Content of some flavonoids in alfalfa and its water extract "Eracond"

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Abstract. The paper states that the extraction of some flavonoids from air-dry raw alfalfa with 70% alcohol-water solution was carried out. The quantitative determination of some flavonoids content in the obtained extracts and dietary supplement "Eracond" by HPLC method was carried out. The content of flavonoids in alfalfa ranges from 0.004 mg/g naringenin to 0.29 mg/g from dry basis mass in naringin, and it ranges from 0.007 mg/g of quercetin to 0.50 mg/g from dry mass in naringin in "Eracond". A higher level of quercetin (0.12 mg/g) was revealed in alfalfa harvested in June (first cut) relative to its content (0.08 ± 0.002) in alfalfa of the second cut. The content of dihydroquercetin (0.007 ± 0.0004 mg/g) and fisetin (0.04 mg/g) in the medicinal plant raw material harvested in August (L-2) decrease in relation to alfalfa harvested in June, where similar indicators were 0.088 ± 0.009 mg/g and 0.08 ± 0.003 mg/g, respectively. The phenolic compounds identified in alfalfa should be considered as promising pathogenetically substantiated agents in the complex therapy of pathologies involving the application of inhibitors of free radical processes.

1. Introduction

Morphofunctional shifts in the human body caused by ionizing radiation are generated in the disturbance of the antioxidant balance in the system of oxidative homeostasis. Free radical processes play an important role in the pathogenesis of acute and chronic radiation pathology. The efficiency of radio protectors depends on their ability to inhibit the physicochemical and chemical stages of energy conversion of ionizing radiation in living tissues, where highly reactive products of water radiolysis are formed, i.e., hydroxyl ions (OH⁻), hydrogen peroxide (H₂O₂), oxygen radical (O₂⁻), hydroperoxyl (O₂H⁺) and hydroxyl (OH⁺) radicals, molecular ions (H₂O⁺) and excited molecules (H₂O⁺) of water [1]. With the prophylactic use of antioxidants of plant origin, the severity of the course of radiation injury decreases and the survival rate of irradiated animals increases [2].

The highly efficient radio protectors include a biologically active additive "Eracond" produced by LLC "Eracond" (certificate of GOS. REG. RF: No. RU 77.99.88.003E.000027.01.18 from 10.01.2018). Alfalfa (Medicago sativa L.) is used as a raw material for the manufacture of this herbal preparation. It exhibits antioxidant properties both in vitro and in vivo [3, 4]. The antioxidant properties of alfalfa have been known for a long time. Phytopreparations based on it are used as preservatives that prolong the shelf life of food products [5]. There is no information in that reveal the mechanisms of implementation of the antioxidant properties of this plant despite a fairly wide range of studies devoted to the study of the chemical composition of alfalfa. Studies aimed at creating new
herbal medicines with antioxidant activity capable of correcting pathophysiological changes in the system of oxidative homeostasis in acute radiation sickness and oncopathology are of a great interest [6-13].

2. Materials and methods

Medical plant raw materials preparation for the production of dietary supplements "Eracond" is carried out from mid-June to September (as it ripens) of the herb alfalfa (Medicago sativa L.). The main requirements for raw materials are color from light green to green; musty, moldy and putrid odors are not allowed; the content of extractives is not less than 8%.

Raw materials after harvesting from the fields are placed in a dry room for the fermentation process. Dried alfalfa hay is then fed into production. Humidity should be no more than 20%, ash content should be no more than 14%.

Thermo-chemical treatment is used at biologically dietary supplements "Eracond" manufacturing. Distilled water according to GOST 6709 and dried alfalfa hay according to TU 9749-006-04863053 are used.

The essence of the technological process is in carrying out preliminary extraction of alfalfa hay crushed on a crusher with paracondensate in a mixer at a temperature of 70°C for 20 minutes, further extraction of hay in an adiabatic autoclave at 85-105°C for 6-8 hours, separating the extract from the solid phase and evaporation of the extract, followed by drying to obtain a finished product with a dry matter content of 40%.

The initial raw materials with a calculated moisture content of 20% are dried using forced ventilation to moisture content of no more than 13%. Then the hay is fed to a KDU-2.0-1 feed grinder, where it is crushed to a fraction of 3-5 mm. The chopped hay is fed into the AZM-0.8A tank through the settling chamber of the feed grinder. Heating steam condensate formed in the TO-1,1 heat exchanger is used as an extraction agent. Next is the process of preparing the pulp. For this, an AZM-0.8A mixer equipped with a stirrer is used. The mixer is fed through a cyclone and a sluice gate. The mixing process is carried out with the mixer turned on.

The finished slurry is fed by an H2-1 pump into one of 6 steam-jacketed reactors of the VLE 1-3-1.0-1.0 type, equipped with pressure and temperature sensors.

The heating of the reactor begins with steam supplied to the jacket of the reactor to a predetermined temperature at a pressure of 5.5-6.0 kgf/cm². The total duration of heat treatment in the reactor is 3 hours, taking into account the time of heating, loading and unloading. After the specified time has elapsed, the extract is separated from the reactor due to the excess pressure in the reactor through lowering it into the tank for sludge, then into the OGSh-321 centrifuge, where the fractions are separated into centrate and raffinate. The product cleared of suspended solids enters the extract dosing tank and from it is pumped to the evaporation system, which includes a tubular evaporator. The end of the evaporation process is controlled by the concentration values in the chemical laboratory of production. The extract brought to the required concentration is poured into canisters.

The paper studies the following samples:

- L-1 is alfalfa hay (a first cut), harvested in June 2020 in the Ishimbay region of the Republic of Bashkortostan.
- Sample E-1.2 is dietary supplement "Eracond" produced from raw materials L-1.
- L-2 is alfalfa hay (a second cut), harvested in August, in the same region of the Republic of Bashkortostan.
- Sample E-1.3 is dietary supplement "Eracond" produced from raw materials L-2.

2.1. Alfalfa extracts Preparation for HPLC

Place 10 g of crushed air-dry raw material; add 100 ml of 70% ethyl alcohol into a conical flask with a capacity of 250 ml. It was extracting for 4 hours with occasional stirring at room temperature. The resulting solution was filtered through a Schott filter with a pore size of 100 μm. The extraction was repeated 2 more times. The combined alcoholic extract was evaporated on a Heidolph rotary
evaporator (Germany). The amount of extracted extractives was determined by the gravimetric method after removing the solvent and reaching a constant mass of the flask with the dried extract.

2.2. Chromatographic analysis conditions
The quantitative determination of flavonoids in dry alfalfa extract was carried out by HPLC on a Waters Breeze chromatograph (Waters, USA) with a spectrophotometric detector. The determination was carried out on a Luna C18 column, 4.6x250 mm, 5 μm (Phenomenex, USA) using a gradient elution mode (water is acetonitrile). The acetonitrile content varied from 5 to 100%. The flow rate of the mobile phase was 0.9 ml/min. Analysis time 60 min. Detection was carried out at an analytical wavelength of 275 nm.

2.3. Standard samples
The calibration curves of standard solutions, quercetin, dihydroquercetin, naringin, naringenin, and fisetin were built. At the first stage of the work, solutions of standard substances with concentration of 0.025, 0.05, and 0.10 mg/ml were prepared by dissolving weighed portions of the corresponding substances in the eluent acetonitrile, water is 50:50 (vol.%) in 25 ml volumetric flasks. For each standard solution, a chromatogram was recorded as it was described above.

Figure 1 presents a chromatogram of a standard naringenin solution as an example.

![Figure 1. Chromatogram of a standard naringenin solution at 275 nm.](image)

A sample of extracts with a volume of 50 μL was introduced into the chromatograph using an autosampler and the chromatogram was recorded. Each sample was analyzed 4 times. The extracts were prepared with a concentration of 5 mg / ml (for alcoholic extracts of alfalfa) and 10 mg / ml (for dietary supplements "Eracond"). The concentration of flavonoids in the extracts was determined from the areas of the peaks of substances with a certain release time using the calibration curves of standard substances. The peak areas of flavonoids were determined as the mean values of parallel determinations and, using calibration plots, their concentrations in the extract were found [6].

The statistical processing was carried out according to the results of 4 determinations of each sample.

3. Results and discussion
It is known that herbal extracts are complex mixtures of organic compounds; therefore, problems arise in their separation. In particular, gradient elution is used to separate substances in aqueous-alcoholic extracts by HPLC.
Gradient elution was also used for chromatographic separation of alfalfa hay extracts and the finished product of the Eracond dietary supplement.

This sample contains a complex mixture of compounds. The desired analytes have been identified among them. Due to the fact that the UV spectrum of most flavonoids contains a maximum in the region of 275 nm, quantitative determination by HPLC was carried out at this wavelength.

Table 1 presents results of the quantitative determination of naringenin, quercetin, naringin, dihydroquercetin and fisetin.

### Table 1. Flavonoid content in extracts.

| Flavonoids        | Flavonoid content, mg/g of dry weight |
|-------------------|--------------------------------------|
|                   | Alfalfa 275 nm | Eracond 275 nm |
| Naringenin        | L1: 0.007 ± 0.0002 | E-1.2: 0.05 ± 0.001 |
|                   | L2: 0.004 ± 0.0001 | E-1.3: 0.0017 ± 0.0001 |
| Quercetin         | L1: 0.08 ± 0.002 | E-1.2: 0.007 ± 0.0005 |
|                   | L2: 0.12 ± 0.002 | E-1.3: 0.040 ± 0.001 |
| Naringin          | L2: 0.29 ± 0.01 | E-1.3: 0.50 ± 0.01 |
| Dihydro-quercetin | L1: 0.088 ± 0.009 | E-1.2: 0.20 ± 0.002 |
|                   | L2: 0.007 ± 0.0004 | E-1.3: - |
| Fisetin           | L1: 0.08 ± 0.003 | E-1.2: 0.20 ± 0.07 |
|                   | L2: 0.04 ± 0.02 | E-1.3: 0.35 ± 0.01 |

According to table 1, reproducible values are observed for all studied flavonoids, with the exception of dihydroquercetin at considering the results of the analysis of extracts from alfalfa hay (L1 and L2). It should be noted that samples L1 and L2 were prepared at different times (June and August). Among the studied compounds in the alfalfa sample, the maximum content was found in naringin (=0.3 mg/g dry weight) and quercetin (=0.1 mg/g dry weight).

In dietary supplements "Ecakond" (E-1.2 and E-1.3) a scatter of values for the same flavonoid in different samples is more significant, however, it can be stated that the content of the studied compounds is 2-5 times higher than in samples L1 and L2.

### 4. Conclusion

The extraction of some flavonoids from air-dry raw alfalfa with 70% alcohol-water solution was carried out. It was found that the content of flavonoids in alfalfa ranges from 0.004 mg/g naringenin to 0.29 mg/g from dry mass in naringin, and in "Eraconda" is from 0.007 mg/g of quercetin to 0.50 mg/g from dry masses in naringin. A higher level of quercetin (0.12 mg/g) was revealed in alfalfa harvested in June (a first cut) relative to its content (0.08 ± 0.002) in alfalfa of a second cut. In the medicinal plant raw material harvested in August (L-2), the content of dihydroquercetin (0.007 ± 0.0004 mg/g) and fisetin (0.04 mg/g) decrease in relation to alfalfa harvested in June, where similar indicators were 0.088 ± 0.009 mg/g and 0.08 ± 0.003 mg/g, respectively. The phenolic compounds identified in alfalfa should be considered as promising pathogenetically substantiated agents in the complex therapy of pathologies involving the use of inhibitors of free radical processes. It is generally accepted that the greatest role in the diverse influence of flavonoids on humans is played by their antioxidant properties.

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