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Hepatoprotective Effect of *Inonotus obliquus* Melanins: In Vitro and In Vivo Studies

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Abstract
The purpose of this study is to identify hepatoprotective properties of melanins from aqueous extracts of *Inonotus obliquus* distinguished by microwave modes used in extraction in both in vitro and in vivo studies. In vitro tests were used in studies of the effect of Chaga mushroom melanins on the vitality of cells of a normal human hepatocyte line *Chang Liver*, as well as their hepatoprotective effect and influence on the cell cycle. The hepatoprotective effect was studied in the context of the influence of the toxicant *d*-galactosamine, at a concentration of 150 mM. The results show that the melanin of the aqueous extract of Chaga, obtained in the process of microwave-assisted extraction at 180 W, at concentrations of 10^{-5} and 10^{-3} g/l, displays a hepatoprotective effect, as it increases the vitality of cells under the toxic influence of *d*-galactosamine by 2–2.5 times. In vivo tests were used in studies of the hepatoprotective properties of the melanin of the aqueous extract of Chaga obtained in the process of microwave-assisted extraction at 180 W on white male Sprague Dawley rats. The melanin was administered to rats for 14 days at a dose of 100 mg/kg. Toxic damage was inflicted on the liver using carbon tetrachloride on days 5 to 12 of administering the melanin; the liver was studied and the blood biochemical parameters were determined on day 15. It was shown that melanin produces a hepatoprotective effect which is expressed in the minimization of liver injury signs such as steatosis, necrosis, fat accumulation, and normalization of the total and unconjugated bilirubin, total protein, serum cholinesterase, and gamma-glutamyl transpeptidase levels.

Keywords Toxic hepatitis · Liver injury · Carbon tetrachloride · Cell line of hepatocytes *Chang Liver* · Melanin of *Inonotus obliquus*

1 Introduction

The liver is the largest parenchymal organ that bears the main weight of attacks made by various toxicants or their metabolites, which often cause damage to the liver tissue. Considering the increasing technogenic impact on human health in the modern world, as well as the frequent use of various pharmaceuticals, liver diseases are among the most common diseases affecting different segments of the population [1]. According to the World Health Organization, around 2 billion people with various hepatic disorders had been registered by 2015. Therefore, studies of new potential hepatoprotectors hold the utmost importance in the modern world.

One of the mechanisms of liver cell injury caused by toxicants, e.g., CCl_4, is the intensification of lipid peroxidation [2]. It has been shown that CCl_4 generates the very reactive trichloromethyl (-CCl_3) and peroxy-trichloromethyl (-$OOCCl_3$) radicals which form alkoxy (R°) and peroxy radicals (ROO°) that generate lipid peroxides [3], covalent interactions with critical target molecules such as DNA, lipids, proteins, and carbohydrates including the generation of reactive oxygen species and alterations of the redox status [4]. Vyshtakalyuk with co-authors [5] show that the antioxidative mechanism is

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integral to the realization of the hepatoprotective effect of the Xymedon conjugate with L-ascorbic acid. Thus, it is reasonable to search for hepatoprotectors in the midst of substances with antioxidant properties.

Nowadays, there is a growing interest in natural hepatoprotective agents, including *Inonotus obliquus,* commonly known as the clinker polypore, or Chaga [6]. Chaga extracts have a wide spectrum of therapeutic applications, as Chaga can be used as an antitoxin, antioxidant, adaptogenic, restorative, immunostimulatory, anti-inflammatory, gastroprotective, and hepatoprotective agent [1, 7–9]. There are also some studies that demonstrate the protective properties of the Chaga melanin fraction in relation to probiotics in vitro [10]. It also has been shown to have a cytotoxic effect on cancer cells of PLP2 U251 and other cell lines [11–13].

The main active component of the aqueous Chaga extract is melanins [14]. Melanins have a form of nanoparticles made from aggregates varying in shape and size [15, 16]. Chaga melanins have the unique property of being in a stable free-radical state and possessing a high content of paramagnetic centers. Their therapeutic effect is based on their high antioxidative activity [14]. More often than not, due to the antioxidative, gene-protective, and membrane-stabilizing properties of Chaga melanins, melanin-based pharmaceuticals exert a hepatoprotective effect [15].

The traditional method of aqueous Chaga extraction is remaceration, which takes over 10 h. It was found that microwave-assisted extraction of the mushroom is equally effective. Moreover, the extraction time is reduced by 2–3.5 times. The melanins that separated from these extractions using hydrochloric acid become their main active component. The melanin output and their antioxidative properties, regardless of the microwave intensity used in the extraction, are equal to or higher than those of the melanins obtained by the classic extraction method, which is remaceration [17].

The relevance of the research conducted here lies in the fact that it studies the hepatoprotective properties of melanins isolated from aqueous extracts of *Inonotus obliquus* distinguished by the microwave ratings used in the extraction, that are the main active component of the aqueous extracts of this mushroom.

The purpose of this work is to identify hepatoprotective properties of melanins obtained from aqueous extracts of Chaga distinguished by the microwave modes used in their extraction.

2 Materials and Methods

2.1 Subject of Research

The subject of research is melanins of *Inonotus obliquus* extracts obtained in the process of microwave-assisted extraction at 90 W, 180 W, and 360 W with hydrochloric acid. One more melanin was obtained in the same manner, but at 180 W with the use of 40% ethanol.

The traditional method of Chaga extraction is remaceration, which includes two stages of the fungus maceration at 70 °C with different ratios of raw material and extracting agent, that is, 1:6 at the first stage and 1:4 at the second stage of maceration. The extraction takes 10 h in total. In order to reduce the extraction time by 3.5 times, the first stage of maceration was replaced by the microwave-assisted extraction stage in three different modes [17].

It should be noted that the experimental use of the main active component of widely used aqueous Chaga extracts, which is melanin, reduces the concentration of its application down to very low doses and allows for comparative analysis of the obtained results with the natural hepatoprotective agent.

Antioxidant activity of the obtained samples of melanins has been measured using two methods—phosphomolybdate and phenanthroline [18, 19]. Particle size, Z potential, and electric conductivity have been defined on nanoparticle Analyzer Malvern Zetasizes Nano ZS using dynamic and electrophoretic light scattering [20].

2.2 In Vitro Testing

The tests were conducted on the human hepatocyte cell line *Chang Liver* obtained from the Russian collection of cell cultures of D.I. Ivanovskii Institute of Virology (Moscow). The cells were cultivated in an IGLA MEM medium with added 10% bovine serum FBS, 1% essential amino acids, and gematicine [21].

2.2.1 In Vitro Determination of Hepatoprotective Properties

The cells were isolated using a mixture of trypsin and versene at a ratio of 1:3. The cell-rich fluid was prepared at a concentration of 10^5 cells/ml. The fluid was poured in drops into a 96-well plate at 200 μl per well and incubated for 24 h. To determine its hepatoprotective effect, according to [22], a toxicant, *d*-galactosamine (GaIN), was put in along with the research subjects at a concentration of 150 mM. To provide a control reference (C), the melanin and *d*-galactosamine solutions were not included in the growth medium during the cell culture. The growth medium of the control cell group (C(GaIN)) was inserted only with *d*-galactosamine. The cells were incubated for another 24 h. For each cell group, the culture process was carried out three times.

2.2.2 Cell Staining and Estimation

In order to determine the number of live and dead cells, we prepared a complete growth medium with fluorescent stains at a rate of 198 μl complete growth medium + 2 μl DAPI or

Hoechst 33342 (concentration 1 mg/ml) + 0.5 μl propidium iodide per well. Then, the culture fluid was replaced with the prepared growth medium with stains and incubated for 45 min. After the incubation, the dead and live cells were calculated on a cell analyzer Cytell Cell Imaging System by GE Healthcare in accordance with the standard protocol.

In order to study the cell cycle, the cells were stained as they were incubating in the growth medium with only one added stain, either DAPI or Hoechst 33342, at a concentration of 1 mg/ml on the basis of 4 μl of stain per 196 μl of growth medium in a well.

2.3 In Vivo Testing

The tests were conducted on adult male Sprague Dawley rats weighing 400–450 g, obtained from the Research and Production Enterprise Laboratory Animal Farm based at the Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences (Pushchino). The animals were kept in accordance with [23, 24] in standard conditions in a vivarium with 12 h of daylight and free access to food and water. The animals were fed with complete feed made according to specification (protein 22%, fiber 4% max., fat 5% max., ash 9% max., humidity 13.5% max., caloric value 295 kcal/100 g).

All animal experimentations and protocols were approved by the Local Ethics Committee of Kazan Federal University (Protocol No. 4 dated 18 May 2017).

The test was conducted according to a preventive procedure over the course of 14 days, according to which, the animals were fed M180 melanin (the first group) or Carsil (the second group) for 7 days at a dose of 100 mg/kg. The third and fourth groups were used as control groups and received the equivalent amount of water. Then, while continuing to administer melanin, Carsil, or water, we induced toxic liver damage by administering CCl_4 in accordance with [22]. CCl_4 was administered for 5 days orally in the form of a 35% oily solution at the dose of solution of 1.5 ml/kg 30 min after the administration of melanin or Carsil under consideration. The third group was used as intact or reference group, received water only, but was not subjected to the toxic effect of CCl_4. The fourth group was fed water instead of the subject substances in a similar way in accordance with the procedure and, similar to the experimental groups, was subjected to CCl_4. There were three animals in each group.

After the induction of the toxic liver damage, the melanin, Carsil, and water under consideration were administered for another 2 days. The day after the last administration of melanin, Carsil, or water, that is, day 15 of the experiment, the animals were euthanized by exsanguination through the carotid artery under anesthesia with chloral hydrate. Euthanasia of animals in all groups was carried out in a similar way.

There are no commercial melanin-based pharmaceuticals on the market, so in this study, we chose Carsil as the most adequate reference substance, as its active ingredient is silymarin, an extract of milk thistle. Sylimarin, like melanins, has pronounced antioxidative properties. Due to its antioxidative properties, silymarin displays membrane-stabilizing properties [25, 26] and exerts hepatoprotective activity.

2.3.1 Sample Preparation

Samples of blood and liver were collected at the time of euthanasia.

Serum was prepared by double centrifugation of the blood at 3000 rpm and at a temperature of + 4 °C. Prior to the analysis, the serum was stored in a refrigerating chamber at − 25 °C.

To conduct a histological inspection, the liver samples were set in 4% buffered formalin. The histological analysis was carried out on an automatic tissue processor Sacura Tissue-Tek® VIP™ 5 Jr. The samples were embedded in paraffin and formed into blocks. 4–5-μm thick sections were made on a microtome SacuraAccu-Cut SRM200 and then were stained with hematoxylin and eosin. In order to detect lipids, the samples were cut frozen using a Sacura Tissue-Tek CriO3 Cryostat into 5–7-μm thick sections, which were then stained with Sudan. The embedding medium was made of a mixture of glycerin and 15% gelatin at a ratio of 1:2, respectively.

2.3.2 Analysis of Tissue Specimen

The specimens were subjected to morphometric analysis on an optical microscope by Nikon with a digital camera using the NISBasicResearch software as shown in [27]. The sections stained with hematoxylin and eosin were studied to calculate the area of necrotic tissue in millimeters and determine the percentage of affected tissue over the total visible area of the section, using formula (1):

$$\text{Affected areas of liver}, \% = \frac{\text{Sum of affected areas}}{\text{Total visible area}} \times 100\% \quad (1)$$

The frozen sections stained with Sudan Black B were similarly studied to determine the area of lipid detection over the total visible area of the section by formula (2):

$$\text{Total area of lipids detection}, \% = \frac{\text{Sum of areas of lipids detection}}{\text{Total visible area}} \times 100\% \quad (2)$$

2.3.3 Blood Chemistry

The biochemical markers were studied on an automatic biochemical analyzer ARD 200 (manufactured by OAO Vitako, Russia) using Chronolab kits (Spain). The analysis determined the following blood values: alanine transaminase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), gamma glutamine transferase (GGT), total and unconjugated bilirubin, albumin, total protein, alkaline phosphatase, serum cholinesterase (CE), and glucose.

3 Results and Discussion

3.1 Properties of Tested Melanins

The study has shown that melanin M90 possesses the most pronounced antioxidant activity upon indicators Total Antioxidant Capacity and Reduction of Ferric Ions. Both tests revealed ME180 having the lowest antioxidant activity. Melanins M180 and M360 have close indicator values (Table 1).

The largest polydispersity of colloidal systems (CS) corresponds to melanins M180 and M360. However, M180 CS mainly consists of small- and medium-sized particles (31% and 51.5%, respectively) and melanin M360—of large particles (72.9%). Other studied melanins consist of large particles only (Table 2).

The highest conductivity and Z potential belong to M360 CS. The lowest Z potential was observed in M90 CS, the lowest conductivity in M180 CS (Table 3).

3.2 Determination of the Cytotoxicity of Chaga Melanins on *Chang Liver* Cell Culture

The purpose of this study was to determine whether the melanins under consideration are cytotoxic to *Chang Liver* cells. The study results are given in Fig. 1.

The obtained data suggest that melanins M90 at a concentration of 10^{-5} g/l and ME180 at a concentration of 10^{-10} g/l inhibit the growth of hepatocytes (statistically significant distinctions from C according to U test, $p < 0.05$) when compared to the control group. The number of live cells in the control group came to 70%. The other samples studied did not show inhibitory activity when compared to the control group (statistically significant distinctions from C according to U test, $p < 0.05$). The inhibitory activity of melanins M90 at a concentration of 10^{-5} g/l and ME180 at a concentration of 10^{-10} g/l, perhaps is related to the fact that they have the greatest negative charge, in comparison to other melanins under consideration, a low polydispersity index and a large number of coarse particles of over 410 nm in diameter (Table 2).

3.3 In Vitro Determination of Cytotoxicity of Chaga Melanins

In vitro testing was used to study the hepatoprotective activity of Chaga melanins on a *Chang Liver* cell line. The results of the hepatoprotective activity study are given in Table 4.

Out of all the melanins under consideration, only melanin M180 at a concentration of 10^{-5} g/l (statistically significant distinctions from GaIN according to U test, $p < 0.05$) displayed hepatoprotective activity. The use of melanin M180 at a concentration of 10^{-10} g/l showed only a tendency to a hepatoprotective effect; however, there were not any statistically significant distinctions from C(GaIN). The hepatoprotective properties of melanin M180 may come from the fact that the solution of this melanin is a colloidal system with low conductivity and high polydispersity, containing particles with a diameter of around 180 nm and an approximate Z potential of 64.3 mV.

For melanin M180, the hepatoprotective properties were studied in a broader range of concentration from 10^{-1} g/l to 10^{-12} g/l. The results are presented in Table 5.

The results suggest that melanin M180 exerted hepatoprotective activity only at a concentration of 10^{-3} g/l (statistically significant distinctions from GaIN according to U test, $p < 0.05$). The other concentrations under consideration did not display pronounced hepatoprotective activity. So, having summed up the results obtained, we may conclude that

Table 1 Identification of melanins recovered using hydrochloric acid from extracts separated by different extraction techniques and their antioxidative properties

Chaga extraction technique	Melanin	Total antioxidant capacity, mg AAE/g melanin[a]	Reduction of ferric ions mg AAE/g melanin[a]
Microwave-assisted maceration extraction at 90 W	M90	246.86 ± 3.20	902.85 ± 9.89
Microwave-assisted maceration extraction at 180 W	M180	220.19 ± 3.77	888.57 ± 4.04
Microwave-assisted maceration extraction at 360 W	M360	221.14 ± 1.96	893.33 ± 6.59
Microwave-assisted alcohol extraction (40% ethanol) at 180 W	ME180	200.06 ± 1.74	820 ± 5.71

[a] AAE, ascorbic acid equivalent

Table 2 Characteristics of colloidal systems of aqueous solutions of Chaga melanins

Melanin	Polydispersity index	Polydispersity, %	Size and quantity of particles						Z potential, mv
			Small		Medium		Large		
			r, nm	% Int	r, nm	%Int	r, nm	% Int	
M90	0.55	74.4	–	–	–	–	212.50	100.0	− 75.7
M180	0.85	92.2	15.83	31.0	89.35	51.5	286.50	17.8	− 64.3
M360	0.45	66.8	6.05	12.7	28.60	14.5	174.20	72.9	− 42.7
ME180	0.40	–	–	–			204.9 590.5	82.1 17.9	− 56.3

M180 displays hepatoprotective activity at concentrations of 10^{-5} and 10^{-3} g/l. Like many other antioxidants [28], for instance, flavonoids, melanin M180 also has a complicated polymodal dose-effect dependence observed in the action of very low doses of biologically active substances (Fig. 2).

3.4 Investigation of the Hepatoprotective Effect of the Chaga Melanins on the Hepatocyte Cell Cycle

Since the tests used in the study of the hepatoprotective activity showed a statistically significant positive effect only for melanin M180 at concentrations of 10^{-3} g/l and 10^{-5} g/l, we studied the *Chang Liver* cell cycle after it was exposed to GaIN at 150 mM and melanin M180 at concentrations of 10^{-3} g/l and 10^{-5} g/l for 24 h. The results are presented in Table 6. Such stages of the cell cycle as G0/G1, S, and M are given in a percentage of the number of cells with a diploid chromosome set (2n).

As Table 6 suggests, cells under the influence of the toxicant GaIN tend to enter the resting phase G0/G1, and since it can be rather difficult for cells to exit the G0 phase, the number of cells in the synthesis and mitotic phases decreases in relation to the control group (C) (statistically significant distinctions from C according to U test, $p < 0.05$). The use of effective doses of melanin M180 promotes expansion in the number of cells in the synthesis and mitotic phases compared to the effect of GaIN at a concentration of 150 mM (statistically significant distinctions from C(GaIN) according to U test, $p < 0.05$). In addition, the use of melanin M180 at a concentration of 10^{-3} g/l brings a statistically significant reduction in the number of cells with a chromosome set <4n + >2n and an increase in the number of cells with a 2n set (statistically significant distinctions from C(GaIN) according to U test, $p < 0.05$). The findings confirm the previously detected hepatoprotective effect of melanin M180 at concentrations of 10^{-3} g/l and 10^{-5} g/l.

3.5 Influence of Melanins on Structural and Morphological Alterations of the Liver

The in vivo tests on animals were used to study the hepatoprotective activity of melanin M180 that showed activity during the in vitro tests.

The photomicrograph 3A (Fig. 3a) demonstrates healthy liver tissues. The photograph (Fig. 3b) taken of a liver specimen of one of the control rats shows the following disorders: enlarged hepatocytes, structural damage (trabeculae cannot be seen), fat dystrophy, necrosis, and ballooning degeneration of hepatocytes. Damages of hepatocytes are evenly distributed in all acinar areas of the liver lobules. Distinctive damage is present when using the Carsil and melanin M180 as well (Fig. 3c, d). The main localizations of damages are in central areas of liver lobules. The difference from the control group that

Table 3 Z potential and conductivity of colloidal systems of aqueous solutions of Chaga melanins

Melanin	Conductivity, mS/cm	Z potential						Z potential, mV
		Low		Average		High		
		Mean, mV	Area. %	Mean, mV	Area. %	Mean, mV	Area. %	
M90	0.45	− 76.9	97.0	− 34.6	3.0	–	–	− 75.7
M180	0.32	− 64.3	100.0					− 64.3
M360	0.59	− 75.9	17.1	− 49.8	25.6	− 29.3	41.5	− 42.7

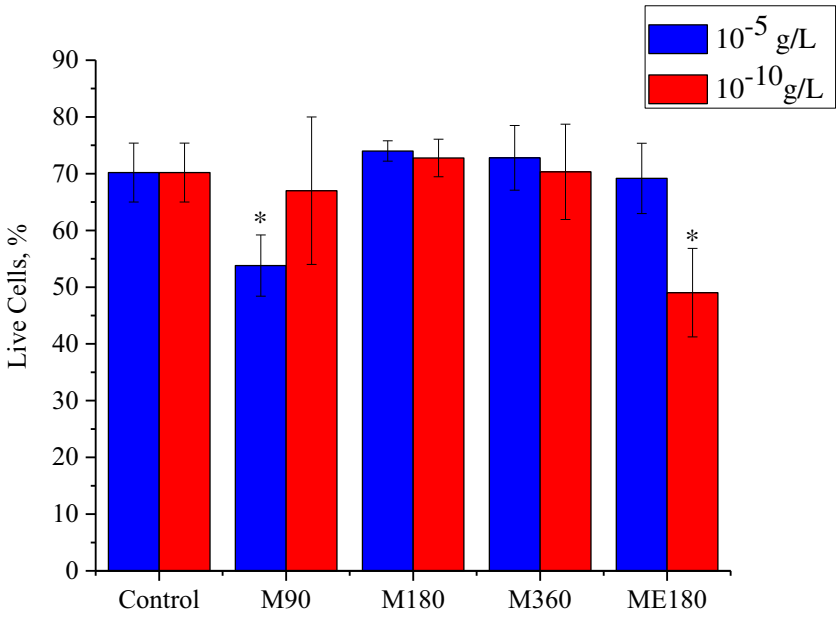

Fig. 1 The number of live cells. * – statistically significant distinctions from Control according to *U* test, $p < 0.05$

was treated only with CCl_4 is that the area of necrotic and other damages is smaller. The results of calculations of the affected areas on liver sections stained with hematoxylin and eosin also showed that melanin M180 has a hepatoprotective effect. The affected area came to 72% versus 85% in the control group. The medicinal drug Carsil delivered a result consistent with that of the Chaga melanin, as the affected area in the group treated with Carsil came to 64% (Fig. 4).

The frozen liver sections stained with Sudan Black B showed that the largest percentage of fat accumulations could be observed in the control group (CCl_4) at 1.6%. The photomicrographs of the sections stained with Sudan Black B are shown in Fig. 1 of the Supplementary material. The area of lipid detection on liver sections when using Carsil and melanin M180 diminished and came to 1.1% and 1.3%, respectively (Fig. 5).

3.6 Blood Chemistry

From all the blood chemistry values studied, the hepatocyte cytolysis markers are transaminases ALT and AST, AST/ALT ratio, and LDH. The total bilirubin, alkaline phosphatase, and gamma glutamine transferase (GGT) levels are markers of

Table 4 Hepatoprotective activity of extracts under consideration determined by percentage of live cells over the number of cells in total

	Extract concentration, g/l		
	10^{-5}	10^{-10}	0
M90 + GaIN[b] 150 mM	1.36 ± 0.10	1.53 ± 0.10	
M180 + GaIN 150 mM	3.50 ± 0.30[a]	3.10 ± 0.40	
M360 + GaIN 150 mM	1.05 ± 0.03	0.92 ± 0.20	
ME180 + GaIN 150 mM	1.24 ± 0.20	1.73 ± 0.20	
C(GaIN 150 mM)[c]			1.31 ± 0.30
C[d]			97.53 ± 0.40

[a] Statistically significant distinctions from C(GaIN 150 mM) according to *U* test, $p < 0.05$

[b] GaIN – *d*-galactosamine

[c] C(GaIN 150 mM), control group, which *d*-galactosamine was added to the growth medium

[d] C, control group, which was not exposed to any substances

Table 5 Cytoprotective effect of M180 melanin on the *Chang Liver* cell line subjected to toxic damage of *d*-galactosamine (GaIN)

	Melanin concentration, g/l	Live cells, %
Experiment 1		
M180 + GaIN 150 mM	10^{-1}	2.19 ± 0.7
	10^{-2}	0.89 ± 0.2
C(GaIN 150 mM)[b]	0	1.1 ± 0.4
C[c]	0	89.3 ± 0.2
Experiment 2		
M180 + GaIN 150 mM	10^{-3}	20.3 ± 0.4[a]
	10^{-7}	13.3 ± 0.4
	10^{-12}	10.6 ± 0.4
C(GaIN)[b]	0	11.1 ± 0.3
C[c]	0	98.2 ± 0.3

[a] Statistically significant distinctions from C(GaIN 150 mM) according to *U* test, $p < 0.05$

[b] C(GaIN 150 mM), control group, which *d*-galactosamine was added to the growth medium

[c] C, control group, which was not exposed to any substances

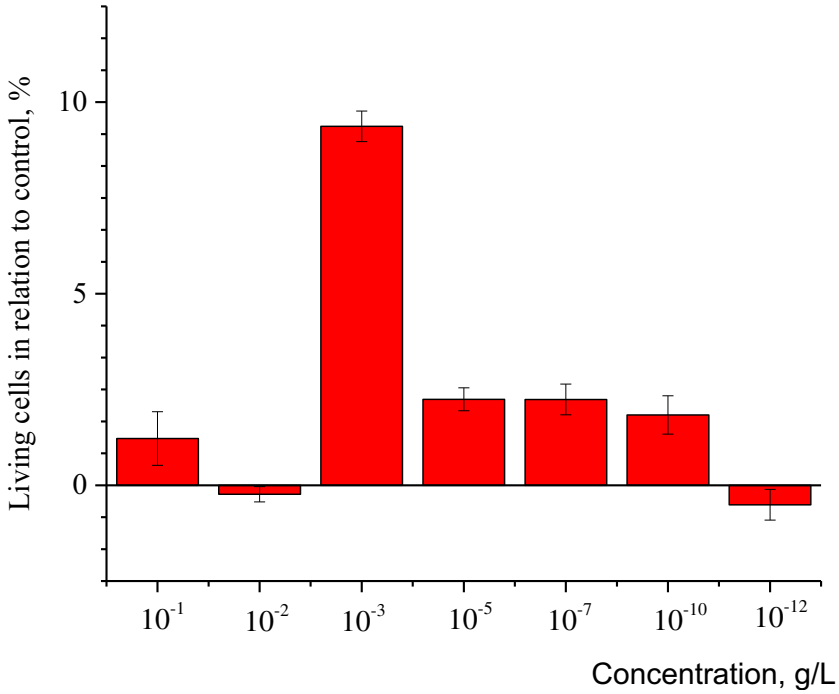

Fig. 2 The number of live cells in reference to the control group

cholestatic liver damage. Such markers, as conjugated and unconjugated bilirubin, speak about the state of the excretory function of hepatocytes.

The ALT level in all the groups of animals did not differ from the intact control to a statistically significant extent, which is attributed to the suspension of the hepatocyte cytolysis process after discontinuation of the CCl_4 administration and spontaneous recovery of this value. After the induction of toxic hepatitis, the AST activity was observed to be a bit higher than in the group where the animals had been treated with Carsil compared to the animals treated with M180. The group of animals that had been treated with the drug Carsil had the LDH level reduced to the level observed in the intact control group. The use of melanin M180 did not bring this value to a normal level.

In the control group, we observed increased levels of GGT, total and conjugated bilirubin, as well as alkaline phosphatase, which is evidence of the development of biliary excretion dysfunction, or cholestasis. Both experimental groups that had been treated with melanin M180 and Carsil had total bilirubin at the same level as the intact control group; moreover, it was observed that the level of the toxic fraction of unconjugated bilirubin had decreased (Table 7). Melanin M180 also normalized the GGT level, whereas in the group that had been treated with Carsil it remained at a high level. Neither melanin M180 nor Carsil had any effect on the alkaline phosphatase activity.

The animals from the control group treated with CCl_4 and water were observed to have low CE activity, which is indicative of hepatic synthetic dysfunction. It was observed that the

Table 6 Effect of the M180 melanin on the cell cycle of the *Chang Liver* cell line subjected to toxic damage of *d*-galactosamine (GaIN)

	Extract concentration, g/l	Cell cycle phases				
		< 4n + > 2n	2n	G1/G0	S	M
C[c]	0	46.31 ± 0.50[b]	53.68 ± 0.20[b]	71.71 ± 0.40[b]	20.92 ± 0.50[b]	7.31 ± 0.30[b]
C(GaIN 150 mM)[d]	0	67.15 ± 2.50[a]	32.85 ± 0.40[a]	91.73 ± 1.60[a]	6.94 ± 1.10[a]	1.31 ± 0.10[a]
M180 + GaIN 150 mM	10^{-3}	54.52 ± 3.30[a,b]	45.47 ± 0.40[a,b]	82.53 ± 1.80[a,b]	14.34 ± 2.30[a,b]	3.12 ± 0.20[a,b]
	10^{-5}	62.64 ± 3.70[a]	37.36 ± 0.80[a]	85.29 ± 3.30[a,b]	12.56 ± 1.20[a,b]	2.14 ± 0.20[a,b]

[a] Statistically significant distinctions from C according to *U* test, $p < 0.05$

[b] Statistically significant distinctions from C(GaIN 150 mM) according to *U* test, $p < 0.05$

[c] C(GaIN 150 mM), control group, which *d*-galactosamine was added to the growth medium

[d] C, control group, which was not exposed to any substances

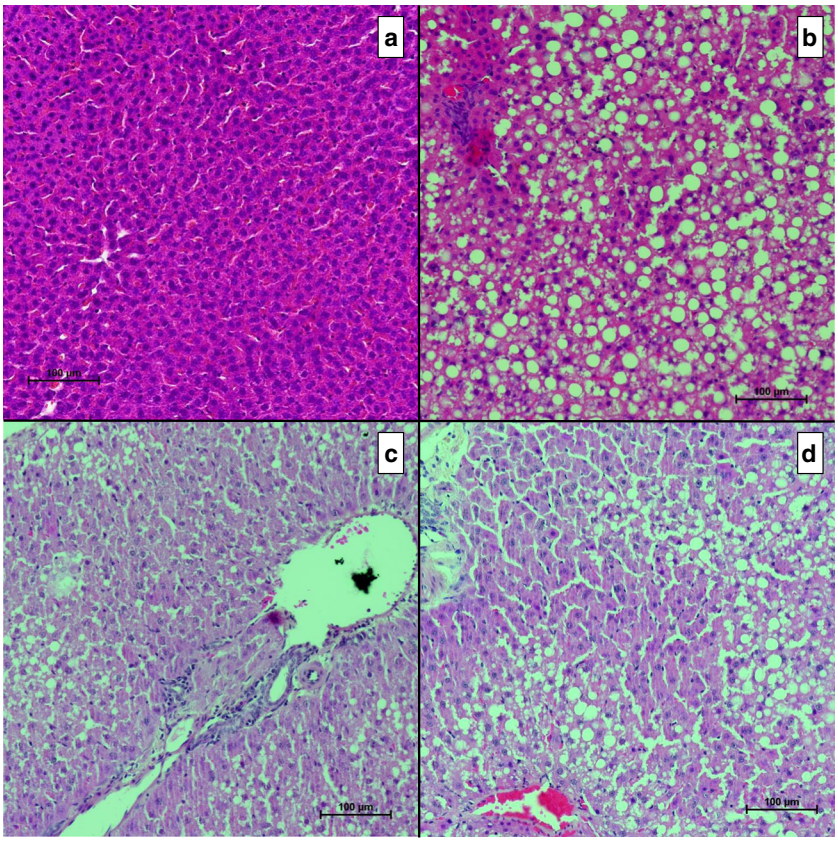

Fig. 3 Photomicrographs of liver (hematoxylin-eosin stain). **a** of a healthy animal; **b** of an animal under CCl_4; **c** of an animal under CCl_4 and Carsil; and **d** of an animal under CCl_4 and melanin M180. Zoom 300×

CE activity in the groups that had melanin M180 and Carsil returned to normal (Table 6). The hepatic synthetic dysfunction observed in the control group also led to a reduced concentration of the total protein, including its albumin and globulin fractions. The groups that had been treated with melanin M180 and Carsil had a higher total protein level than the control group, however, this did not differ significantly from that of the intact control group, in which case the total protein level returned to normal due to the albumin fractions. These findings also confirm normalization of the hepatic synthetic function.

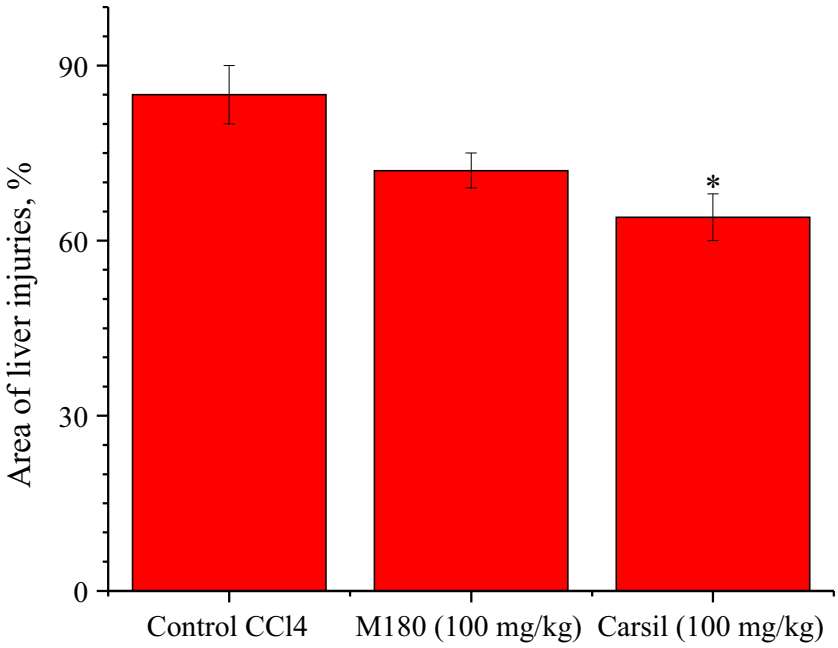

Fig. 4 Area of liver damage (in % over the total area), *: statistically significant distinctions from the control CCl_4 group according to *U* test, $p < 0.05$

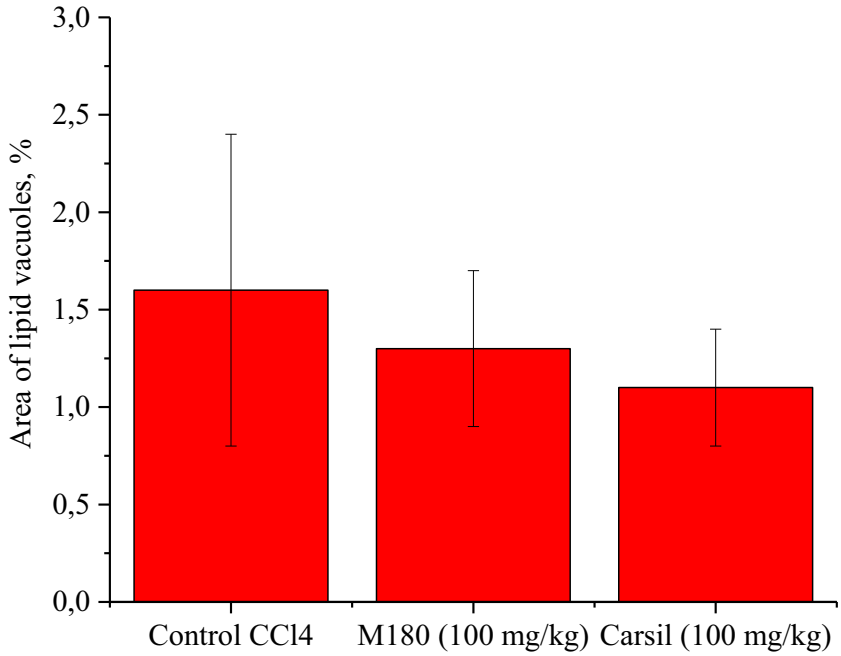

Fig. 5 Area of lipid vacuoles (in % over the total area)

The results of the in vivo tests conducted on rats verify that the hepatoprotective properties of the Chaga melanins M180 are consistent with those of the drug Carsil.

4 Discussion

Four specimens of the *Inonotus obliquus* melanins were received for the study of their hepatoprotective activity. They were first checked for cytotoxicity on a culture of normal human cells *Chang Liver*, after which their hepatoprotective activity was rated in vitro.

The colloidal systems (CS) of melanins M90 and ME180 in aqueous solutions are similar—they have the same conductivity rating and coarse particles in the dispersed phase (Tables 2 and 3). Perhaps the presence of larger particles and their variation in size in the CS of melanin M180 bring a greater variety of surface charges from − 106.0 to − 42.9 mV compared to the CS of melanin M90. This might also explain the difference in their antioxidative properties, as the CS of

Table 7 Influence of melanin M180 on blood chemistry values

Value	Intact	Control CCl_4	M180	Carsil
ALT, U/L	76.00 ± 5.51	82.33 ± 4.33	82.50 ± 5.87	83.67 ± 1.86
AST, U/L	62.34 ± 12.50	88.00 ± 4.36[a]	88.00 ± 7.47[a]	99.67 ± 2.96[a]
AST/ALT ratio	1.33 ± 0.12	1.06 ± 0.02	0.80 ± 0.30	1.19 ± 0.05
LDH, U/L	1192.00 ± 6.56	2086.33 ± 301.43	2314.25 ± 422.78	1148.00 ± 84.60
GGT, U/L	0.67 ± 0.30	1.67 ± 1.20	0.50 ± 0.50	1.33 ± 0.33[a]
Total bilirubin, μM/L	2.79 ± 0.16	3.46 ± 0.27[a]	2.86 ± 0.31	2.75 ± 0.03
Conjugated bilirubin, μM/L	0.69 ± 0.18	1.11 ± 0.13[a]	1.49 ± 0.43	0.92 ± 0.10
Unconjugated bilirubin, μM/L	2.10 ± 0.27	2.35 ± 0.18	1.37 ± 0.13[a,b]	1.83 ± 0.15
Alkaline phosphatase, U/L	170.67 ± 8.69	217.67 ± 8.88[a]	212.00 ± 10.46[a]	211.67 ± 16.25[a]
Total protein, g/L	56.00 ± 0.58	50.67 ± 1.20[a]	53.25 ± 2.17	52.00 ± 0.00
Albumin, g/L	32.00 ± 0.58	28.00 ± 1.00[a]	31.50 ± 0.87	31.00 ± 0.58
Globulins, g/L	24.00 ± 1.00	22.67 ± 0.33	21.75 ± 1.31	21.00 ± 0.50
Glucose, mM/L	12.58 ± 0.06	12.56 ± 0.01	12.37 ± 0.20	12.66 ± 0.08
Cholinesterase, U/L	338.67 ± 0.33	273.00 ± 9.71[a]	320.50 ± 14.65	340.33 ± 49.00

[a] Statistically significant distinctions from the intact group according to *U* test, $p < 0.05$

[b] Statistically significant distinctions from the control CCl_4 group according to *U* test, $p < 0.05$

melanin M90 has a 23% higher Total Antioxidant Capacity and a 10% higher Reduction of Ferric Ions than the CS of melanin ME180 (Table 1). The distinction of these colloidal systems from those of melanins M180 and M360 is attributed to the fact that the CS of melanin M90 was formed in an aqueous medium with longer exposure to microwaves (at 90 W, 5 min; 180 W, 2 min; 360 W, 1 min), although less intensive, whereas the CS of melanins ME180 was formed with the presence of 40% ethyl alcohol in its dispersed medium, which reduced its polarity. The factors described reinforced the processes of particle aggregation in the dispersed phase in these CS, which led to the formation of large melanin particles. The presence of large particles in the CS of melanins M90 and ME180 and their negative charge of over − 75 mV affected their ability to inhibit the vital activity of hepatocyte cells (Fig. 1).

Although the CS of melanins M180 and M360 exhibit similar antioxidant properties in terms of Total Antioxidant Capacity and Reduction of Ferric Ions, they are considerably different in terms of the structure of their CS. The differences observed in the CS of melanins M180 and M360 do not affect the vital activity of hepatocytes (Fig. 1), however, they have a significant impact on the expression of their hepatoprotective properties. The M180 CS has the lowest conductivity and the highest polydispersity with a high content, up to 51.5%, of medium-sized particles that are around 180 nm in diameter with a surface charge close to − 64.3 mV compared to all the other melanins considered in the study. The M360 CS has the highest conductivity and has primarily larger particles, up to 72%, that are approximately 350 nm in diameter, as well as particles with a charge close to − 75 mV. The average size of particles in the dispersed phase of the M180 CS, which comes approximately to 180 nm, their charge, which is lower than − 75 mV, and their low conductivity and high polydispersity might have a positive impact on the hepatocyte cells, which increases their vitality under the toxic influence of *d*-galactosamine by 2–2.5 times as compared to data from the control group (Table 4).

Based on the obtained data, we chose melanin M180 with hepatoprotective activity derived from the aqueous extract of Chaga using microwaves at 180 W. An expanded analysis of hepatoprotective properties in a wider range of concentrations has shown that M180 exhibits hepatoprotective properties at concentrations of 10^{-5} g/l and 10^{-3} g/l.

It should be noted that by using exclusively the main active component of the aqueous extracts of *Inonotus obliquus* in the experiments, which is melanin, we were able to reduce its concentration to extremely low doses. According to [25], the use of very low doses of antioxidants is characterized by a complex, non-monotone polymodal dose-effect dependency, hence, it is extremely important to determine the specific concentration of the biologically active subject of the analysis.

In vitro tests were conducted to study the effect of melanin M180 on a hepatocyte cell cycle. This enabled us to confirm the hepatoprotective properties of this melanin at concentrations of 10^{-5} g/l and 10^{-3} g/l and establish that it stimulates the cell transition from the G0 phase to the synthesis phase when compared to the control group, where the cell culture was injected only with GaIN.

After it had been discovered in vitro that melanin M180 has hepatoprotective properties, it was tested in vivo at a concentration of 100 mg/kg. According to the results of the animal testing, it was established that melanin M180 has a hepatoprotective effect expressed in the reduction of necrotic and steatotic changes in liver cells and the improvement of the blood chemistry values when compared to the control group of animals that had received water and CCl_4.

The hepatoprotective properties of the Chaga melanins extracted using hyperbranched polymers have already been demonstrated and proven to be more pronounced than those of Carsil. However, the biochemical parameters did not stabilize to the level of the intact animals. This research conducted in accordance with a therapeutic procedure has shown that melanin does not fully recover the total serum bilirubin level, as it remains 2–2.5 times higher than that of the intact group. The alkaline phosphatase level did not return to normal, and neither did it in the present research under the influence of M180, remaining almost two times higher [8]. In the present research conducted according to a preventive experimental procedure and with melanin obtained in the process of microwave-assisted aqueous extraction of the Chaga mushroom at 180 W, the total bilirubin level did not increase compared to the intact animals. Moreover, we observed a statistically significant decrease in toxic fractions of unconjugated bilirubin. On that basis, it follows that hepatoprotective properties of melanin obtained in microwave-assisted extraction at 180 W are more pronounced when compared to the hepatoprotective activity of the previously tested Chaga melanin extracted using hyperbranched polymers.

5 Conclusion

The in vitro studies of melanins obtained from aqueous extracts of *Inonotus obliquus* in a microwave-assisted process at different modes determined melanin extracted in a microwave-assisted process at 180 W to have the most pronounced hepatoprotective activity. Subsequent in vivo tests of this melanin on animals confirmed its hepatoprotective properties expressed in the decrease of the signs of liver tissue damage such as steatosis, necrosis, fat accumulation, and normalization of the levels of total and unconjugated bilirubin, total protein, serum cholinesterase, and gamma-glutamyl transpeptidase. Thus, the effect of melanin on the pathomorphological changes in the liver is comparable to

the effect of Carsil. However, melanin M180 promotes normalization of a greater number of biochemical values of blood when compared to Carsil, which points to the advantages of the melanin studied.

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