



**Effect of extraction solvent on total phenolic content,
total flavonoid content and antioxidant activity of
*Cetraria islandica***

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Abstract : Oxidative stress may lead to a number of diseases such as atherosclerosis, nephrotoxicity, liver cirrhosis, cancers, diabetes, and Alzheimer disease. Medicinal plants are an important source of antioxidants. Therefore, the antioxidant potential of *Cetraria islandica* was evaluated in this work. The coarse powder of leaf of *Cetraria islandica* was extracted in Soxhlet apparatus, with ethanol (90%) and ethanolic extract of *Cetraria islandica* (EECI) was further processed for phytochemical screening, total phenol content, total flavonoid content, and various in vitro antioxidant assays. The phytochemicals present in EECI were glycosides, carbohydrates, triterpenoids, proteins and amino acids, gums and mucilages, and flavonoids. The content of extractives was 8.3 mg/ml for *Cetraria islandica* extract 70%. The content of polyphenolic compounds in terms of per head acid was the highest for the sample of 70% and amounted to 0.586 mg/ml, and the content of flavonoids per standard solution quercetin - 0,012 mg/ml - also for 70% extract. By DPPH, the percentage of radicals of absorbing activity for 70% of the extract was 86%, and the antiradical activity of 0,417, indicating a sufficiently high rate of *Cetraria islandica* antioxidant activity at such a concentration of ethanol. The tested extracts showed next results by FRAP assay: 486 µmol/L (EE96) 135 µmol/L (EE70) and 158 µmol/L (EE40). ABTS method showed the highest result of the extract of *Cetraria islandica* 40%. The obtained results confirmed the high potential of the extracts as a source of phenolic compounds, in particular flavonoids.

Keywords : *Cetraria islandica* phenolic compounds, flavonoids, antioxidant activity, phytochemical screening.

Introduction

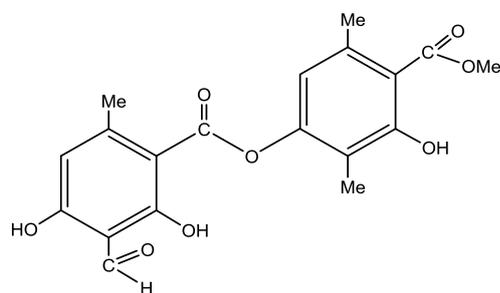
Drugs, derived from medicinal plants, commit a complex of the biochemical effect on the human body. Therapeutic effects, which are observed with using plant extracts, rich in phenols, due to the intake of natural substances in the human body, which affect the redox processes at the cellular level, tissue regeneration, the negative impact of environmental factors, processes, infectious diseases. It is known that natural antioxidants are divided into two groups: the first group is formed by enzymatic substances (superoxide oxidase, etc.), the second group includes tocopherols, vitamins C, A, D, E, K, quercetin, gallic acid ^[1].

Medicinal herbal raw materials despite the centuries-old period of using in traditional medicine and multifaceted researching of modern phytochemical scientists-pharmacists remain unsolved. These can be viruses, bacteria, adverse environmental factors. Traditional and official medicine have significant plant resources for the treatment of respiratory diseases. Analyzing the recipes of traditional medicine, we can conclude that many medicinal plants are used in the treatment of the aforementioned diseases ^[1,2].

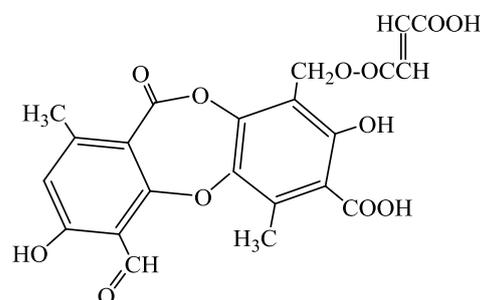
The using of these plants for the treatment and prevention of diseases of the respiratory system is due to the presence in them of active substances that have a certain pharmacological effect. In particular, polysaccharides explain the antitussive effect; essential oils, depending on the type of plant, have antimicrobial, bronchodilator, expectorant action; saponins - an expectorant effect. Pharmacologically active in respiratory diseases are alkaloids, which have a calming effect on the cough center and reduce the frequency of attacks of bronchial asthma ^[2].

One of the plants that take place in the treatment of respiratory diseases is the moss Iceland *Cetraria islandica*, a plant of the family Parmeliaceae, it belongs to the deciduous-shrub lichens and grows almost all over Ukraine in pine forests in swamps and mountains. Distributed in all high mountains, occurs in northern and central Europe (in the southern part of the mountains). Medicinal plant material is the elephant (*Lichen islandica*), which resembles a bushy, upright, irregularly branched formation. The thalamus has the shape of a bush, with incorrectly branched erect lobes. Particles of ribbon-shaped, leathery-cartilaginous, flat, fringed by short hairs on the edge, greenish-brown or olive-green above, grayish-whitish below, red spots are often seen at the base, very flat-branched on the tips of the tops are very branched round, disc-shaped apothecia. Raw materials have a slight peculiar smell and bitter-mucous taste ^[2,3,4].

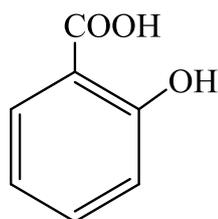
A number of substances have been found in the raw materials of *Cetraria islandica*, which in one way or another determine the pharmacotherapeutic effect of the plant. The following secondary metabolites have been identified: atranorine (1.2%), fumaroprocetar (0.5-1.5%), salicylic (4-6%), usinic (0.2-4%) and lecanic acid (more than 36%) ^[5]. The highest content in raw materials have polysaccharides - up to 50%, which are presented lichenin and isolichenin. Researching have found that boiling *Cetraria islandica* with water leads to the formation of a large amount of sticky gelatin product, "moss starch". About 16 chemical elements have been found in the *Cetraria islandica* that can be arranged in increasing order depending on the quantitative content: N> Si> Ca> K> Fe> S> Mg> Na> P> Cl> Mn> Zn> Cu> B> Co> Mo ^[4,5].



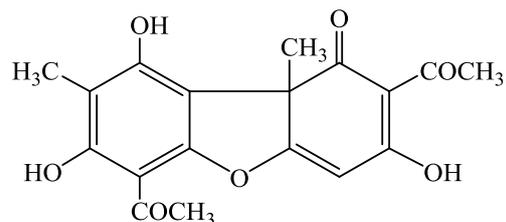
Atranorine



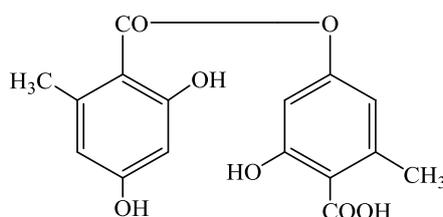
Fumaropro-acetic acid



Salicylic acid



Usnic acid



Lecanoic acid

Despite its widespread using in folk medicine, as of September 2019, *Cetraria islandica* in the pharmaceutical market of Ukraine is presented only in the formulation of Herbion® Icelandic moss syrup (KRKA, DD, Novo mesto), Isla-mint (Engelhard Artznimittel GmbH & Co. KG, Germany), Isla-Moos (Engelhard Artznimittel GmbH & Co. KG, Germany) [7].

For the manufacturing of medicines of *Cetraria islandica* are used aqueous extracts, which are a complex of polysaccharides and mainly for the treatment of diseases of the respiratory system are used as an expectorant [2,7]. To expand the using of this raw material, we investigated alcohol-water extracts of *Cetraria islandica*.

Materials and Methods

Preparation of raw materials and obtaining alcohol-water extracts

Air-dry crushed to a size of 2-3 mm industrial raw materials of *Cetraria islandica* was used for or the research [8].

The choice of the extractant

In the preparation of the extract, ethyl alcohol of various concentrations is widely used in the pharmaceutical industry. As an extractant, it has a wider range of extraction of biologically active substances compared to water, its extractive ability depends on the concentration, the higher the alcohol concentration, the less hydrolytic processes are possible in its environment and enzymes are inactivated. Extracts obtained using ethyl alcohol at a concentration of $\geq 70\%$ are free of biopolymers (proteins, slides, pectins).

It is characterized by preserving properties. The canning effect starts at 15-18%; in extraction (new galenic and galenic preparations) - at a concentration of 20% (in alcoholic mixtures more than 20% do not develop microorganisms and mold). The best antiseptic properties are 70% ethanol, which is important for long-term storage of tinctures and extracts, so most often for the extraction of vegetable raw materials as the extractant use mixtures of ethanol and water with a percentage of from 30 to 70%.

Three concentrations of alcohol-aqueous mixtures were chosen for the studies, namely 96% ethanol, 70% and 40% solution to find the optimal concentration, which will provide the maximum yield of substances with antioxidant properties.

Preparation of water-alcohol extract

Extracts for research were prepared by the method of classical maceration of the ratio of raw material extractant 1:10, the infusion time is 14 days. This method of extraction allows to obtain a complex of BAR in

unchanged natural form, since it does not involve the use of additional heat supply. The crushed raw material was poured with alcohol-water mixtures with a corresponding content of ethyl alcohol until the raw material is completely coated. Infused at room temperature for two weeks and poured tinctures from green to yellow-brown color depending on concentration^[8].

Preliminary Phytochemical Screening

This involves the analysis and screening of EECI for different phytochemical compounds. The preliminary screening gives a general idea regarding the presence of different compounds possessing therapeutic values. The phytochemical screening was done as per the WHO guidelines. The results are shown in Table 1.

Determination of the content of extractives in the extract

The content of the extractives was determined by gravimetric method. 1 ml of the test extract was made in a glass beaker. The solvent was blown off under a stream of nitrogen at 40° C on a RapidVap® Vertex Evaporator. The content of extractives one milliliter of the extract was re-weighed and calculated^[8].

Estimation of Total Phenolic Content

Total phenolic content was estimated using the Folin-Ciocalteu method. An aliquot of the extract was mixed with a Folin-Ciocalteu reagent in water. The solution was kept in the dark at room temperature for 5 minutes. After that samples were mixed thoroughly with solution of 300 µL sodium carbonate in water. The solution was kept in the dark at room temperature for 20 hours. The absorbance was measured on a Hitachi U-2810 spectrophotometer at a wavelength of 760 nm with a layer thickness of 10 mm^[8,9].

As a comparison solution, a solution consisting of 20 µl of 70% methanol P, 1.58 ml of purified water and 100 µl of Folin-Chokalteu reagent and 300 µl of Na₂CO₃ solution in purified water was used.

Total phenolic content was expressed as a milligram of gallic acid equivalents (GAE) per 1 g of extract samples (mg GAE / g)^[8,9].

Estimation of Flavonoids

Working solution. To 160 µl of the test tincture was added 1680 µl of 70% alcohol-aqueous mixture and 160 µl of aluminum chloride (3% solution). After 40 min of exposure in the dark place measure the optical density of the solution on a spectrophotometer Hitachi U-2810 at a wavelength in the range of 360-420 nm in a cell with a layer thickness of 10 mm.

As a comparison solution, a solution consisting of 160 µl of the test tincture and 1840 µl of 70% alcohol-aqueous mixture is used. The content of flavonoids in the tincture (in terms of quercetin) was calculated using a calibration graph of the optical density versus the concentration of quercetin acid in solution. A series of dilutions of 1 mg / ml quercetin stock solution was prepared for its construction. The studies are carried out similarly as described above. The repetition rate is three.

Determination of the radical of absorbing activity by means of a stable free radical of 2,2-diphenylpicrylhydrazyl

The antioxidant activity of the extract was measured with the DPPH method. A solution of DPPH was freshly prepared by dissolving 4 mg DPPH in 100 mL ethanol. The extract (0,2 mL) and DPPH solution (1,8 mL) was mixed together in a test tube. The test tube was then incubated in the dark for 30 minutes at room temperature. As a control solution was prepared 0,2 ml of ethanol and 1.8 ml solution of DPPH in ethanol. The decrease in absorbance was measured at 517 nm using a spectrophotometer. The percentage inhibition of radicals was calculated using the following formula^[8,11,12]:

$$\% \text{ inhibition} = 100 \times (A_0 - A_1) / A_0,$$

where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in the presence of all of the extract samples and reference. All the tests were performed in triplicates.

The half-maximal inhibitory concentration (IC50) was reported as the amount of antioxidant required to decrease the initial DPPH concentration by 50%. All tests were performed at least in triplicate^[13,14].

Calculated antiradical activity by the following formula:

$$ARA=1/IC50.$$

The total antioxidant capacity was also expressed in mg of Trolox (mg AAE/g of extract), and was calculated as follow:

$$AAE = ARP \text{ extract}/ARP \text{ Trolox}$$

Ferric reducing antioxidant power (FRAP) assay

To determine the antioxidant activity used the method FRAP (reduces the antioxidant power of iron - iron reducing antioxidant power).

Mixed 1500 μ l of FRAP solution, 150 μ l of water and 50 μ l of the test extract. After stirring, the samples were kept for 4 minutes^[8,11].

For the preparation of the control solution was mixed 50 μ l of 70% ethanol and 1650 μ l of FRAP solution. The absorption coefficient of the spectrophotometer was measured at a wavelength of 593 nm. The experiment was performed in three replicates^[12,13].

The free-radical-scavenging activities of the extracts and purified compounds were evaluated through 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) method

Scavenging activities of the extracts and purified compounds from *Cetraria islandica* towards ABTS radical were also measured. Briefly, a stock solution of ABTS radical cation was prepared by dissolving ABTS (7 mM, 25 mL in deionized water) with potassium persulfate (K₂S₂O₈) (140 mM, 440 μ L). The mixture was left to stand in the dark at room temperature for 15-16 h (the time required for formation of the radical) before use. For the evaluation of ABTS radical scavenging activity, the working solution was prepared by the previous solution and diluting it in ethanol to obtain the absorbency of 0.700 ± 0.02 at 734 nm (ABTS working solution should be replaced every five days at least because the free radical degrades easily). The solvent extracts and purified compounds (0.1 mL) at different concentrations were mixed with the ABTS working solution (1.9 mL) and the reaction mixture was allowed to stand at 30 °C for 6 min, then the absorbance was measured by using a UV-visible spectrophotometer at 734 nm, at which point the antioxidants present in the extracts and purified compounds began to inhibit the radical, producing a reduction in absorbance, with a quantitative relationship between the reduction and the concentration of antioxidants present in the tested sample. The radical scavenging activity is given as ABTS radical scavenging effect that is calculated by equation (2):

$$\text{ABTS radical scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

At the same time a standard curve was obtained using Trolox standard solution at various concentrations (ranging from 0 to 100 μ g/mL) in 95% ethanol. Scavenging activities of the purified compounds towards ABTS radical were expressed as TEAC (Trolox equivalent antioxidant capacity). Different concentrations of each purified compound were chosen to test the ABTS radical scavenging activity. The results were compared with the standard curve for calculation of TEAC. Ascorbic acid (VC) and gallic acid were used for positive controls, respectively. All the tests were performed in triplicate.

Results and Discussion

Preliminary Phytochemical Screening

The results of the extractives are presented in Table 1.

Determination of the content of extractives in the extract

The results of the extractives are presented in Table 2.

Table 1: Phytochemical screening of *Cetraria islandica* extracts

S. No.	Test	Phytochemicals present in EECI
1	Alkaloids	-
2	Glycosides	+
3	Carbohydrates	+
4	Steroids	-
5	Triterpenoids	+
6	Fixed oils and fats	-
7	Tannins	+
8	Mucilages	+
9	Flavonoids	+
10	Saponins	+
11	Phenols and Phenolic compounds	+
12	Lichen acids	+
13	Polysaccharides	+
14	Vitamins	+
15	Organic acids	+
16	Proteins	+
17	Fats	+
18	Microelements	+

Table 2: The results of the extractives

Object	ES, mg/ml	TPh, mg/ml	FL, mg/ml	RAA*, %	IC50	FRAP, $\mu\text{mol/L}$	ARA	ABTS
Extract 96 %	3,8	0,137	0,005	25	-	158	-	263
Extract 70 %	8,3	0,586	0,012	86	2,40	135	0,417	218
Extract 40 %	5,4	0,328	0,006	69	2,45	486	0,408	824

«-» - the result is absent; at the maximum concentration of substances inhibition of 25% is achieved.

* Determination of the radical of the absorbing activity by the DPPH method.

Estimation of Total Phenolic Content and Total Flavonoid Content

After measuring the radical of the absorbing activity of *Cetraria islandica* liquid extracts diluted to a concentration of 1 mg/ml, it should be noted that the content of polyphenolic compounds in terms of per head acid was the highest for the sample of 70% and amounted to 0.586 mg/ml, and the content of flavonoids per standard solution - comparison - quercetin - 0,012 mg/ml - also for 70% extract (Table 2).

Determination of the radical of absorbing activity by means of a stable free radical of 2,2-diphenylpicrylhydrazyl

During the interaction with the stable free radical DPPH, the tested extracts showed activity at 25% (EE96) 86% (EE70) and 69% (EE40), however, after determination of IC50 it was found that EE70 and EE40 have very close values respectively 2.40 mg/ml and 2.45 mg/ml, which clearly indicates the close quantitative content of the active antiradical substances in these extracts, which is confirmed by the value of the antiradical activity of 0.417 and 0.408.

The AOA dependences on concentrations of the corresponding extracts is shown in Figures 1-4.

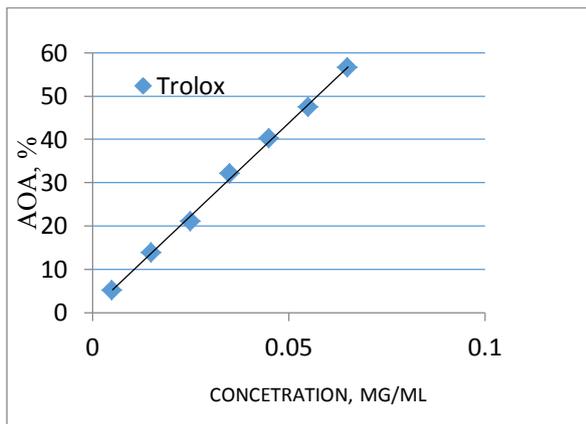


Figure 1: Standard curves of Trolox

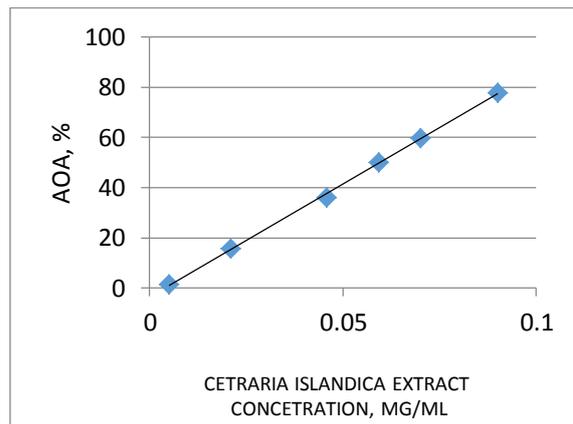


Figure 2: 1, 1-diphenyl-2-picryl-hydrazyl radical scavenging activity of the ethanolic 40% extracts of *Cetraria islandica*

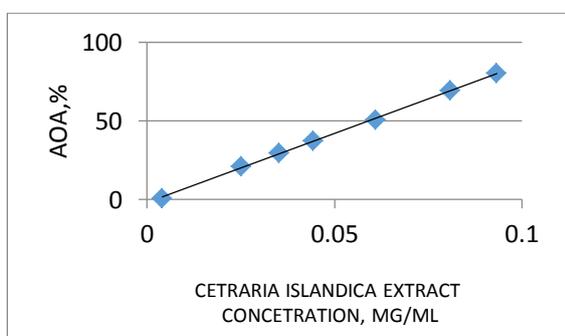


Figure 3: 1, 1-diphenyl-2-picryl-hydrazyl radical scavenging activity of the ethanolic 70% extracts of *Cetraria islandica*

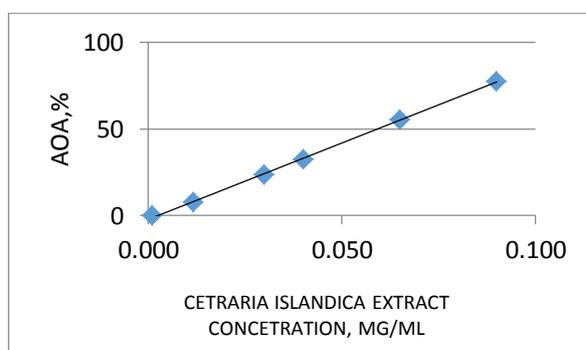


Figure 4: 1, 1-diphenyl-2-picryl-hydrazyl radical scavenging activity of the ethanolic 96% extracts of *Cetraria islandica*

Ferric reducing antioxidant power (FRAP) assay

The results of Ferric reducing antioxidant power assay are presented in Table 2. The tested extracts showed next results: 486 $\mu\text{mol/L}$ (EE96) 135 $\mu\text{mol/L}$ (EE70) and 158 $\mu\text{mol/L}$ (EE40).

The free-radical-scavenging activities of the extracts and purified compounds were evaluated through 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) method

The highest result showed the extract of *Cetraria islandica* 40%, which makes it possible to talk about the possibility of using extracts to create new drugs. The results are presented in Table 2.

Conclusion

Our experimental research shows the feasibility of obtaining *Cetraria islandica* extract to remove a complex of biologically active substances (flavonoids and polyphenols). The results of our studies of antioxidant activity with different concentrations of ethanol confirm the prospect of using *Cetraria islandica* for the manufacturing of extracts and the creation of pharmaceutical and cosmetic products based on them.

The antioxidant activity of the three extracts of *Cetraria islandica* by the DPPH method and the total content of polyphenolic compounds by the Folin-Ciocalteu method was determined. Research have shown that the antioxidants found in these extracts have a radical absorbing and antioxidant activity. However, the high

correspondence between the antioxidant properties and the total phenol content indicates that phenolic compounds are the major contributors to the antioxidant activity of these extracts. This again proves the effectiveness of 70% alcohol-water extract as an extractant for phenolic compounds.

The obtained results confirmed the high potential of the extracts as a source of phenolic compounds, in particular flavonoids, and the feasibility of further studies the phytochemical composition and biological activity of moss elephants.

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