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Research article

EXTRACT OF EUROPEAN SPRUCE STROBILES AS A PROMISING TOOL TO MINIMIZE THE RISKS OF INFLAMMATION

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The article is devoted to examining anti-inflammatory activity of dry aqueous extract of European spruce (Picea abies) strobiles using different routes of administration.

Strobiles of European spruce for extracts were harvested on the territory of the Perm region of the Russian Federation in a mixed forest with a predominance of European spruce and Scots pine (Pinus sylvestris). Dry aqueous extract was obtained according to the original patented method. Procyanidins content in spruce strobiles and dry extracts was determined by using acid cleavage of procyanidins to anthocyanidins according to the Porter method. Anti-inflammatory activity was established by using carrageenan-induced paw edema in rats. White laboratory outbred Wistar rats were used in the experiment.

According to the results of the study, the procyanidin content was found to equal approximately 13 % in the samples of spruce strobiles. Intraperitoneal administration of dry extract of strobile spruce at a dose of 100 mg/kg was established to induce pronounced anti-inflammatory activity. Intraperitoneal administration of smaller doses of strobile extract resulted in pronounced anti-inflammatory activity at a dose of 50 mg/kg. A dose of 10 mg/kg successfully suppressed inflammation (50 % edema suppression) 1 and 3 hours after carrageenan administration ($p < 0.05$) according to hydrometric data, but this was not confirmed by photometric data. Oral administration of the extract showed no anti-inflammatory activity. With the rectal route of administration, no pronounced anti-inflammatory activity was found in the studied extract.

The extract of spruce strobiles obtained by the original method contains 56 % procyanidins and exhibits pronounced anti-inflammatory activity when administered intraperitoneally. The use of the extract in oral and rectal routes of administration requires more in-depth study.

Keywords: *European spruce, strobiles, dry extract, procyanidines, intraperitoneal administration, oral administration, rectal administration, anti-inflammatory activity.*

In the Russian Federation, coniferous strobiles are harvested to obtain seeds for forest regeneration. Forest regeneration includes forest seed industry and reforestation. Seed yield from strobiles is only about 2 %. After seeds have been extracted, strobiles remain in forestry in huge quantities. The rich chemical composition of common spruce strobiles is the basis for searching for promising pharmacologically active substances.

We have developed a method for obtaining a dry aqueous extract of common spruce strobiles where one of the leading groups of biologically active substances are condensed tannins or procyanidins.

Proanthocyanidins were first examined by Jacques Masquelier in the 1940s in a study of

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pine bark, which Native Americans boiled to treat scurvy [1]. Procyanidins are derivatives of flavan-3-ols that have a typical C6-C3-C6 flavonoid backbone. In total, about 15 subclasses of proanthocyanidins have been identified, of which procyanidins are the most common. There are dimeric, trimeric, tetrameric and polymeric procyanidins [2].

Procyanidins are of medical interest because of their multiple beneficial properties [3, 4]. Procyanidin D1 has been shown to be effective in rheumatoid arthritis when administered orally for 23 days. The antiarthritic effect was mediated by the regulation of Th17 (T-helpers producing interleukin-17) / Treg (T-suppressors) cell balance; the regulatory effect, in turn, was associated with the inhibitory effect of procyanidin D1 on aromatic hydrocarbon receptor expression [5]. Procyanidin B1 increases the influx of mesenchymal stem cells to wounds and accelerates wound healing in a diabetic mouse model [6]. Procyanidin C1 exhibits antitumor properties by inducing apoptosis in breast cancer cells [7].

Literature sources describe different effects of procyanidins on the immune system. Using a splenocyte proliferation model, it has been shown that procyanidin D1 exhibits an immunosuppressive effect reducing the levels of interferon-α and interleukin–2 in a dose-dependent manner [8].

An aqueous extract of black spruce bark contains a significant amount of procyanidins and exhibits antiradical and anti-inflammatory activity [9]. Type A procyanadins isolated from cinnamon bark – *Cinnamomum verum J. Presl, (Lauraceae family)* exhibit anti-inflammatory activity in rat models of carrageenan edema and adjuvant-induced arthritis [10].

According to the WHO, chronic inflammatory diseases are one of the most important causes of death in the world. Their prevalence in the world is expected to grow steadily over the coming years. Inflammatory diseases include stroke, chronic respiratory disease, heart disease, cancer, obesity and diabetes, etc.[1] [11].

Considering high pharmacological activity of procyanidins isolated from plant sources, it is of interest to identify anti-inflammatory activity of spruce strobile extracts.

The purpose of the research was to study procyanidins levels in a dry aqueous extract of European spruce strobiles and evaluate its anti-inflammatory activity using different routes of administration.

Materials and methods. The object of the study is an extract of European spruce (*Picea abies (L.) Karst., Pinaceae*) strobiles growing in the Perm region (Russia). To obtain the extract, spruce strobiles were harvested on the territory of the Ilyinsky district of the Perm region in a mixed forest with a predominance of European spruce and Pinus sylvestris. After harvesting, raw materials were subjected to air-shadow drying.

The method of obtaining the extract. About 50 g of spruce strobiles crushed to particles passing through a sieve with a hole diameter of 2 mm, are placed in a flask, are added 1.5 liters of water (hydraulic ratio 1:30) and extracted with stirring for 1.5 hours while heating at 80–85 °C. The extraction completed, the raw material is separated from the extract by filtration. The resulting extract is concentrated under vacuum at a temperature of 80–85 °C 10 times from the original volume. Next, the ballast, inactive fraction is separated by cooling the evaporated extract at a temperature of -18 °C for 15 minutes. In this case, a precipitate falls out, which contains a part of the polysaccharide complex, proteins, tannins, and resinous substances. The precipitate is compacted by centrifugation and discarded. Next, the supernatant is evaporated in a vacuum evaporator to a thick mass and dried in an oven at a temperature of 50 °C[2].

The extract is a light brown amorphous powder with a specific odor, soluble in water,

[1] Rukovodstvo po provedeniyu doklinicheskikh issledovanii lekarstvennykh sredstv. Chast' pervaya [Guidelines for conducting preclinical studies of medicines. Part One]. In: A.N. Mironov ed. Moscow, Grif i K, 2012, 944 p. (in Russian).

[2] Pat. RF RU 2756009C1. Sposob polucheniya sredstva, obladayushchego protivovospalitel'noi aktivnost'yu [Method of obtaining a drug with anti-inflammatory activity]: patent for an invention. D.Yu. Apushkin, A.I. Andreev, D.K. Gulyaev, V.D. Belonogova, I.P. Rudakova, V.V. Novikova, date of publication: September 24, 2021 (in Russian).

partially soluble in 50 % and 70 % ethyl alcohol, insoluble in diethyl ether, ethyl acetate, or chloroform.

Obtaining microcapsules in a shell of cellulose acetate phthalate (CAP). Cellulose acetate phthalate (CAP) coated microcapsules are obtained by evaporating a highly volatile solvent in a liquid medium. Initially, 2 g of the powder is ground in a mortar in a dry form, placed in 20 ml of a polymer solution (5 % solution of cellulose acetate phthalate) in a chemical beaker and dispersed for 10 minutes on a magnetic stirrer. Then, 150 ml of vaseline oil is poured into the reactor (chemical beaker). The installation with an anchor stirrer is lowered into the glass and the equipment is turned on. The resulting drug suspension is poured into the reactor in a thin stream with the anchor mixer running and mixed for 15 minutes at 20 °C and a rotation speed of 800 rpm, preventing the mixture from being released. To cure the shells (remove the volatile solvent), the temperature is raised to 40 °C based on 5 °C after 20 minutes with a constantly running stirrer. After hardening (microcapsules do not flatten when pressed with a glass rod on filter paper), the stirrer is turned off. The resulting microcapsules are separated from the dispersion medium (vaseline oil) using a grid with a hole size of 0.2 mm. The microcapsules separated from the oil are washed 3 times with hexane (15–20 ml per portion). The finished microcapsules are left to air dry at room temperature.

Determination of procyanidins content. The procyanidin content in spruce strobiles and its dry extracts was determined using acid cleavage of procyanidins to anthocyanidins according to the Porter method [12, 13].

About 0.2 g of the extract (accurately weighed) (weighed amount of 1.0 grams of strobiles with a particle size that goes through a sieve size of 0.5 mm) was placed in a 100 ml round-bottom flask. 20 ml of 60 % ethyl alcohol was added, closed with a cork and weighed with an error of ± 0.01 g. Next, the flask was attached to a reflux condenser and heated in a water bath at a temperature of 80 °C for 15 minutes (40 minutes for the strobiles). After cooling to room temperature, the flask with stopper was weighed and brought to the initial mass with 60 % ethyl alcohol. The contents of the flask were centrifuged for 10 minutes at a speed of 2000–3000 rpm; 0.1 ml of the obtained extract was transferred into a 50 ml round-bottom flask, 0.9 ml of 60 % ethyl alcohol, 6 ml of acid butanol, 0.2 ml of an iron-containing reagent were added. The resulting mixture was attached to a reflux condenser and heated in a water bath at 80 °C for 50 minutes. The resulting solution was cooled at room temperature.

The optical density of the solution was measured on a SF 2000 spectrophotometer at a wavelength of 540 nm in a cuvette with a layer thickness of 10 mm using a solution consisting of 1 ml of 60 % ethyl alcohol, 6 ml of acid butanol, and 0.2 ml of an iron-containing reagent as a reference one.

The procyanidin content in terms of cyanidin chloride (%) was calculated by the formula:

$$X = \frac{A \cdot 20 \cdot 7.2 \cdot 100}{136 \cdot m \cdot 0.1 \cdot (100 - W)},$$

where

A is optical density of the test solution;

136 is specific absorption rate $E^{1\%}_{1\text{см}}$ of cyanidin chloride;

m is mass of raw materials (extract), g;

W is weight loss on drying, %.

White laboratory outbred Wistar rats and white laboratory outbred ICR (CD-1) mice were used in the experiment. Animal preparation included selection by sex, age and health status. Within the selected subpopulation, randomized selection was performed using a random number generator in the experimental groups, the reference group (diclofenac sodium) and the control group. Each group included at least 6 animals[3]. Animals in groups

[3] Rukovodstvo po eksperimental'nomu (doklinicheskomu) izucheniyu novykh farmakologicheskikh veshchestv [Guidelines for the experimental (preclinical) study of new pharmacological substances], 2nd ed. In: R.U. Khabriev ed. Moscow, Meditsina Publ., 2005, 826 p. (in Russian).

were labeled by applying through individual labels. Randomization quality control was performed on the basis of testing the significance of mass shifts and the homogeneity of variances before the experiment.

Design of animal experiments. The dry strobile extract was dissolved in 0.9 % NaCl solution or 2 % food starch solution, and administered to animals intraperitoneally, orally or rectally at doses of 100 (intralaboratory standard screening dose), 50 or 10 mg/kg 40 minutes before administration of a 1 % carrageenan solution (Sigma Aldrich, USA). Enteric-coated tablets of diclofenac sodium, 0.05 g, produced by OOO Ozon, Zhigulevsk, were used as the reference drug. The reference drug was dissolved in 0.9 % physiological NaCl solution or 2 % food starch solution and administered to animals. Animals in the control group received 0.9 % physiological NaCl solution or 2 % starch solution as an equistress effect. There was no reference group for the rectal form, and the control group received a 2 % starch solution in an equal (with the experimental group) volume. A solid dosage form of a plant extract (microcapsules) was administered orally after suspension in a 2 % food starch solution. The dose for the animal was determined based on the active substance (spruce strobile extract) contained in the dosage form. An acute inflammatory reaction was induced by subplantar administration of 0.1 ml 1 % carrageenan. An increase in foot volume, indicating the development of edema, was assessed using an aqueous plethysmometer and an anhydrous plethysmometer, where an optical three-dimensional measuring system is used [14, 15]. The anti-inflammatory effect was evaluated by reduction in the volume of edema in the experimental groups compared to the control group. To assess the anti-inflammatory activity of substances, the following indicators were used:

1) The value of the growth percentage. It characterizes the degree of increase in the volume of the paw in the experimental group compared with the control calculated by the formula:

$$X = \frac{a}{b} \cdot 100\ \%,$$

where X is the value of the growth percentage; a is the background value of the paw volume; b is the value of the paw volume 1 / 3 / 5 hours after administration of carrageenan.

2) The percentage of edema inhibition (characterizes the ability of a substance to inhibit the development of inflammation or reduce the amount of exudate that comes out of the blood vessels into the area of inflammation). It is calculated according to the formula:

$$Y = \frac{c}{d} \cdot 100\ \%,$$

where Y is the percentage of edema inhibition; c is the median percentage increase in the paw volume in the control group 1 / 3 / 5 hours after the administration of carrageenan; d is the median percentage increase in the paw volume in the experimental group 1 / 3 / 5 hours after the administration of carrageenan.

To analyze the experimental data, we used the nonparametric two-sided Wilcoxon signed-rank test for independent samples; corrections for multiple comparisons were not introduced[4] [16]. Outliers were identified and excluded according to the 1.5 IQR rule (outliers removed using 1.5*interquartile range rule).

Results and discussion. At the first phase of the study, we identified the content of procyanidins in strobiles of European spruce and two types of extracts, in the extract obtained by the method described in patent № 2756009C1, and in the extract obtained by conventional hot-water extraction without sediment removal. The presence of a condensed group in the extract of tannins was previously proven using qualitative reactions: a reaction with a 1 % solution of iron-ammonium alum and a Stiasny reaction. For quantification of procyanidins, a modified Porter method [12] was used, which is based on the acid cleavage of procyanidins to anthocyanidins. The results of the study are presented in the Table 1.

[4] Hollander M., Wolfe D.A., Chicken E. Nonparametric Statistical Methods. Canada, John Wiley & Sons, 2013, 848 p.

Table 1

The content of procyanidins in strobiles of European spruce and hot-water extracts

Sample	Content of procyanidins, %
Strobiles of European spruce	13.21 ± 1.57
Hot water extract with sediment removal (Patent RU № 2756009C1)	56.75 ± 2.53
Hot water extract without sediment removal	18.61 ± 0.65

According to the results of the study, the content of procyanidins was found to equal approximately 13 % in samples of common spruce strobiles. The scheme for obtaining a dry aqueous extract of common spruce strobiles, specified in patent № 2756009C1, makes it possible to obtain a substance with a content of procyanidins more than 3 times higher than the content in the extract of common spruce strobiles without removing the sediment. A significant increase in the content of procyanidins can affect the pharmacological activity since many activities are associated with procyanidins, including anti-inflammatory effects [9].

The results of studying the anti-inflammatory activity of the obtained extract when administered intraperitoneally are presented in Table 2.

Table 2 presents data on the experiment, the purpose of which was to determine the presence of pronounced anti-inflammatory activity of dry spruce strobiles extract. Spruce strobile extract showed pronounced anti-inflammatory activity when administered intraperitoneally to rats. Moreover the effect was so strong that the carrageenan edema model itself could not develop unlike the control or the reference groups. Diclofenac was used as a reference drug, as it is one of the most popular NSAID today. Diclofenac showed a pronounced anti-inflammatory activity, but the anti-inflammatory effect of the spruce strobile extract was higher, especially during the third hour of the experiment. Such observations were noted both in photometric and hydrometric measurements.

The RR (relative risk) of developing an increase in rat paw edema in the group receiving diclofenac relative to the group receiving spruce strobile extract is 2.94 at the first hour; at the third hour, 3.94; at the fifth hour, 4.88. OR (odds ratio) that an increase in inflammation will occur in the group receiving diclofenac, in comparison with the group receiving the extract of spruce strobiles, equals 5 at the first hour, 10.15 at the third hour, and 24.4 at the fifth hour. This indicates a more pronounced anti-inflammatory effect of spruce strobile extract in comparison with diclofenac. The use of spruce strobile extract minimizes the risks of developing an inflammatory reaction.

To confirm the effectiveness of spruce strobile extract as an anti-inflammatory agent, it was of interest to investigate anti-inflammatory activity in different animal species. CD-1 mice were used as the second model animal. The results of exploring the anti-inflammatory activity of spruce strobile extract in mice are presented in Table 3.

Table 2

Anti-inflammatory activity of spruce strobile extract when administered intraperitoneally to rats

Substance code / dose	Route of administration	Assessment method	Edema inhibition (%)					
			1 h	p-value	3 hrs	p-value	5 hrs	p-value
Spruce strobile extract 100 mg/kg	ip	Photo	> 95	**0.020**	99.6	**0.004**	> 95	**0.005**
Spruce strobile extract 100 mg/kg	ip	Hydro	84.6	**0.004**	>95	**0.004**	93.8	**0.004**
Diclofenac 10 mg/kg	ip	Photo	67.9	0.2403	61.8	**0.0411**	55.3	0.0651
Diclofenac 10 mg/kg	ip	Hydro	86.1	**0.0022**	72.1	**0.0050**	45.1	**0.0022**

Note: * ip – intraperitoneally; photo – measurement with an anhydrous plethysmometer, where an optical three-dimensional measuring system is used; hydro – measurement with a water plethysmometer, *p* (Wilcoxon signed-rank test) – *p*-value according to the Mann – Whitney test. **Bold indicates *p*-values < 0.05**.

Table 3

Anti-inflammatory activity of spruce strobile extract when administered intraperitoneally to mice

Group	1 h			4 hrs		
	Median Growth Percentage, %		Paw edema inhibition (%)	Median Growth Percentage, %		Paw edema inhibition (%)
Control	26.67	*p*-value	-	31.29	*p*-value	-
Spruce strobile extract	12.75	**0.0021**	52.2	10.63	**0.0021**	66.0
Diclofenac	11.49	**0.0297**	56.9	21.26	0.1244	32.0

Note: **p* (Wilcoxon signed-rank test) – *p*-value according to the Mann – Whitney test. **Bold indicates *p*-values < 0.05**.

The results of the experiment indicate pronounced anti-inflammatory activity of the spruce strobile extract, which was confirmed in screening experiments on two animal species, when assessing the volume of edema by two mutually independent instrumental methods.

Table 4 presents data on an experiment aimed at determining the minimum effective dose (to reduce the amount of substance used) that would also effectively suppress the inflammation model. For this, a small dose range was taken: 50 mg/kg and 10 mg/kg (data on a dose of 100 mg/kg are present in the previous experiment).

Table 4 shows that intraperitoneal administration of spruce strobile extract at a dose of 50 mg/kg to rats resulted in a considerable and statistically significant suppression of the inflammatory response. A dose of 50 mg/kg of spruce strobile extract can be used as a substitute for a dose of 100 mg/kg. A dose of 10 mg/kg, according to hydrometric data, suppresses inflammation (50 % suppression of edema) at the 1st and 3rd hour after the administration of carrageenan ($p < 0.05$), but this is not confirmed by photometric data.

The next stage of our work involved exploring anti-inflammatory activity of the spruce strobile extract when administered orally. Influence of the aggressive environment in the stomach was reduced by using microencapsulation. Since many anti-inflammatory drugs are used as suppositories, another experiment was conducted using this route of administration. The results of experiments with oral and rectal routes of administration are presented in table 5.

Oral administration of the extract had no significant effect on the inflammatory response. When assessing the oral exposure to a dry aqueous extract of common spruce strobiles from the first to the fifth hour, we did not establish any statistically significant difference

Table 4

Anti-inflammatory activity of spruce strobile extract when administered intraperitoneally (dose reduction)

Substance code	Route of administration	Assessment method	Edema inhibition (%)					
			1 h	*p*-value	3 hrs	*p*-value	5 hrs	*p*-value
Spruce strobile extract 50 mg/kg	ip	Hydro	> 95	**0.004**	> 95	**0.004**	> 95	**0.004**
Spruce strobile extract 10 mg/kg	ip	Hydro	58.5	**0.001**	50.2	**0.001**	17.9	**0.001**
Spruce strobile extract 50 mg/kg	ip	Photo	> 95	**0.032**	> 95	**0.008**	> 95	**0.008**
Spruce strobile extract 10 mg/kg	ip	Photo	28.2	0.329	39.4	0.082	5	0.792

Note: * ip – intraperitoneally; photo – measurement with an anhydrous plethysmometer, where an optical three-dimensional measuring system is used; hydro – measurement with a water plethysmometer, *p* (Wilcoxon signed-rank test) – *p*-value according to the Mann – Whitney test. **Bold indicates *p*-values < 0.05.**

Table 5

Anti-inflammatory activity of spruce strobile extract after oral and rectal administration

Substance code	Route of administration	Assessment method	The difference between the experimental and control groups, *p*-value		
			1 h	3 hrs	5 hrs
Spruce strobile extract 50 mg/kg	Orally	Photo	0.862	0.728	0.281
Spruce strobile extract in microgranules 50 mg/kg	Orally	Photo	0.731	0.731	0.731
Spruce strobile extract 50 mg/kg	Rectally	Photo	0.429	0.177	0.247

Note: * Photo – measurement with an anhydrous plethysmometer, where an optical three-dimensional measuring system is used, *p* (Wilcoxon signed-rank test) – *p*-value according to the Mann – Whitney test.

from the control group in the inhibition of inflammation. Similarly, as a result of the experiment with rectal administration, there was no statistically significant difference with the indicators of the control group in terms of the level of anti-inflammatory activity. Oral administration of microcapsules with spruce strobile extract also did not reduce the severity of carrageenan edema.

This study established that the concentration of procyanidins occurs when using a technique involving a stage of sediment removal by a sharp change in temperature. The content of procyanidins obtained by this method is higher than by using standard hot water extraction.

Procyanidins are one of the main groups of biologically active substances in spruce strobile extract. The anti-inflammatory effect of procyanidins is associated with the ability to reduce the production of reactive oxygen species in the focus of inflammation. Reactive oxygen species are involved in the activation of the NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling system and the MAPK (mitogen-activated protein kinase) signaling pathway. The MAPK signaling pathway determines the expression of various genes by regulating several pro-inflammatory factors [17].

Intraperitoneal injection of spruce strobile extract leads to a significant inhibition of the inflammatory response in comparison with the control. The anti-inflammatory activity of spruce strobile extract was confirmed in two animal species at three dose levels (100, 50, 10 mg/kg) using an aqueous and anhydrous plethysmometer for measurement. This study may be a starting point in the development of anti-inflammatory drugs for use in medical and veterinary practice.

There are inconsistent data in the literature on the absorption of procyanidins from the gastrointestinal tract. Some studies state that procyanidins are able to be absorbed from the gastrointestinal tract and undergo glucuronidation and sulfation [18, 19]. In contrast, other studies have shown that procyanidins have very low bioavailability [20]. Bioavailability can also influence the severity of the anti-inflammatory effect of an extract. Our study showed that oral administration of the studied extract did not lead to suppression of the inflammatory response. However, it should be noted that the extract was administered to the animals once; long-term administration of the extract has not been evaluated. The literature reports that the administration of procyanidin D1 for 23 days led to the suppression of the inflammatory response against the background of rheumatoid arthritis [5]. This indicates that the studied extract rich in procyanidins does not show a rapid and pronounced anti-inflammatory effect when taken orally, but may be effective with long-term oral use.

In the environment of gastric juice, many substances are destroyed or change their structure. To reduce the effect of gastric juice on the extract of spruce strobiles, microcapsules with CAP were obtained. Microcapsules are capsules consisting of a thin shell of CAP, spherical or irregular in shape. The use of this approach makes it possible to obtain enteric microcapsules. Our study established that mi-

croencapsulation did not lead to an increase in the anti-inflammatory activity of the spruce strobile extract when administered orally. This indicates that the absence of pronounced anti-inflammatory activity in the spruce strobile extract is not associated with possible changes in the structure of substances in the gastrointestinal tract.

Conclusion. Our study established that a dry aqueous extract of European spruce strobiles, obtained by an original method, contains a significant amount of procyanidins. The experiments on laboratory animals established that the studied extract has pronounced anti-inflammatory activity when administered intraperitoneally. The studied extract is active in two animal species. No pronounced anti-inflammatory activity has been established after oral or rectal administration of the extract. The features of absorption of common spruce strobiles extract and oral use as an anti-inflammatory agent require more in-depth study.

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Competing interests. The authors declare no competing interests.

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