CHEMICAL AND PHARMACOLOGICAL PROPERTIES OF DRY EXTRACT FROM BLACK BIRCH FUNGUS

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Black birch fungus (*Inonotus obliquus f. sterillis*, known in Russia as "chaga") is used in folk medicine for the treatment of gastrointestinal tract disorders. Since 1955, a dense extract of this fungus has been produced on the commercial level and used in medical practice as the drug befungin.

The purpose of this work was to study the chemical composition and pharmacological properties of the total dry black birch fungus extract (DBFE), prepared according to a new patented technology [1], and the individual biologically active fractions.

EXPERIMENTAL CHEMICAL PART

For the chemical analysis, a chromogenic polyphenol complex was isolated from DBFE by acidification with hydrochloric acid to pH 2.2 – 2.5. According to the published data [2, 3], the polyphenol complex of DBFE was then hydrolyzed with a 10% hydrochloric acid solution in an autoclave at a pressure of 1.5 atm and a temperature of 126°C.

Neutral substances isolated from the hydrolyzate were used for the extraction of phenols and acids with chloroform in a neutral medium. Then acids were separated from phenols by using sodium bicarbonate, and flavonoids were isolated from the neutral medium with ethyl acetate, leaving carbohydrate in the aqueous layer [4, 5].

Thus, we have obtained the fractions of bound phenols, bound carbohydrates, flavonoids, carboxylic acids, and neutral compounds (Table 1). The free phenols and free carbohydrates were determined in an aqueous solution upon precipitation of the polyphenol complex (Table 1).

The total content of free and bound phenols was determined by spectrophotometry at a wavelength of 460 nm using a 2% solution of 4-aminoantipyrine and a 1% solution of potassium hexacyanoferrate [6]. The content of phenols was determined with the aid of a calibration plot constructed for the phenol solutions. Note that the content of phenols in the DBFE preparations studied in this work (0.25%) is close to that reported for concentrates (0.28%) [7].

The phenol and acid fractions were analyzed by gas chromatography (GC) using a Khrom-5 instrument with a plasma

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TABLE 1. Composition of the New DBFE Preparation

Fraction	Contents, %
Phenols (free)	0.62
Phenols (bound)	0.19
Carboxylic acids	6.64
Flavanoids	0.09
Carbohydrates (free)	3.88
Carbohydrates (bound)	14.00
Other compounds	75.14

TABLE 2. Data of GC Analyses for the Phenol Fractions Obtained from the New DBFE Preparation

Standard compounds	Retention temperature, °C		Phenol assignment
	standard compounds	phenols of pigment complex	
2,6-Xylenol	150	133	–
Phenol	156	142	–
o-Cresol	157	162	*p*-Cresol
p-Cresol	162	195	
m-Cresol	164	203	Pyrocatechol
2,3-Xylenol	168	212	–
Thymol	170	223	α-Naphthol
3,4-Xylenol	173	233	Hydroquinone
Pyrocatechol	203	237	Resorcin
α-Naphthol	224	244	–
β-Naphthol	228	...	...
Hydroquinone	233	...	...
Resorcin	237	...	...

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TABLE 3. Data of GC Analyses for the Acid Fractions Obtained from the New DBFE Preparation

Standard compounds	Retention temperature, °C		Acid assignment
	standard compounds	acids of pigment complex	
n-C_3	126	181	*n*-C_{10}
i-C_4	127	196	—
n-C_6	147	201	*n*-C_{12}
n-C_8	164	212	*i*-C_{13}
n-C_{10}	181	218	*n*-C_{14}
n-C_{12}	200	227	*i*-C_{15}
i-C_{13}	212	...	...
n-C_{14}	217	...	...
i-C_{15}	227	...	...
n-C_{16}	231	...	...
n-C_{18}	246	...	...

ionization detector and a glass column (filled with a modified packing material Chromaton N-AW-DMCS + 15% Carbovax 20M) with a length of 1.2 m and a diameter of 0.3 cm. The carrier gas (helium) was supplied at a rate of 40 ml / min. The chromatograms were measured in a programmed-temperature mode. The phenol components were identified on the basis of retention temperatures known for the standard compounds (Table 2). This method allowed us to determine individual phenols such as *p*-cresol, pyrocatechol, hydroquinone, resorcin, and α-naphthol.

According to the published data, the hydrolyzate of the pigment complex of DBFE contains aromatic acids (syringic, vanillic, *p*-hydroxybenzoic, pyrocatechuic, and pyrogallic) as well as volatile acids and oxalic acids [4, 5].

We have also studied the fatty acid components. Carboxylic acids of the pigment complex were isolated in the form of salts (upon treating the phenol – acid fraction with a sodium bicarbonate solution), purified using a KU-2 cation exchanger in the H^+-form, and concentrated on a water bath.

The qualitative and quantitative GC analyses were performed under the conventional conditions described for phenols. We have detected for the first time the higher fatty acids having normal structures (C_{10}, C_{12}, and C_{14}) and isostructures (C_{13}, C_{15}) (Table 3).

Flavonoids isolated from DBFE were classified into several groups. The presence of flavonoids was confirmed by cyanidine reaction [8]. The solution of isolated flavonoids acquired a pink color upon reduction with magnesium in the presence of hydrochloric acid. The cyanidine reaction is characteristic of flavones, flavanones, and catechols. The assignment of flavonoids to various groups was based on the 2D chromatography patterns obtained with an FN-12. The longitudinal runs were made using a solvent system of *n*-BuOH–CH_3COOH–H_2O (4 : 1 : 5), and the perpendicular direction was run with a 15% acetic acid. The chromatograms were dried in air, treated with ammonia vapors and a 5% aqueous sodium carbonate, and viewed under visible or UV illumination. The spots had yellow, green-yellow, and brown colors. Comparison with the published data [9] and the patterns of reference compounds allowed us distinguish between the flavonoids belonging to flavones, flavanones, and catechols (Table 4).

There are various methods that can be used for the quantitative analysis of carbohydrates. The most widely employed technique is based on the reaction with a phenol – sulfuric acid mixture [10]. This method allows the total amount of carbohydrates to be determined without preliminary hydrolysis of polysaccharides in the presence of humic substances and inorganic components.

To 1 ml of an aqueous solution obtained upon separation of the chromogenic polyphenol complex were added 1 ml of a 5% aqueous phenol solution and 5 ml of concentrated sulfuric acid. The mixture was cooled to room temperature. The solution gradually acquired a pink color that was stable during 24 h [11]. The intensity of coloration was evaluated by spectrophotometric measurements at 490 nm. The samples were measured in 1-cm cells and referenced to a mixture of 1 ml distilled water, 1 ml of a 5% aqueous phenol, and 5 ml of concentrated sulfuric acid. The content of carbohydrates was determined from a calibration plot constructed for the standard D-glucose solutions.

The total (free and bound in the polyphenol complex) carbohydrates were determined by the phenol – sulfuric acid method, and the

TABLE 4. Data on the Flavonoid Determination by Paper Chromatography

Sample	Chromatogram processing	Spot color	
		visible	UV
Apigenin	–	pale-yellow	brown
	NH_3 vapors	greenish-yellow	green-yellow, bright
	5% aqueous Na_2CO_3	bright-yellow	–
Naringenin	–	–	–
	NH_3 vapors	pale-yellow	–
	5% aqueous Na_2CO_3	green-yellow	–
Morin	–	pale-yellow	green-yellow
	NH_3 vapors	green-yellow	green-yellow, bright
	5% aqueous Na_2CO_3	yellow	–
Quercetin	–	pale-yellow	green-yellow
	NH_3 vapors	green-yellow	green-yellow, bright
	5% aqueous Na_2CO_3	yellow	–
Flavonoids of pigment complex	–	pale-yellow	brown, green-yellow
	NH_3 vapors	pasle-yellow, greenish	green-yellow, bright
	5% aqueous Na_2CO_3	yellow, green-yellow	–

bound carbohydrates were determined upon hydrolysis of the chromogenic polyphenol complex.

Carbohydrates were identified by column chromatography using a KU-2 cation exchanger in the Ca^{2+} form, eluted with distilled water at a column temperature of 54°C. The samples were taken as 1 ml aliquotes and analyzed by spectrophotometry at 490 nm using the phenol–sulfuric acid method. An analysis of the fractions taken revealed the presence of glucose, galactose, xylose, and sorbit. The components were identified by comparing the retention volumes of the individual carbohydrates to those determined using the reference compounds measured under the same experimental conditions. The amounts of identified carbohydrates were 2.32% in the free state and 8.95% in the bound state.

Nonidentified compounds can be tentatively assigned to glycosides of various nature.

EXPERIMENTAL PHARMACOLOGICAL PART

The acute toxicity of DBFE was determined by single injections to mice at doses ranging from 1000 to 6000 mg/kg in a 1 ml volume. Each dose was studied in a group of ten animals.

The acute ulceration of the mucous membrane of the stomach in Wistar rats was induced in mongrel mice by single intraperitoneal injections of reserpine, noradrenaline, or butadion at a dose of 5.0, 2.5, and 300 mg/kg, respectively, or by an acute stressor action (hanging for 18–20 h by the neck skin folder) [12]. The chronic ulceration was reproduced as described in [13]. The efficacy of drug preparations was evaluated by inhibition of the extent of ulcer damage in the mucous membrane of the stomach (measured by the average number of ulcers per animal in the group), percentage of animals with ulcers, the Pauls index (the degree of ulceration multiplied by the percentage of animals with ulcers), and the antiulcerogenic activity (the ratio of the Pauls indices in the control and test groups) – preparations with the latter ratio exceeding two were considered active.

The oncologic tests were performed using the model of metastatic Lewis lung carcinoma induced in BIac mice. The experimental tumors were inoculated by subcutaneous injections of 1×10^6 tumor cells in a 0,1 ml volume of physiological solution. The tumor development was evaluated 25 days after inoculation by determining the weight of the primary tumor focus, the percentage of animals with lung metastases, and the average number of damaged areas on the lung surface per animal in the test group. The antitumor activity was characterized by percentage inhibition of the tumor growth or metastasis, calculated by dividing the difference of values in the control and test groups by the control value and multiplying by 100. The test mice were killed by dislocation of the cervical part of the vertebral column, and the test rats by ether action.

We have studied the specific activity of the total DBFE obtained by the new method [1] and its separate fractions, including the chromogenic (pigment) polyphenol complex, carboxylic acids, and carbohydrates. The reference preparation was represented by the dense commercial fungal extract befungin. The optimum therapeutic doses established in the preliminary experiments using the acute stress model were 0.45 ml/kg for befungin and 90 or 180 mg/kg for DBFE. The separate DBFE fractions (carbohydrates, flavonoids, phenols, and carboxylic acids) were administered at the doses approximately corresponding to their contents in the complex preparation (30, 2, 4, and 12 mg/kg, respectively). The drugs

TABLE 5. Antiulcer Activity of DBFE Preparations

Ulceration model	Test group	Number of test animals	Drug dose (mg/kg or ml/kg)	Extent of ulcer damage	Animals with ulcers, %	Pauls index	Antiulcer activity
Noradrenalin model	control	6	–	10.5 ± 3.5	100	10.50	–
	befungin	6	0.45	1.6 ± 0.6	83	1.32	7.95
	DBFE	6	90.00	0.8 ± 0.5*	33	0.26	40.40
Reserpine model	control	6	–	6.5 ± 0.7	83	5.39	–
	befungin	6	0,45	1.3 ± 0.4*	68	0.88	6.13
	DBFE	6	90.00	2.5 ± 0.2*	83	2.07	2.60
	DBFE	6	180.00	0.8 ± 0.1*	50	0.40	13.45
Butadion model	control	10	–	19.6 ± 6.8	100	19.60	–
	befungin	8	0.45	7.2 ± 3.8	100	7.20	2.72
	DBFE	7	45.00	12.7 ± 2.7	100	12.70	1.54
	DBFE	7	90.00	2.1 ± 0.7*	71	1.90	10.30
	DBFE	7	180.00	7.1 ± 2.2	71	5.00	3.92
Neurogenic model	control	10	–	4.7 ± 1.5	100	4.70	–
	befungin	10	1.00	2.2 ± 0.5	70	1.54	3.05
	DBFE	10	180.00	1.2 ± 0.2*	80	0.96	4.89

* Difference against control is statistically reliable for $p \leq 0.05$.

were introduced into the stomach via a gastric pipe for five days and 1 h before the induction of ulcers. In the oncologic tests, the DBFE and reference preparations were introduced for 15 – 20 days after the model tumor inoculation.

The experimental data were processed using the methods of variation statistics.

RESULTS AND DISCUSSION

Study of the acute toxicity of the DBFE preparations showed that single injections of the drug at a dose of 1000 – 6000 mg / kg did not lead to a loss of test mice, produced no changes in the body weight, state of hairs, or motor activity, and did not induce diarrhea. Therefore, DBFE exhibits no acute toxicity in the dose range of 1000 – 6000 mg / kg.

It was found that DBFE possesses a pronounced antiulcerous activity in the noradrenalin, reserpine, and butadion stomach pathology models in rats and the model of neurogenic stressor ulcers in mice (Table 5). The protective effect of DBFE was comparable with that of befungin; in some cases, DBFE was even more effective than the reference drug befungin.

The efficacy of DBFE was also confirmed on the model of chronic ulceration process (the Pauls index for DBFE is reduced by a factor of 8 – 10 against control). Note that befungin was 2 – 2.5 times less active than DBFE.

Study of the antiulcerous action of the individual biologically active fractions isolated from DBFE showed a significant protective effect of DBFE carbohydrates and flavonoids with respect to the butadion and neurogenic models. In the latter model, the antiulcerogenic activity of carbohydrates and flavonoids was measured by the indices 6.6 and 5.1, respectively. The antiulcerous action of these fractions was manifested for the most part by a decreased number of damaged areas in the mucous membrane of the stomach and by a lower number of animals with ulcers in the test groups.

As for the Lewis lung carcinoma model in BIac mice, the DBFE preparation produced a moderate antitumor action, comparable with that of befungin, with respect to the primary focus: the effect of both phytopreparations amounted to 20 – 30%. However, the antimetastatic properties are more pronounced in DBFE than in befungin. Indeed, the metastases in lungs were observed in 100% of animals in the control group, in 57% of mice receiving befungin, and in 16% of mice treated with DBFE. The average number of metastatic manifestations per mouse was 14.0 ± 5.2 in the control group, 5.1 ± 2.7 in mice receiving befungin (percentage inhibition, 64%), and as low as 0.17 ± 0.1 (99% inhibition) in the group treated with DBFE.

The antimetastatic properties of DBFE were also confirmed in the model of "neglect" tumor damage, whereby the drug administration began on the tenth day after inoculation. Neither befungin nor DBFE significantly affected the development of the primary tumor focus, showing only a weak protective trend. The antimetastatic effect of DBFE was nevertheless more pronounced than that of befungin: the average number of metastases was 2.2 ± 1.2 and 12.5 ± 3.8 (14.2 ± 4.0 in the control group) and the proportion of animals with metastases was 40 and 60% (100% in the control), respectively. Thus, the dry black birch fungus extract is advantageous to befungin in the model of neglected malignant process.

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