COMPOSITION OF BIOLOGICALLY ACTIVE SUBSTANCES ISOLATED FROM THE FRUITS OF RUSSIAN OLIVE (*ELAEAGNUS ANGUSTIFOLIA*) INTRODUCED IN THE EUROPEAN PART OF RUSSIA

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A phytochemical study of the fruits of *Elaeagnus angustifolia* L. occurring in the Central Nonchernozem region of the Russian Federation has been performed. Qualitative reactions and quantitative instrumental methods of analysis proved the presence of polysaccharides, flavonoids, coumarins, phenolcarboxylic acids, amino acids, saponins, carotenoids, vitamins, and tannins. The quantitative content of the biologically active compounds has been estimated. It is shown that the fruits of *Elaeagnus angustifolia* occurring in Russia are a promising source of compounds presenting considerable interest for the pharmacy and food industry.

Representatives of the genus *Elaeagnus* L. are studied in many countries in order to expand the raw material base for food, agricultural, and pharmaceutical products. In Russia, *E. angustifolia* L. (Russian olive) is most well known as a medicinal plant. Its fruits contain a complex of biologically active substances [1].

The valuable therapeutic and prophylactic properties of Russian olive have been noted in the folk medicine of many Asian and Trans-Caucasus countries. Fruits of local species are used for diseases of the gastrointestinal tract because they exhibit astringent, anti-inflammatory, and encapsulating activity. They are used as cholegogics (for bronchitis), diuretics (for edema), antihelmintics, and vitamins. Tincture of fruit exhibits hypotensive and slight analgesic action. Baby food is prepared in Central Asia from pericarp powder.

The drug pshatin, which is a concentrate of polyphenolic compounds, is prepared from *E. angustifolia* fruits in Armenia and is used for cholitis and other diseases of the GI tract [2, 3].

This all indicates that a phytochemical study of *E. angustifolia* is promising in order to determine the active principles of interest to the pharmacy and food industries.

A study of the chemistry of *E. angustifolia* introduced to the Central Nonchernozem region of the RF is especially interesting.

EXPERIMENTAL PART

We studied dried ripe fruits of *E. angustifolia* collected from a seven-year-old plant growing in the botanical garden of Sechenov Medical Academy and from Samara and Belgorod Oblasts.

Drying was carried out in air and shade. A Mikma laboratory mill was used to grind the fruits.

Qualitative reactions for biologically active compounds according to modified methods described in the SP XIth Ed. were used to determine the chemical composition [4].

Tests for polysaccharide content. Polysaccharides were isolated by precipitation with ethanol from the aqueous extract obtained from the dried and ground fruits. Isolated total polysaccharides were hydrolyzed for 10 h in a sealed ampul with H_2SO_4 solution (2 N) [3]. The filtrate was evaporated to a volume of 2 mL and used for identification of monosaccharides by descending paper chromatography (FN-1, Germany; C, Russia) using BuOH:Py:H_2O (6:4:3) for separation of galactose and glucose; BuOH:CH_3CO_2H:H_2O (4:1:5), for xylose and fructose; PhOH:H_2O (1:1), for mannose and glucose; EtOAc:Py:CH_3CO_2H:H_2O (5:5:1:3), for rhamnose and the aforementioned monosaccharides. Compounds were detected with anilinium phthalate and urea

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solution (5.0 g urea dissolved in 100 mL ethanol and 20 mL 2 N HCl).

The total sugar content calculated as saccharose and reducing sugars calculated as glucose were determined by the Bertran method as modified by Berry and Il'in [1]. For this an accurately weighed portion (~5.0 g) of ground fruit was ground in a mortar with a small amount of ground glass. The ground mass was transferred to a 250-mL volumetric flask. The mortar and funnel were washed with hot water (150 mL). The mixture was cooled, treated with lead acetate solution (3 mL, 10%), mixed well, and adjusted to the mark with water. The precipitate was allowed to settle and was filtered off. The filtrate was used to determine monosaccharides.

Tests for flavonoid content. Moistening ground raw material with aqueous ammonia (5%) produced an intense yellow color.

Total flavonoids were determined by grinding an average sample of raw material to a particle size passing through a sieve with 1-mm openings. Each sample (accurate weight, ~1 g) was extracted with ethanol (95%) in a Soxhlet apparatus for 4 h. The extract was evaporated in a rotary evaporator, dissolved in heptane (10 mL) to remove lipophilic substances, dissolved in hot water (20 mL), transferred to a separatory funnel, and cooled. The aqueous solution was extracted for 12 h at room temperature with *n*-BuOH (50 mL). The organic phase was separated, concentrated in a rotary evaporator, and placed with a capillary at the origin of the chromatographic plate (Kieselgel 60 F_{254}). The plate was dried and placed in a chamber containing *n*-BuOH:CH_3CO_2H (glacial):H_2O (4:1:5). Spots of flavonoids were detected using ammonia vapor and Wilson reagent. This method was used as a rapid method for determining flavonoids.

The TLC data were confirmed using HPLC to identify flavonoids. We used a Waters (USA) chromatograph, Lambda-Max Model 481 LC Spectrophotometer detector, and recording using the Multichrom program set for Windows 1.47a (ZAO Ampersend, Moscow, Russia). Data obtained by TLC with a standard were confirmed by comparing widths, areas, and retention times of peaks in chromatograms of the studied samples with analogous parameters of standard solutions. The method was used to determine quantitatively the flavonoid content.

Tests for coumarin content. A weighed portion (~2 g) of ground fruit was treated with ethanol (20 mL), refluxed for 15 min, cooled after 20 min, and filtered through filter paper (solution A).

The alcohol extract (10 mL) was treated with methanolic NaOH (20 drops, 10%) and heated on a water bath at 80°C for 5 min. The solution turned yellow (solution B). The resulting solution (5 mL, calibrated pipette) was placed into a 10-mL conical flask. Then, solution A remaining in the first flask was treated with distilled water (15 mL). The pH was adjusted to 3 – 5 using HCl (10%). A fluffy white precpitate formed. Freshly prepared diazo-reagent (Pauli, Kutaczek) was added (5 drops) into the second flask, after which the solution turned red.

Tests for saponin content. Steroidal glycosides (saponins) in fruit were detected using a foam-forming reaction and precipitation by lead acetate (Lieberman-Burchard reaction, Lafont and Salkovskii).

Tests for amino-acid content. An accurately weighed portion (50 mg) of finely ground fruit powder was placed in a 50-mL flask with a ground-glass stopper, treated with sodium dodecylsulfate solution (25 mL, 0.25%) in phosphate buffer (500 mM, pH 6.5), incubated in a thermostat for 60 min at 45°C, and transferred to a column (15 × 1.5 cm) packed with Toyopearl HW-55 F gel. Fractions were collected at 25°C using sodium dodecylsulfate solution (0.25%) in phosphate buffer (50 mM, pH 6.3) as eluent. The chromatography was performed using a Multirac (LKB) fraction collector equipped with a detecting device, recorder, and peristaltic pump at 280 nm in a cuvette (L = 0.2 cm). The isolated protein fractions were concentrated using Minicon CS 15 membrane cells. The amino-acid composition of a protein fraction was determined on a Keltec 1030 instrument (Sweden). The concentrate solution in buffer [80% CH_3CN, 0.1% trifluoroacetic acid (TFA) in triply distilled water, 10 nM] was placed in an ampul for hydrolysis using a microsyringe and evaporated in vacuo (10 torr) with an acetone-slush trap. The ampul was treated with HCl (0.3 mL, 5.7 N). Air was removed using a vacuum pump and freeze-thaw cycles. The ampul with the frozen sample was sealed under vacuum, stored in a thermostat for 24 h at 105°C, opened, evaporated, treated with water (0.5 mL), dried again, dissolved in a mixture (0.7 mL) of TFA (0.1%) and CH_3CN (5%) (pH 1.8), centrifuged for 5 min at 2,000 rpm, and transferred by microsyringe into the autosampler of an amino-acid analyzer. The integrator readings were interpreted using standrad chromatograms of known amino acids.

Tests for vitamin content. Powdered leaves (0.5 g) were treated with water (5 mL), stirred, left for 15 min, and filtered. The aqueous extract (1 drop) was placed on a plate (Merck G-60-TLC, 10 × 10) with a standard (pure ascorbic acid). The plate was placed into a chromatography chamber [EtOAc:CH_3CO_2H (glacial), 80:20], developed in descending mode for 20 min (solvent front 13 cm), and dried. The plate was treated with aqueous sodium 2,6-dichlorophenylindophenolate (0.04%). A white spot on a pink background was observed.

Tests for carotinoid content. 1) Ground leaves (1 g) were treated with $CHCl_3$ (5 mL) in a 25-mL flask, extracted for 2 h, and filtered. The solution was placed on a plate (Merck G-60-TLC, 10 × 10) with β-carotene standard, placed in a chamber (cyclohexane:ether, 80:20), developed in descending mode for 20 min (solvent front 13 cm), dried in air for 5 min, treated with phosphomolybdic acid solution (10%) in ethanol, and heated to 60 – 80°C. Blue spots on a greenish-yellow background appeared.

Tests for tannin content. A sample of ground leaves of *E. angustifolia* (30 g) was treated with C_6H_6:$CHCl_3$ (1:10) in a Soxhlet apparatus for 12 h to remove resin, chlorophyll, and other ballast substances; dried in air, and extracted again in the Soxhlet apparatus using acetone for 20 h. The acetone was evaporated in vacuo. The solid of total catechins (0.8 g) was dried in a vacuum desiccator. Chromatography was carried out on plates (Merck G-60-TLC, 10 × 10) with known standards of pure catechins using vanillin solution (1%) in conc. HCl for detection.

UV spectra of the compounds in ethanol (70%) were recorded on a SF-16 spectrophotometer in a 10-mm cuvette. IR spectra in mineral oil and KBr disks were recorded on an IK-10 spectrophotometer (with NaCl and LiF prisms in the range 800 – 3600 cm^{-1}) with 0.5% concentrations. Specific rotation was determined on a circular polarimeter (CM model) in 0.5-dm cuvettes [1].

RESULTS AND DISCUSSION

Glucose, mannose, galactose, fructose, xylose, and rhamnose were identified in the polysaccharide complex. Their R_f values were 0.18, 0.20, 0.21, 0.23, 0.25, and 0.41, respectively, using BuOH:CH_3CO_2H:H_2O (4:1:5). Galacturonic acid (R_f = 0.271) was identified chromatographically using solvent systems EtOAc:HCO_2H:CH_3CO_2H:H_2O (18:1:4:3) and EtOAc:Py:CH_3CO_2H:H_2O (5:5:1:3). The content of reducing sugars in fruit of *E. angustifolia* was 50.67 – 55.75%; total sugar (saccharose), 60.0 ± 5.0%. Total pectin content (water-soluble and water-insoluble) in fruit was determined gravimetrically and reached 3.58 ± 0.3%.

Rutin, quercetin, isorhamnetin, isorhamnetin-3-*O*-β-galactopyranoside, eleagnoside, kaempferol, and caffeic acid were found in the flavonoid fraction of the fruit. The content of total flavonoids and phenolcarboxylic acids was determined gravimetrically as 1.35 ± 0.15%.

Coumarins were represented by two compounds that were oxycoumarin derivatives, including *p*-coumaric acid.

The foam-forming reaction that was carried out by the Fontan-Candel method (depending on the pH) suggeseted the presence of saponins in fruit of Russian olive. The isolated saponins did not cause hemolysis of erythrocytes (2% suspension) in isotonic saline. TLC on Silufol plates (Czech Rep.) using $CHCl_3$:CH_3OH:C_2H_5OH (20:5:5) detected a yellow band with R_f 0.67 using Sanier reagent. The content of total saponins in the fruit was determined gravimetrically as 1.96 ± 0.52%.

The amino acids aspargic acid, threonine, serine, glutamine, proline, glycine, alanine, valine, methoinine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine, tryptophan, cysteine, and cysteinic acid were observed and identified in fruit of *E. angustifolia*.

Vitamins in the raw material were ascorbic acid (vitamin C) and vitamins A (or provitamin A, β-carotene), E, and K. It was found that the fruit contained ascorbic acid (5.6 mg%) and β-carotene (17.5 mg%).

The presence of tannins (catechins and tannins) in the fruit was also proved using qualitative reactions and chromatography. The content of tannins in the fruit was determined by the pharmacopoeic method (SP, XIth Ed.) as 5.03 ± 0.05%.

Thus, biologically active substances occurring in fruit of *E. angustifolia* introduced to the Central Nonchernozem region of the RF were determined quantitatively.

The results can be used to develop regulations for producing food and pharmaceuticals from this raw material.

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