Flavonoid Composition of *Juniperus oblonga Bieb*

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Juniperus oblonga Bieb is widely spread in the Caucasus Mountains, particularly in its eastern and southern regions. Diuretic effect of juniper berries is determined by the presence of volatile oils and polyphenol complex, particularly flavonoids. Flavonoids were extracted from raw material with 70% ethanol and then with ethyl acetate. Column chromatography of ethyl acetate fraction on polyamide yielded 5 compounds, which were identified on the basis of physicochemical constants of parent compounds and products of acid hydrolysis and alkaline degradation of aglycones and on the basis of UV-spectroscopy as apigenin, isoquercitrin, apigenin-7-glucoside, quercetin-3-rutinoside, and scutellarin-7-glucoside. Quantitative composition of flavonoid in equivalent to rutin concentration in *Juniperus oblonga Bieb* was 0.910±0.007% (UV-spectrophotometry data).

Key Words: *juniper; flavonoids; glycosides; aglycones; UV-spectrophotometry*

Juniperus oblonga Bieb. (*J. communis var. oblonga Bieb.*), small tree or gross bush 3-5 m high, with long needlelike leaves (16-20 mm long, three in whorl) with white stomatal line, galberries are black, spherical or elliptical in shape, with glaucousness. This species is widely spread in the Caucasus Mountains, particularly in eastern and southern regions [1].

Currently, long-needle juniper is not used in medical practice because its chemical and pharmacological properties are poorly studied. However, it is used in traditional medicine together with common juniper. This prompted us to perform a detailed chemical analysis of long-needle juniper to prove the possibility of its use in scientific medicine along with officinal form.

Numerous studies confirmed that diuretic effect of juniper berries is associated with the presence of volatile oils and a polyphenol complex, particularly flavonoids [4]. Composition of relative oil from long-needle juniper fruits was established previously [2], therefore the objective of this study is to evaluate flavonoid composition of its galberries.

Increasing interest to flavonoids is their antioxidant activity and wide spectrum of biological activities. Immunostimulatory, antitumor, cardioprotective, hepatoprotective and gerontoprotective, antiplatelet, antiallergic and antiviral effects of flavonoids are currently known [3].

MATERIALS AND METHODS

For isolation of flavonoids, air-dried long-needle juniper galberries (500 g) were separated from lipophilic substances with chloroform in a Soxhlet apparatus. Dried raw material was extracted with 70% ethanol, evaporated in rotating evaporator IR-1M3 to thickening, diluted with 4× volume of water, and left in a refrigerator for 24 h. Lade-down residuum was filtered. The filtrate was transferred into a separating funnel and washed with several additional portions of chloroform. After chloroform treatment, the stock solution was extracted with ethyl acetate. Ethyl acetate extract was dried using anhydrous sodium sulfate and evaporated on a rotatory evaporator.

Then, ethyl acetate extract was fractionated on the column with polyamide gel. Eluate composition was controlled by its staining in cyanidine probe and by paper chromatography in butyl alcohol-concentrated acetic acid-water (4:1:5; System I) and acetic acid 15% (System II). Substances F_I-F_V were thus isolated

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TABLE 1. Qualitative Reactions of Flavonoids on Paper Chromatograms

Flavonoids	Spot color after staining with reagents							Detected
	UV	UV+NH_3	$AlCl_3$, 5% alcohol solution in UV light	sodium carbonate, 5% water solution	Wilson reagent	Diazo reagent	Benedict reagent	
F_I	Deep-brown	Brown	Yellow	Yellow	+	+	—	Flavone
F_{II}	Dark-yellow	Canary-yellow	Canary-yellow	Yellow	+	+	+	Flavonol
F_{III}	Green-yellow	Green	Yellow	Yellow	+	—	—	Flavone
F_{IV}	Dark-yellow	Canary-yellow	Yellow	Yellow	+	+	+	Flavonol
F_V	Dark	—	Pale-yellow	Yellow	+	—	—	Flavone

and identified as flavonoids on the basis of qualitative tests and chromatography results (Table 1).

Analysis of chromatograms using chromogenic agents revealed certain features in the structure of these compounds:

1) all compounds appeared to be flavonoids, what was demonstrated by positive reaction with aluminum chloride, sodium carbonate, zirconium (III) oxychloride (Horhammer-Hansel agent) and ammonia (except F_V);

2) compounds F_{II} and F_{IV} demonstrating positive Horhammer reaction, lost jaundice fluorescence after treatment with 5% citric acid, which indicated glycosidation in position 3;

3) all compounds included free OH-group at C_5, which was demonstrated by positive reaction with Wilson reagent;

4) compounds F_I, F_{II}, F_{IV}, and F_V demonstrated positive reaction with diazo reagent (orange staining), which is characteristic of free OH-group in C_7;

5) negative reaction of compounds F_I, F_{III}, and F_V with Benedict reagent attested to the absence of ortho-dioxy-groups in ring B.

Identification of the purified compounds F_I-F_V was performed on the basis of physicochemical constants for parent compounds and products of acid hydrolysis and alkaline degradation of aglycones and on the basis of UV-spectroscopy (Table 2).

For identification of glycosides, acid hydrolysis was performed under mild conditions. For this purpose, 10 ml 15% acetic acid or 10% sulfuric acid were added to the studied compounds and heated on boiling water bath, the hydrolysate was than cooled, transferred to the separation funnel, and the reaction product was extracted with diethyl ether. Residual hydrolysis fluid was neutralized with barium carbonate and used to detect sugar content. To this end, the residue was diluted with 70% ethanol and analyzed by chromatography in System I with sugar "testificators". Sugar component was detected using aniline-phthalate reagent with subsequent drying of the chromatogram in a drying chamber at 105°C.

Alkaline hydrolysis of aglycones was carried out in 20% potassium hydroxide water solution in boiled water bath for 1 h. The hydrolyzate was cooled, adjusted to pH 4-5 with 20% sulfuric acid; hydrolysis

TABLE 2. UV-Spectra of Extracted Flavonoids

Compound	C_2H_5OH λ_{max}, nm	CH_3COONa λ_{max}, nm	$CH_3COONa+H_3BO_3$ λ_{max}, nm	C_2H_5ONa λ_{max}, nm	$AlCl_3$ λ_{max}, nm	$AlCl_3$+HCl λ_{max}, nm
F_I	336, 268, 294_{melt}	376, 274, 301	338, 268, 302_{melt}	392, 275, 324	384, 348, 276, 301	381, 340, 299, 276
F_{II}	356, 258	361, 272	375, 262	387, 275	430, 276, 333	401, 272, 353
F_{III}	336, 268	—	338, 268	392, 275	384, 276	381, 276
F_{IV}	359, 259, 266_{melt}, 299_{melt}	393, 271, 325	387, 262, 298	410, 272, 327	433, 275, 303_{melt}	402, 271, 300, 364_{melt}
F_V	335, 285	—	344, 289	384, 334, 308	375, 304	361, 302
F_{VI}	350, 256	372, 272	367, 260	393, 270	430, 333, 276	401, 353, 272

products were extracted with diethyl ether. Ether extract was evaporated to dry, diluted with 96% ethanol, and chromatographically separated in System I. Chromatograms were stained with sulfanilic diazotized acid and bromphenol blue.

Final conclusion on the identity of the extracted compound was made by the absence of melting point depression in the mixture of the analyzed component with the "witness".

Compound F_I, yellow crystals yielding positive results in Briant cyanidine probe, which attests the aglycone nature of the compound, t_{melt} 350-353°C (ethanol), R_f 0.91 (System I), λ_{max} 268, 294_{melt}, 336 nm, alkaline hydrolysis yields phloroglucinol and p-hydroxybenzoic acid. On the basis of these results, the compound was identified as apigenin (5,7,4'-trihydroxyflavon).

Compound F_{II}, yellow crystals, t_{melt} 220-222°C (ethanol), positive cyanidine reaction, does not pass into octyl alcohol (*i.e.* is a glycoside), R_f 0.6 (System I), 0.38 (System II), test with zirconium (III) oxychloride in the presence of citric acid is negative, which attests to hydroxyl group substitution in position C_3; λ_{max} 258, 356 nm, addition of diagnostic reagents (sodium acetate and sodium ethylate) leads to bathochromic shift of both absorption bands, which indicates the presence of free oxygroups at C_5, C_7, C'_3, C'_7. Acid hydrolysis (15% CH_3COOH, 2 h) yields quercetin and D-glucose. Phloroglucinol and protocathechuic acid were detected after alkaline hydrolysis. These findings together with the absence of t_{melt} depression for mixed probes with authentic sample suggest that the studied compound is isoquercitrin (quercetin-3-glycoside).

Compound F_{III}, yellow crystals, t_{melt} 178-180°C (ethanol), positive cyanidine probe, R_f 0.65 (System I), 0.25 (System II), λ_{max} 268, 336 nm, UV-spectrum data with diagnostic supplements indicated that free hydroxyl groups are located in positions C_5 and C'_4. No bathochromic shifts under the influence of sodium acetate were observed, which indicates the absence of free-OH-group at C_7. Aglycone apigenin and D-glucose were detected after acid hydrolysis (15% CH_3COOH, 4 h). Thus, compound F_{III} was identified as apigenin-7-glycoside.

Compound F_{IV}, light-yellow crystals, t_{melt} 195-198°C (ethanol), positive cyanidine probe, R_f -0.45 (System I), 0.51 (System II), λ_{max} 259, 266_{melt}, 299_{melt}, 359 nm. In the presence of diagnostic additives, a bathochromic shift of absorption band I (sodium acetate +25 nm, boric acid and sodium acetate anhydrous +28 nm, aluminum chloride +45 nm) is observed, which is characteristic of free OH-groups at C_7, C_5, C'_3, C'_4. The bathochromic shift decreased (+30 nm) after addition of aluminum chloride with hydrochloric acid, which is characteristic of free hydroxyl group at C_5 and substitution at C_3. Aglycone quercetin and disaccharide rutinosa were obtained after hydrolysis under mild conditions (15% CH_3COOH, 30 min). The test of mixture of F_{IV} with the authentic sample did not cause depression of t_{melt}, therefore the compound was identified as quercetin-3-rutinoside (rutin).

Compound F_V, yellow crystals, t_{melt} 233-235 °C (ethanol), positive cyanidine probe and reaction with strontium chloride, which indicates the presence of ortho-dioxy-group in positions 5 and 6. R_f -0.47 (System I), 0.2 (System II), λ_{max} 285, 339 nm. UV-spectrum with diagnostic additives showed the absence of free OH-group in position 7 (no bathochromic shift with sodium acetate) and the presence of free hydroxyl groups in positions 5 and 4'. Hydrolysis (10% H_2SO_4, 3 h) yields aglycone scutellarin and glucose. Thus, the compound was identified as scutellarin-7-glucoside.

Quantitative evaluation of flavonoid composition in the raw material was based on spectrophotometric analysis of direct extract from raw material based on

TABLE 3. Results of Quantitative Analysis of Flavonoids in *Juniperus oblonga Bieb* Fruits

X (%)	$\bar{X}$-X_i	$(\bar{X}-X_i)^2$	Metrological characteristics
0.912	-0.002	0.000004	$\bar{X}$=0.91
0.915	-0.005	0.000025	$\sum(\bar{X}-X)^2 = 0.000214$
0.91	—	—	$S_x = \sqrt{\frac{\sum(\bar{X}-X_i)^2}{n(n-1)}} = 0.00267$
0.917	-0.007	0.000049	
0.9	0.01	0.0001	$\Delta X = S_x \cdot t_x = 0.007$
0.916	-0.006	0.000036	ε=2.5%
$\bar{X}$=0.91		$\sum(\bar{X}-X)^2 = 0.000214$	

chemical interaction of flavonoids with anhydrous aluminum chloride in the medium of hydrochloric acid diluted with formation of chelate complexes.

Maximum absorbance in UV band for alcohol extract from the fruits after addition of $AlCl_3$+HCl supplementation was at 405-412 nm.

Optimal conditions for flavonoid extraction from raw material were chosen. It was found that 70% ethanol is the best solvent for flavonoids at a 1:100 raw material–solvent ratio, optimal time for extraction 30 min, and the number of extractions 5. The spectra were recorded using spectrophotometer SF-46 in a 10-mm cuvette (in absolute ethanol).

Flavonoid content was calculated as the content of rutin as a component of the mixture (Table 3).

The analysis showed that flavonoid composition of *Juniperus oblonga Bieb* includes at least 5 compounds: apigenin, isoquercitrin, apigenin-7-glucoside, rutin, scutellarin-7-glucoside. Quantitative composition of flavonoid in equivalent to rutin was 0.910±0.007%.

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