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In vitro EFFICACY OF EXTRACTS OF *Arctostaphylos uva-ursi* L. ON CLINICAL ISOLATED *Escherichia coli* AND *Enterococcus faecalis* STRAINS

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ABSTRACT. Antibacterial activity, total phenolic content and flavonoid concentrations of extracts from the leaves of *Arctostaphylos uva-ursi* L. were performed. Tested bacterial strains are 20 clinical isolates, including different strains of *Escherichia coli* and *Enterococcus faecalis*. The total phenolic content was determined using Folin-Ciocalteu reagent. The concentration of flavonoids in extracts were determined using spectrophotometric method with aluminium chloride.

The value of minimum inhibitory concentration (MIC) was in the range from 0.625 mg/mL to 10 mg/mL. The most active plant extracts were aqueous and ethanol extracts. Ethanol extract had the highest amount of the total phenolic content (300.23 mg GA/g) and concentration of flavonoids (73.46 mg RU/g).

Keywords: *Arctostaphylos uva-ursi*, bearberry, antibacterial activity, phenols, flavonoids.

INTRODUCTION

The use of plant extracts with antibacterial property can be of large significance in the treatment of many infections. In recent years a number of papers on this topic is increasing (ELUMALAI *et al.*, 2011; JOHNSON *et al.*, 2011; KHAN *et al.*, 2011).

Bearberry (*Arctostaphylos uva-ursi* L., fam. Ericaceae) leaves and preparations made from them are used in traditional and modern medicine for urinary tract infections (SARIĆ, 1989).

Bearberry is an evergreen shrub. The leaves are oval, up to 3 cm long, leathery. The flowers are collected in small clusters. The fruit is a red berry. This plant grows in dry and rocky habitats at high mountains up to 2400 meters above sea level (SARIĆ, 1989).

Bearberry contains many active compounds such as arbutin, methylarbutin, tannins, ursolic acid, gallic acid, syringic acid, as well as some flavonoids (PEGG *et al*., 2008). Medicinal substances from leaves of this plant exhibit strong antibacterial (SCHINDLER *et al*., 2002), anti-inflammatory (SHANMUGAM *et al*., 2008), antioxidant (AMAROWICZ *et al*., 1999) and diuretic (BEAUX *et al*., 1999) effects.

Relevant literature review has shown a little data about the biological activity of different extracts of *A. uva-ursi*, originated from Bosnia and Herzegovina. The aim of our study was to investigate the antibacterial effect of aqueous, ethanol and ethyl acetate extracts of bearberry which is growing on Vranica mountain, on 20 selected urinary tract pathogens. In addition, a total phenol and flavonoid content in the extracts were studied.

MATERIAL AND METHODS

Plant material

Leaves of *A. uva-ursi* were collected from natural populations on Vranica mountain in central Bosnia and Herzegovina, in autumn of 2010. The plant material was dried at room temperature.

Chemicals

Gallic acid, rutin hydrate and aluminium chloride hexahydrate ($AlCl_3$) were purchased from Acros Organics, New Jersey, USA. Chlorogenic acid and Folin-Ciocalteu phenol reagent (Sigma Chemicals Co, St Louis, MO, USA), nutrient liquid medium, a Mueller-Hinton broth (Liofilchem, Italy) and antibiotic amoxicillin (Panfarma, Belgrade, Serbia).

Preparation of plant extracts

Prepared plant material (10 g) was placed in bottles with 200 ml of solvent (water, ethanol, ethyl acetate) and saved at room temperature. After 24 h, infusions were filtered through filter paper and extracted again with same amount of solvents. After 48 h, 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 contents in the plant extracts

The total phenolic content was determined using by the Folin-Ciocalteu procedure (WOOTTON-BEARD *et al*., 2011). The reaction mixture was prepared by mixing 0.2 ml of methanolic solution of extract (1 mg/mL) and 1.5 ml of 1:10 Folin-Ciocalteu reagent dissolved in water. The mixture was allowed to equilibrate for 5 min and than mixed with 1.5 ml 6% $NaCO_3$ solution. After incubation for 90 min 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 GA/g of extract).

Determination of flavonoid concentrations in the plant extracts

The concentrations of flavonoids was determined using spectrophotometric method with aluminium chloride (QUETTIER-DELEU *et al*., 2000). 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 strongly shaken, and after 10 min 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 RU/g of extract).

In vitro antibacterial assay

Bacterial strains

Twenty bacterial strains were used to evaluate the antibacterial properties of plant extracts, including ten Gram-positive (*Enterococcus faecalis*) and ten Gram-negative bacteria strains (*Escherichia coli*). All clinical isolates were 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 1:10 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 of the extracts had been determined by the tube dilution method through the series of dilutions (NCCLS, 1997). In the test tubes filled with the Mueller Hinton broth, the solution of the extracts is added and the series of double dilutes have been made. In each of the test tubes the 100 μL of the suspension of the tested bacteria was added. The 24 hours incubation at the temperature of 37 ºC was conducted. MIC has shown to be the lowest concentration of the extract which inhibits the growth of the bacteria. The minimum bactericidal concentration (MBC) is the lowest concentration of the tested substance which has the bactericidal effect. These values have been collected by inoculation the Mueller Hinton agar with the test tube content. It was the content from the test tubes in which the MIC has been found and all the test tubes more than MIC were found. Amoxicillin was used as a positive control. The same method was used to identify the value of the MIC for the amoxicillin. 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

The calculate the mean and standard deviation values was performed using the SPSS package.

RESULTS AND DISCUSSION

Total phenolic content and flavonoid concentrations

The phytochemical analysis of different extracts of *A. uva-ursi* showed total phenolic content and concentration of flavonoids (Table 1). The ethanol extract had the highest phenolic content with 300.23 mg of GA/g, while similar content was measured for the aqueous extract with 250.48 mg of GA/g of extract. Quantities of flavonoids identified in the tested extracts are presented in Table 1. Total flavonoid content was obtained as the highest in the ethanol extract (73.46 mg RU/g).

The antibacterial activity of the extracts showed that the phenolic compounds and flavonoids were responsible for the growth inhibition of bacterial strains.

A. uva-ursi contains many active compounds such as arbutin (5-15%) and methylarbutin (up to 4%) (LEIFERTOVA *et al*.,1974). DOMBROWICZ *et al*. (1991) reported that *A. uva-ursi* contains triterpenes and phenolic acids, gallic acid, ursolic acid, malic acid, quinic acid.

Antibacterial activity of plant extracts

Effect of different extracts from leaves of bearberry on ten *Enterococcus faecalis* and *Escherichia coli* strains were tested, by macrodilution method. Antibacterial activity of tested extracts was evaluated by determining MICs and MBCs values. The results of *in vitro* activities of extracts on strains of Gram-positive and Gram-negative pathogenic bacteria are presented in Table 2. The aqueous extract showed the most of the antibacterial effect than other tested extracts. The ethanol and ethyl acetate extracts were showed a result similar to that of aqueous extract, against strains of *Enterococcus faecalis*. Extracts were showed a stronger antibacterial activity against Gram-positive strains, in generally.

The antibacterial effect of plant extracts of leaves of *A. uva ursi* on different bacteria was studied by a very large number of researches. MOSKALENKO (1986) determined that this plant has strong bacteriostatic activity on *Bacillus subtilis, Escherichia coli, Helicobacter pylori, Shigella sonnei* and *Shigella flexneri*. The inhibitory effects of bearberry extracts on *Arcobacter butzleri*, *A. cryaerophilus* and *A. skirrowii* were reported by CERVENKA *et al*. (2006).

Antibacterial effect is caused by the presence of hydroquinone, originated from arbutin. Analysis of the arbutin metabolites, from leaves of bearberry, was investigated by QUINTUS *et al.* (2005). Antibacterial effect of arbutin examined on *Pseudomonas aeruginosa*, *Ureaplasma urealyticum* and *Mycoplasma hominis* (ROBERTSON and HOWARD, 1987) and also on *Listeria monocytogenes* (PARK, 1994). These studies have confirmed the claim of a strong antibacterial activity of arbutin.

CONCLUSIONS

This study presents the antibacterial activity and phytochemical analysis of different extracts of leaves *A. uva-ursi*. We found that all tested extracts of bearberry leaves have a good antibacterial activity on all tested bacterial strains causing urinary tract infections. Aqueous extract has shown a strong antibacterial activity; the ethanol extract also. The content of total phenols indicate that ethanol and aqueous extracts have very high level of these ingredients.

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Table 1. Phytochemical screening on the aqueous, ethanol and ethyl acetate extracts of leaves of *A. uva ursi*

Type of extract	**Total phenolic content[1] (mg GA/g of extract)**	**Flavonoid concentration[1] (mg RU/g of extract)**
Aqueous	250.48 ± 0.00	51.25 ± 0.11
Ethanol	300.23 ± 0.00	73.46 ± 0.27
Ethyl acetate	49.40 ± 0.06	12.85 ± 0.05

[1]All the data are mean values of three replicates of each samples ± standard deviation.

Table 2. Antibacterial activities of aqueous, ethanol and ethyl acetate extracts from leaves of *A. uva ursi* against tested strains of bacteria based on macrodilution method

	Aqueous extract		**Ethanol extract**		**Ethyl acetate extract**		**Amoxicillin**	
Species	**MIC[1]**	**MBC[2]**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
E. faecalis MFBL-Ef1	1.25	5	1.25	2.5	1.25	10	0.488	125
E. faecalis MFBL-Ef2	2.5	2.5	2.5	5	2.5	20	0.488	125
E. faecalis MFBL-Ef3	1.25	5	2.5	5	2.5	20	0.977	> 125
E. faecalis MFBL-Ef4	1.25	2.5	2.5	5	2.5	10	0.488	125
E. faecalis MFBL-Ef5	2.5	5	2.5	5	2.5	10	0.977	> 125
E. faecalis MFBL-Ef6	1.25	5	1.25	2.5	1.25	5	0.977	> 125
E. faecalis MFBL-Ef7	2.5	5	2.5	10	2.5	20	0.977	> 125
E. faecalis MFBL-Ef8	1.25	5	2.5	5	2.5	20	0.244	125
E. faecalis MFBL-Ef9	1.25	5	2.5	5	1.25	10	0.244	125
E. faecalis MFBL-Ef10	1.25	5	2.5	5	2.5	20	0.977	> 125
E. coli MFBL-Ec1	0.625	0.625	5	5	10	10	2000	4000
E. coli MFBL-Ec2	1.25	1.25	5	10	10	20	2000	4000
E. coli MFBL-Ec3	2.5	2.5	10	>40	10	20	2000	4000
E. coli MFBL-Ec4	2.5	2.5	10	>40	10	20	2000	4000
E. coli MFBL-Ec5	2.5	2.5	10	20	10	20	3.906	7.813
E. coli MFBL-Ec6	1.25	1.25	10	20	10	20	5000	> 4000
E. coli MFBL-Ec7	5	5	10	10	10	20	4000	> 4000
E. coli MFBL-Ec8	1.25	1.25	10	>40	10	10	3.906	7.813
E. coli MFBL-Ec9	5	5	10	20	10	20	4000	> 4000
E. coli MFBL-Ec10	5	5	5	5	10	20	3.906	7.813

[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.