The Escherichia coli strains and ***Proteus vulgaris*** strains represented Gram-negative bacteria. Bacterial strains of ***Enterococcus faecalis*** were Gram-positive. These pathogens are the most frequent cause of urinary tract infections [15]. All clinical isolates were a generous gift from the Institute of Public Health, Banja Luka.

Suspension preparation

The original density of the bacterial suspension was 0.5 Mc Farland after which the additional dilution in saline at the proportion of 110 is made. The final concentration of the bacteria in the test tubes was 10^6 colony forming units (CFU)/ml.

Macrodilution method

The minimum inhibitory concentration (MIC) of the extracts had been determined using the tube dilution method through the series of dilutions [16]. In the test tubes filled with the Mueller Hinton broth, the solution of the extracts is added

and the series of double dilutes was made. In each of the test tubes 100µl of the suspension of the tested bacteria was added. The mixture was incubated for 24 hours at the temperature of 37 °C. The same method was used to identify the MIC value for amoxicillin. These values have been determined by inoculating the Mueller Hinton agar with the test tube content. Amoxicillin was used as a positive control. Whereas the extracts were dissolved in 10% DMSO, solvent control test was performed to study the effects of 10% DMSO on the growth of bacterial strains. It was observed that 10% DMSO did not inhibit the growth of bacteria.

Statistical analysis

Data are presented as means ± standard deviations where appropriate. All statistical analyses were performed using SPSS package (IBM Corp. Chicago, USA).

RESULTS

Total phenolic content and flavonoid concentrations

The results of total phenolic content and flavonoid concentrations in tested extracts from *C. vulgaris* are presented in Table 1. The total phenolic content was expressed as gallic acid equivalents. The aqueous extract had the highest phenolic content with 142.46 mg of GAE/g of extract. The ethanol extract was richer in phenolic active compounds than ethyl acetate extract. The content of flavonoids was expressed as rutin equivalent. Total flavonoid content in plant extracts ranged between 42.11 to 63.68 mg RUE/g of extract. High concentration of flavonoids was measured in ethyl acetate extract from *C. vulgaris*.

Antibacterial activity

In vitro antibacterial activities of aqueous, ethanol and ethyl acetate extracts of *C. vulgaris* leaves and flowers were studied on strains of Gram-positive and Gram-negative bacteria. The results of antibacterial activities of extracts against 30 strains of pathogenic bacteria are presented in Table 2. Antibacterial activity of tested extracts was evaluated by determining MICs and MBCs values. All tested extracts from *C. vulgaris* inhibited urinary pathogens extracted from urine samples.

TABLE 1. Total phenolic contents and concentrations of flavonoids in leaves and flowers of *C. vulgaris* extracts

Type of extract	Total phenolic content[1] (mg GAE/g of extract)	Flavonoid concentration[1] (mg RUE/g of extract)
Water	142.46±0.50	42.11±0.32
Ethanol	81.86±0.95	43.03±0.11
Ethyl acetate	67.55±0.38	63.68±0.19

[1]Values represent mean±standard deviation.

The strongest antibacterial activity was detected on *P. vulgaris* while the activities on *E. coli* and *E. faecalis* strains were similar. Aqueous extract showed the strongest effect against all tested bacterial strains. In general, the tested extracts demonstrated selective antibacterial activity and the activity depended both on the species of bacteria and on the type of extract. Ethyl acetate extract showed excellent activity on strains of *P. vulgaris*.

DISCUSSION

Previous research has indicated that phenolic compounds have antibacterial properties [17].

Phenolics belong to the group of secondary plant compounds and have various bioactivities such as antioxidat [18], anti-ulcer [19], and anti-inflammatory [20]. The interest in possible health benefits of flavonoids has increased due to their powerful antimicrobial activities [21].

Extract of *C. vulgaris* was found effective against *M. tuberculosis* bacillus [21] and the results of this study provide scientific basis for the traditional use of this plant in the treatment of tuberculosis [22].

Huttunen et al. [23] investigated antimicrobial activity of five Finnish honey products against important human pathogens *Streptococcus pneumoniae*, *S. pyogenes* and *Staphylococcus aureus*. In this research, heather (*C. vulgaris*) honey showed significant antimicrobial activity against all tested pathogens. Heather honey was tested against *Staphylococcus aureus* and *C. vulgaris* was shown it could be a source that could provide honey with high antibacterial activity [24].

Our previous studies have shown that species belonging to the family *Ericaceae* are rich in phenolics [25,26]. The antimicrobial activity of phenolic compounds from heather is also significant. Antibacterial effects of different extracts of *C. vulgaris* showed that phenolic compounds and flavonoids were responsible for the growth inhibition of bacterial strains.

This study indicated that extracts of *C. vulgaris* have antibacterial activity and may have potential medical use. Studies of medicinal plants are important sources of knowledge for development of less harmful and more effective antimicrobial agents for treatment of urinary infections.

CONCLUSIONS

The results of this research suggest that aqueous, ethanol and ethyl acetate extract from *C. vulgaris*, tested *in vitro*, show great potential as a natural antibacterial agents. Thus, they may be useful in the treatment of infectious diseases caused by urinary tract pathogens. Aqueous extract may have a significant effect on the prevention of the infections

TABLE 2. Antibacterial activities of aqueous, ethanol and ethyl acetate extracts from leaves and flowers of *C. vulgaris* against tested strains of bacteria.

Species	Aqueous extract		Ethanol extract		Ethyl acetate extract		Amoxicillin	
	MIC[1]	MBC[2]	MIC	MBC	MIC	MBC	MIC	MBC
E. coli Mf-Ec1	20	40	20	40	40	>40	1000	2000
E. coli Mf-Ec2	20	40	20	40	40	>40	4000	8000
E. coli Mf-Ec3	10	20	20	40	40	>40	4	8
E. coli Mf-Ec4	20	40	20	40	40	>40	2	4
E. coli Mf-Ec5	10	20	20	40	40	>40	2000	4000
E. coli Mf-Ec6	10	20	20	40	40	>40	2000	4000
E. coli Mf-Ec7	10	20	20	40	40	>40	4	4
E. coli Mf-Ec8	10	20	20	40	40	>40	1000	2000
E. coli Mf-Ec9	10	20	20	40	40	>40	4000	8000
E. coli Mf-Ec10	10	20	20	40	40	>40	1000	2000
E. faecalis Mf-Ef1	20	40	20	>40	40	>40	0.977	125
E. faecalis Mf-Ef2	10	20	20	>40	40	>40	0.488	>125
E. faecalis Mf-Ef3	10	20	20	>40	40	>40	0.488	>125
E. faecalis Mf-Ef4	10	20	20	>40	40	>40	0.488	>125
E. faecalis Mf-Ef5	20	40	20	>40	40	>40	0.488	>125
E. faecalis Mf-Ef6	20	40	20	>40	40	>40	0.488	>125
E. faecalis Mf-Ef7	10	20	20	>40	40	>40	0.977	>125
E. faecalis Mf-Ef8	10	20	20	>40	40	>40	0.488	125
E. faecalis Mf-Ef9	10	20	20	>40	40	>40	0.488	>125
E. faecalis Mf-Ef10	10	20	20	>40	40	>40	0.488	>125
P. vulgaris Mf-Ef1	2.5	5	10	40	5	10	>4000	>4000
P. vulgaris Mf-Ef2	2.5	5	10	40	5	20	>4000	>4000
P. vulgaris Mf-Ef3	2.5	5	10	40	5	10	>4000	>4000
P. vulgaris Mf-Ef4	2.5	5	10	40	5	10	0.977	7.812
P. vulgaris Mf-Ef5	2.5	5	10	40	5	20	>4000	>4000
P. vulgaris Mf-Ef6	2.5	5	10	40	5	20	0.977	7.812
P. vulgaris Mf-Ef7	2.5	5	10	40	5	10	>4000	>4000
P. vulgaris Mf-Ef8	2.5	5	10	40	5	20	2000	>4000
P. vulgaris Mf-Ef9	2.5	5	10	40	5	20	>4000	>4000
P. vulgaris Mf-Ef10	2.5	5	10	40	5	20	>4000	>4000

[1]Minimum inhibitory concentration (MIC) and [2]minimum bactericidal concentration (MBC) values are given as mg/ml for plant extracts and μg/ml for antibiotic.

of urinary tract. The levels of the total phenolic content indicated that *C. vulgaris* extracts are rich source of the phenolic compounds.

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DECLARATION OF INTEREST

The authors declare that they have no competing interests.

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