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DETERMINATION OF ISOCYTISOSIDE AND ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACT FROM *AQUILEGIA VULGARIS* L.

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Abstract: The leaves and stems of *Aquilegia vulgaris* L. yielded an ethanolic extract in which the content of isocytisoside, its main component, was determined by authors' method. The method consisted in chromatographic separation of the extract, elution of the isocytisoside spot and measurement of its absorbance using a UV/VIS spectrophotometer. The isocytisoside content was 3.00%. Antimicrobial activity of the ethanolic extract was also determined.

Keywords: *Aquilegia vulgaris* L., ethanolic extract, quantitative determination of isocytisoside, antimicrobial activity.

Aquilegia vulgaris L. has been used in folk medicine against liver disorders, especially in the therapy of jaundice, and also in the treatment of scurvy, dermatitis and as a diaphoretic agent (1). The herb is a component of the immunostimulating preparation Padma 28. It is also used as a cure for neurosis.

The studies carried out in the Department of Pharmacognosy on compounds present in columbine have included the isolation and identification of flavonoids and analysis of phenolic acids. It has been shown that the leaves and stems contain 4'–methoxyapigenin 6–C–glucoside (isocytisoside) as the predominant constituent, and also flavone C– and O–glycosides, whereas kaempferol 3,7–diglucoside and kaempferol 3,7,4'–triglucoside, are the main components of the flowers accompanied by other kaempferol derivatives and flavone: isovitexin (2, 3, 4). Furthermore, the presence of phenolic acids, namely caffeic, ferulic, p–coumaric, protocatechuic, vanillic, α–resorcylic, synapic, chlorogenic and p–hydroxybenzoic acids, has been confirmed in the leaves and flowers (5, 6, 7). Tannins and cyanogen glycoside were also reported for this species (8).

Flavonoids and phenolic acids are compounds of multidirectional activity and are known for their antimicrobial properties (9, 10, 11, 12, 13). The antimicrobial activity has been tested both on the extracts contained flavonoids and on pure flavonoids. The conclusions concerning relationship between their structure and activity have been limited due to insufficient systematic investigations. Waage et al. (9) provided some data on the activity of seven flavonoid glycosides against Gram–negative bacteria *Pseudomonas maltophilla* and *Enterobacter cloaceae*. His studies revealed that the highest activity had quercetin 3–O–rhamnoside, whereas the remaining 3–O– and 7–O–glucosides were weaker inhibitors, and quercetin 3'–O–glucoside had no activity. Mori et al. (10) examined 28 compounds from various chemical groups, i.e. flavones, flavonols, flavanones, flavanonols and catechins, for their activity against *Proteus vulgaris* and *Staphylococcus aureus*. The MIC was 50–200 μg/ml for six flavonoids active against *Proteus vulgaris* and five compounds against *Staphylococcus aureus*. The authors concluded that the presence of hydroxyl groups at the rings A and B was essential for the activity. Particularly active were compounds with hydroxyls at C–3', 4' and 5'. Flavonoids lacking the C–2,3 double bond were ineffective. Flavonoids exert their antimicrobial action by inhibiting DNA and RNA synthesis. Lipophilic flavonoids have increased potency against Gram–positive bacteria. Flavonoids tested by Basile et al. (11), namely apigenin, vitexin, saponarin, lucenin, apigenin and luteolin 2,7–O–di– and triglucosides, as well as biflavonoid compound, were more effective against Gram–negative bacteria.

The aim of the present study was the preparation of an ethanolic extract from leaves and stems of *Aquilegia vulgaris* L. (**AvEE**), elaboration of the method for its standardization for isocytisoside content, and evaluation of the antimicrobial activity of the extract.

EXPERIMENTAL

1. Plant material

The leaves and stems of *A. vulgaris* L. were collected in May/June 1998 at the Botanical Gar-

den of the A. Mickiewicz University, Poznań. Voucher specimens are deposited at the Department of Pharmacognosy of K. Marcinkowski University of Medical Sciences, Poznań.

2. Preparation of the ethanolic extract (AvEE)

The air dried and coarsely powdered leaves and stems (200 g) were extracted twice with refluxing 70% ethanol. The extraction took six hours in each case. The combined extracts were concentrated to about 150 ml and treated with 150 ml hot water. The insoluble part was filtered off and the filtrate was condensed to yield 55 g of a homogenous brown mass (**AvEE**).

3. Quantitative determination of isocytisoside in the AvEE

The determination was done using our method elaborated for the assay of the isocytisoside content in the leaves and stems of various species of the *Aquilegia* genus (Bylka, in press).

Preparation of the calibration curve

The calibration curve was established using isocytisoside of mp 219–220°C, that was obtained by a previously described method (2). The purity of the compound was checked by thin–layer chromatography.

Isocytisoside (0.0100 g) was dissolved in methanol p.a. and diluted to 25 ml with this solvent. The isocytisoside solutions in volumes of 0.05, 0.1, 0.15, 0.20 and 0.25 ml, that equalled to 0.02, 0.04, 0.06, 0.08 and 0.1 mg of isocytisoside, were applied onto plates (DC–Fertigplatten Kieselgel 60, Schichtdicke 0.25 mm, Merck, Art. 5721). The plates were run in *n*–butanol – glacial acetic acid – water 4:1:2 to 18 cm and, after drying at room temperature for 12 hours, were viewed under $UV_{366\,nm}$ light. The spot corresponding to isocytisoside (Rf=0.63) was scraped off the plate with a tolerance of 1 cm and transferred to a centrifuge tube.

The silica gel samples were extracted with 4 ml of methanol during 15 min. with occasional stirring, and the silica was centrifuged at 3000 rpm. The absorbance was measured at 274 nm in the presence of a reference solution in 1 cm quartz cuvettes using a UV–VIS spectrophotometer.

The calibration graph had straight–line course at concentration from 5 to 20 $\mu g \cdot cm^{-3}$. The correlation coefficient for the regression line was r=0.99974 as well as advantageous values of regression equation $[(y=a \cdot x \pm b) y = 0.005503 \cdot x \pm 0.001]$ were found.

Determination of isocytisoside in the extract

The **AvEE** (0.2000 g) was dissolved in 10 ml of 70% methanol in a volumetric flask and diluted to 25 ml with methanol p.a.. The extract (0.1 ml) was applied onto plates.

Further procedure was the same as for the calibration curve. Spectrophotometric measurements were made as for the calculation of analytical curve.

The results of statistical analysis of six measurements are given below: mean $\bar{x}$=3.00%; standard deviation s=0.125; average deviation $s_{\bar{x}}$ =0.051; variation coefficient VC=4.16%; confidence interval for 95% probability μ=3.00±0.131.

4. Microbiological investigations

The following reference strains were used in the tests:

Staphylococcus aureus ATCC 6538; *Micrococcus luteus* ATCC 9341; *Escherichia coli* NCTC 8196; *Proteus mirabilis* NCTC 6635; *Pseudomonas aeruginosa* ATCC 27853; *Bacillus subtilis* ATCC 6633; *Candida albicans* ATCC 10231

Culture media:

Mueller–Hinton medium for bacteria, Sabouraud medium for fungi. 24–hour cultures of the reference strains were diluted according to the McFarland scale 0.5 in a sterile solution of normal saline.

Determination of MIC and MBC

A suspension of the reference strain was added to each concentration of the AvEE extract (from 0.312 mg/ml to 10 mg/ml) diluted with the liquid medium and the mixture was incubated at 35–37°C (bacteria) or 25°C (fungi) for 18–24 hours, then the medium was examined. A clear medium indicated that the microorganism did not grow, a turbid one confirmed its growth. The MIC (minimal inhibitory concentration) was the lowest concentration of a substance at which the microorganism multiplication was not observed.

Next 0.1 ml aliquots of each dilution were transferred with a pipette on Petri plates containing freshly prepared culture media. The plates were inspected after incubation as above. The concentration at which the microorganism did not show any growth was designated as the MBC (minimal bactericidal concentration).

The MIC and MBC values were shown in the Table 1.

RESULTS AND DISCUSSION

The leaves and stems of *A. vulgaris* L. were extracted twice with boiling 70% ethanol. The

extracts were combined, concentrated and treated with hot water. The precipitate was filtered off and the filtrate was concentrated to a syrupy mass (**AvEE**).

The concentration of isocytisoside, a principal flavonoid component, was determined in the extract. The assay was done by our method consisted of chromatographic separation of the extract and measurement of the absorbance of isocytisoside, eluted from the spot, using a UV VIS spectrophotometer (Bylka, in press). The results were analysed statistically. The concentration of isocytisoside in the **AvEE**, determined by this method, was 3.00%.

Table 1. Antimicrobial activity of extract from leaves and stems of *Aquilegia vulgaris L.* (**AvEE**).

Bacteria	MIC mg/ml	MBC mg/ml
S. aureus	> 5	> 10
M. luteus	0.625	5
E. coli	> 10	> 10
P. mirabilis	5	10
Ps. aeruginosa	10	> 10
B. subtilis	7.5	7.5
C. albicans	7.5	10

Explanation:
MIC – minimal inhibitory concentration
MBC – minimal bactericidal concentration

The **AvEE** was tested for antibacterial activity using strains of Gram–positive bacteria: *Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Candida albicans* and Gram–negative bacteria, namely *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa.*

Minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations were determined. The antimicrobial activity was determined by broth dilution test according to NCCLS (14).

The AvEE extract showed bacteriostatic activity against all strains, except *E. coli.* The MIC for *Ps. aeruginosa* was 10 mg/ml, for *C. albicans* and *B. subtilis* – 7.5 mg/ml, for *S. aureus* – 5 mg/ml, and, for the most sensitive strain, *M. luteus* – 0.625 mg/ml.

The bactericidal activity (MBC) ranged from 5 mg/ml for *M. luteus* and 7.5 mg/ml for *B. subtilis* to 10 mg/ml for *C. albicans* and *P. mirabilis.* At the concentrations used the extract did not show bactericidal activity against *E. coli, S. aureus* and *Ps. aeruginosa.*

Although some range of biological activities, like the hepatoprotective effect described in our recent paper (15), have been established for *Aquilegia vulgaris* L., there have been no reports concerning antimicrobial activity of this species.

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