

Studying the effects of *Cantharellus cibarius* fungi on *Opisthorchis felineus* trematode and on parasite host – C57BL/6 inbred mice

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Opisthorchiasis is a dangerous parasitic disease caused by trematodes in the family Opisthorchiidae. One of the causes of this infection is the species *Opisthorchis felineus*, which is common in the Russian Federation and Western Europe. The disease has a large number of complications and relatively few effective treatments, so nowadays it is relevant to look for new drugs for the treatment of opisthorchiasis, with the maximum antiparasitic and minimal side effect. In this work, a potentially anthelmintic effect of the methanol extract of the golden chanterelle mushroom (*Cantharellus cibarius*) was investigated. In *in vitro* experiments, the significantly reduced mobility and survival rates of juvenile *O. felineus* specimens with increasing concentrations (10–1000 µg/ml) of the *C. cibarius* extract were shown. In *in vivo* studies, administration of the *C. cibarius* extract on the first day after parasitic infection of inbred C57BL/6 mice resulted in a decrease of the number of helminths in the bile ducts of the liver, evaluated 6 weeks after infection. In another series of experiments, administration of the *C. cibarius* extract for 7 days to mice infected with *O. felineus* for five weeks had no anthelmintic effect. In both cases, the state of the infected hosts, evaluated by a number of physiological and biochemical parameters (relative weight of organs, blood indices), did not deteriorate, indicating that there was no adverse effect of the *C. cibarius* extract. The results obtained suggest that the *C. cibarius* extract might have anthelmintic properties if applied as parasite larvae excyst.

Key words: *Opisthorchis felineus*; methanolic extract of *Cantharellus cibarius*; C57BL/6 mice; blood biochemical markers.

Исследование эффектов грибов *Cantharellus cibarius* на трематоду *O. felineus* и на хозяина паразита – инбредных мышей C57BL/6

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Описторхоз – опасное паразитарное заболевание, вызываемое трематодами семейства Opisthorchiidae, в том числе видом *Opisthorchis felineus*, распространенным на территории Российской Федерации и Западной Европы. В наше время остается актуальным поиск новых препаратов для лечения описторхоза с максимальным противопаразитарным и минимальным побочным действием. В работе исследованы потенциальные антигельминтные эффекты экстракта грибов Лисичка обыкновенная (*Cantharellus cibarius*). В экспериментах *in vitro* показано, что при повышении концентрации экстракта *C. cibarius* (10–1000 мкг/мл) подвижность и выживаемость ювенильных особей *O. felineus* значительно снижается. В исследованиях *in vivo* экстракт *C. cibarius*, вводимый мышам инбредной линии C57BL/6 в первые сутки после инфицирования, приводил к снижению количества гельминтов в желчных протоках печени, оцениваемому через шесть недель после инфицирования. В другой серии экспериментов введение экстракта в течение семи дней мышам с пятидневным сроком инфицирования не оказывало антигельминтного эффекта. В обоих случаях состоянии хозяина паразита, оцениваемое по ряду физиологических и биохимических параметров, не ухудшалось, что говорит об отсутствии какого-либо негативного действия экстракта *C. cibarius*. Полученные результаты свидетельствуют в пользу возможных антигельминтных свойств экстракта *C. cibarius* при применении на стадии экцистирования личинок паразита.

Ключевые слова: *Opisthorchis felineus*; метанольный экстракт *Cantharellus cibarius*; мыши C57BL/6; биохимические показатели крови.

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Opisthorchiasis is a disease of hepatobiliary system and pancreas, caused by trematodes of the Opisthorchiidae family including *Opisthorchis felineus*. The Ob-Irtysh region is the biggest area of *O. felineus*-induced opisthorchiasis in Russia. Infection of urban population is up to 80 %, in rural areas – more than 90 % (Mordvinov et al., 2012; Yurlova et al., 2017). Opisthorchiasis is characterized by inflammatory processes in liver and bile ducts (Sripa, 2003; Nair et al., 2011). *O. felineus* infection results in multiple pathologies of liver and pancreas: cholangitis, cholecystitis, strictures of bile ducts, gallstones, hepatitis-like symptoms (jaundice, hepatomegalia), pancreatitis (Paltsev, 2005; Saltykova et al., 2016). Some authors have noted a correlation between *O. felineus* infection and oncology of human hepatobiliary system and pancreas (Brazhnikiova, Tolkaeva, 2002).

Due to the large-scale Opisthorchiasis distribution, duration of the disease and severe consequences caused in the infected humans and animals, constant search for drug treatment methods is conducted on laboratory animals. To this purpose, Opisthorchiasis is frequently modeled on golden hamsters – *Mesocricetus auratus* (Pinlaor et al., 2009; Pakharukova et al., 2015). However, these animals are not natural hosts for this trematode. Some Opisthorchiasis studies are performed on knock-out (Nair et al., 2011) and C57BL/6 inbred mice (Zelentsov, 1974; Avgustinovich et al., 2016; 2017a, b, 2018). For *O. felineus*-infected C57BL/6 mice, a prolonged (up to six weeks) juvenile marita stage is typical, which distinguishes them from hamsters. It gives particular advantages in studying the chronic effect of agents on immature *O. felineus* maritae.

Praziquantel is the main and most effective agent for human opisthorchiasis treatment. Some authors assume that parasites develop resistance to this agent (Greenberg, 2014). Praziquantel also has side effects (Erko et al., 2012) and considerable cytotoxic influence on liver cells (Sripa et al., 2011). Therefore, researching new compounds, effective against opisthorchiasis remains important. Fungi of *Cantharellus* genus, particularly *C. cibarius*, are considered as potential source of such agents. These fungi demonstrate anti-nematode properties in nature (Muszyńska et al., 2016). They are broadly used in traditional medicine (Cieniecka-Rosłonkiewicz et al., 2007), because the fungus contains substances with potential therapeutic properties against various diseases (Valverde et al., 2015; Nyman et al., 2016). *In vitro* and *in vivo* studies revealed high antioxidant and anti-inflammatory properties of fungi extracts, more pronounced in alcohol extracts than in aqueous extracts (Vamanu, Nita, 2014). Methanolic fungi extract has an advantage over aqueous extract due to its antimicrobial effect (Kozarski et al., 2015; Muszyńska et al., 2016), while antimicrobial activity of methanolic extract against *Escherichia coli* is 7 times higher than ethanolic extract (Aina et al., 2012). Single-dose or chronic intraperitoneal introduction of *C. cibarius* methanolic extracts are used for mice, the dose range – 200–800 mg/kg (Khalili et al., 2014, 2015). No studies of *C. cibarius* efficacy in treating opisthorchiasis infections have been performed.

Thus, the study objective was to determine anthelmintic properties of *C. cibarius* methanolic extract in two modes of administering to mice of C57BL/6 inbred line: at the stage of introducing *O. felineus* (the first day of infection) and at the stage of parasites attached to biliary ducts (5 weeks of

infection). Fungi extract effect upon *O. felineus* was also assessed *in vitro*. In addition, we intended to obtain and compare the chemical compositions of *C. cibarius* methanolic and ethanolic extracts.

Materials and methods

Animals. Mature male mice of C57BL/6 line were obtained from the Center for Genetic Resources of Laboratory Animals (RFMEFI61914X0005 and RFMEFI61914X0010) of the Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences (Novosibirsk, Russia). The animals were kept in groups (3–6 animals) in standard cages 36 × 23 × 10 cm with light conditions – 12:12 h (day:night), air temperature 24 °C, food and water *ad libitum*. All procedures were performed in accord with the Directives of the European Communities Council of 24 November 1986 (86/609/EEC), and in line with the standards of the Bioethics Commission of the “Institute of Cytology and Genetics” Federal Research Centre (No. 26 Protocol of 13.03.2015).

O. felineus metacercariae were separated from naturally infected *Leuciscus idus*, inhabiting the Ob river within the boundaries of Novosibirsk. Metacercariae were washed with 0.9 % NaCl sterile solution and contained at 4 °C no longer than 24 h for *in vivo* experiments and up to 7 days in sterile phosphate buffer with added kanamycin antibiotic (25 µg/ml) for *in vitro* experiments.

Obtaining of *C. cibarius* extract. The extracts were obtained from *C. cibarius* fungi collected in the Novosibirsk region. The extracts were made using the method of T. Kukina with co-authors (2016). Fungi, dried at the temperature not exceeding 40 °C, were powdered on an electric mill, and then the raw material was sieved through 2 mm holes. A 50 g sample was loaded in a Soxhlet extractor for 20 h. Methanol, ethanol and ethanol with methanol reextraction were used as the extraction agents. The obtained substances were evaporated to dryness on a Buchi rotor evaporator (Switzerland) with water bath at 40 °C and reduced pressure (20–30 mm Hg) due to use of a waterjet pump. Methanolic extract was used in *in vivo* and *in vitro* experiments according to recommendations of other researchers (Aina et al., 2012). For *in vivo* studies *C. cibarius* extract was suspended in 10 % Tween 80, which is used in animal experiments as a solvent (Teufack et al., 2017). For *in vitro* studies, the extract was dissolved in dimethyl sulfoxide (DMSO).

***In vitro* studies.** Excystation of juvenile worms was achieved by adding 0.06 % trypsin solution (Sigma, USA) to metacercariae, keeping it for 15 min at 37 °C. Then the worms were washed five times with incubation medium [the composition: RPMI 1640, L-glutamine (Life Technologies, USA), antibiotics (100 µg/ml streptomycin + 100 unit/ml penicillin, Sigma, USA), an antimycotic agent (25 µg/ml amphotericin B) and 1 % glucose] and put in wells of a standard cultural plate, containing 990 µl of the medium and 10 µl of the studied agent. Dry fungi extract was diluted to the required concentration in 100 % DMSO so that adding 10 µl to the medium made the concentration of DMSO 1 %, and the extract – 10, 100 or 1000 µg/ml. There were two wells per concentration, each containing 60–80 juvenile *O. felineus* specimens. 1 % DMSO was added to the control wells. The plate was put in CO₂-incubator (37 °C, 5 % CO₂) for 24 h.

Mobility of juvenile worms was analyzed in 24, 72, 120 and 168 h after adding the agent based on the method of J. Keiser with co-authors (2013). Mobility was assessed visually using a Axiovert 40CFL microscope (Zeiss, Germany) according to a 4-score scale (4 – active continuous worm movement, 3 – poorly pronounced, slow movements of the entire body, 2 – very rare movements, mostly limited to a single body part, 1 – complete immobility), and then normalized by the control group (1 % DMSO). A day after adding the agents, the average effective agent concentration, when complete immobility occurred in 50 % of all worms (IC_{50}), was calculated using CompuSyn 1.0 software (ComboSynInc).

In vivo studies. Two schemes of introducing *C. cibarius* extract to the infected animals were used. In the first series of experiments at 6 pm metacercariae in 0.1 ml saline were injected to half of the animals (100 larvae/mouse), and 0.9 % NaCl to the other half. On the next day at 10 am a single dose of *C. cibarius* extract solution or a solvent (10 % Tween 80) was introduced. *C. cibarius* extract was administered to half of the animals in each group, and the solvent to the other half. Each subgroup had 7–14 mice. In the second series of the experiments *C. cibarius* extract (or solvent) were administered to the infected animals daily during 7 days, 5 weeks after introducing *O. felineus* metacercariae. Two groups of 14–15 mice were used. The substances were administered to the mice intragastrically via special probes (Braintree Scientific, Inc., USA). A 600 mg/kg dose was chosen based on the literature data (Khalili et al., 2014).

During both experiments the animal body mass was measured every ten days. In 6 weeks after infection the mice were euthanized by decapitation and the biomaterial for further studies was taken. Liver, spleen and thymus were weighted and the relative organ mass was calculated to gram of body mass. Liver was put in 0.9 % NaCl solution to calculate the number of parasites in biliary ducts and gall bladder and to determine their maturity state. For exposing worms and analyzing the state of biliary ducts, an Axiovert 40CFL light microscope (Zeiss, Germany) with $\times 4$ magnification was used. The blood collected during decapitation was centrifuged at 3000 rpm, 4 °C, 20 min. Serum was separated and stored at –70 °C until performing the biochemical studies.

Biochemical studies. Activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactic dehydrogenase (LDH) enzymes, the content of glucose (GLU), total protein (TP), cholesterol (CHOL), triglycerides (TG) in blood serum was determined with a standard reagents kit (Biocon, India). The measurements were performed using a Photometer-5010 biochemical semiautomatic analyzer (Boehringer Mannheim, Germany).

Studying biochemical composition of *C. cibarius* extracts. The content of protein, polysaccharides, phenol compounds, flavonoids and carotinoids in *C. cibarius* extracts were determined applying an earlier described method (Protsenko et al., 2018).

Statistics. Statistical processing of the data was made in Statistica 6.0 (StatSoft) software suit. Two-way analysis of variance (ANOVA) was carried out for the first scheme of administration: the first factor was “infection” (infected – non-infected), the second factor was “agent” (*C. cibarius*

extract, solvent). To analyze the extract effect in the second administration scheme, one-way ANOVA was used. Body mass changes were evaluated by the Wilcoxon test criteria for paired comparison. The data were presented as the mean value \pm error of mean. The results were considered statistically significant at $p \leq 0.05$ and as a trend at $0.05 < p < 0.1$. Parasite survival in the *in vitro* experiment was estimated with Kaplan–Meier method, the survival curves were compared using χ^2 . The curves differed at $p \leq 0.05$.

Results

In vitro study

In the first minutes after introducing the extract or 1 % DMSO no changes in worm appearance or activity were observed. In 24 h worm mobility in wells with 10, 100 and 1000 $\mu\text{g/ml}$ extract concentration was 6.15 %, 7.69 % and 41.15 % below the control mobility respectively. Further on (in 72–120 h) worm mobility was gradually decreasing. In 120 h the group with 10 $\mu\text{g/ml}$ of extract had 64 % less mobility than in the control group; in 100 $\mu\text{g/ml}$ group – 71.2 % less than in the control group; and in the 1000 $\mu\text{g/ml}$ group complete termination of activity was observed. In 168 h most worms died. In 24 h after adding *C. cibarius* extract IC_{50} was 1.58 $\mu\text{g/ml}$.

At each stage of observation the number of dead (immobile, non-transparent) worms was calculated. Mortality in the control group was 0 % 24 h after the beginning of the experiment, 9.41 % – in 72 h, 37.65 % – in 120 h and 87.65 % – in 168 h; in the group with concentration of *C. cibarius* extract 10 $\mu\text{g/ml}$ the results were 2.08, 29.86, 70.14 and 97.92 %, respectively. With 100 $\mu\text{g/ml}$ extract concentration the values were 3.96, 37.62, 77.23 and 91.19 %, respectively, while with 1000 $\mu\text{g/ml}$ extract concentration – 12.08, 87.25 and 100 % in 120–168 h. In all cases the parasite survival diagrams upon administering *C. cibarius* extract were significantly different from the control values (10 $\mu\text{g/ml}$: $\chi^2 = 41.57$, $p = 1.14 \cdot 10^{-10}$; 100 $\mu\text{g/ml}$: $\chi^2 = 17.33$, $p = 3.13 \cdot 10^{-5}$; 1000 $\mu\text{g/ml}$: $\chi^2 = 227.48$, $p = 0.000$).

The appearance of parasites had changed under the agent influence: many worms in wells with 10 and 100 $\mu\text{g/ml}$ extract doses had enlarged excretory bladder in 72 h (Fig. 1, a, b), maintained further on. No such changes were observed in the control worms during the entire 168-hour period (Fig. 1, d) and specimens with 1000 $\mu\text{g/ml}$ extract concentration that had nearly all died by that time (Fig. 1, c).

In vivo study

Single-dose *C. cibarius* extract administered on the first day after infection led to a statistically significant ($F_{1,20} = 4.36$, $p = 0.048$) decrease of the number of parasites in biliary ducts of liver 6 weeks after infection (18.1 ± 1.5), in comparison with the control group (23.5 ± 2.2). No considerable changes in the appearance of the parasites separated from mice liver with and without introducing *C. cibarius* extract were found microscopically. The worms were mostly immature, with two properly visualized intestine branches. Both groups had an animal with one mature *O. felineus* specimen.

No significant effect of the “infection” and “agent” factors on the relative mass of liver and spleen in the analyzed groups was found; there was no interaction between the factors (Table 1). Thymus mass increased in *O. felineus*–

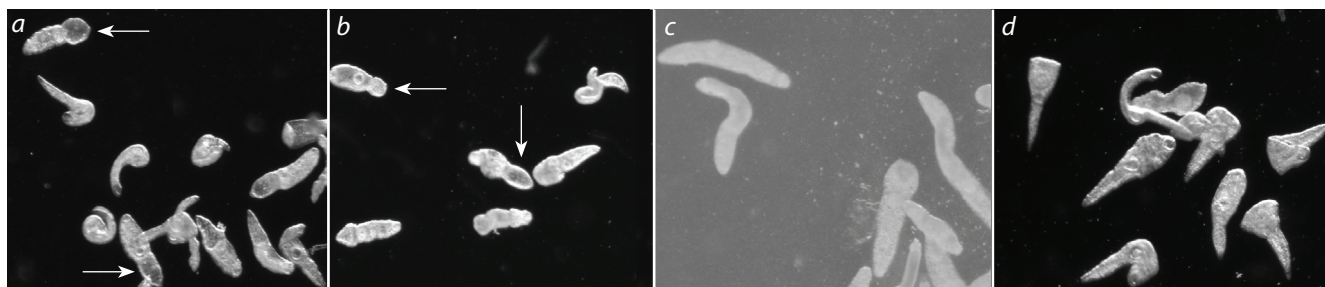


Fig. 1. Appearance of juvenile *O. felinus* specimens in 72 h after exposure to 10 µg/ml (a), 100 µg/ml (b) and 1000 µg/ml (c) *C. cibarius* extract and the solvent (d).

The arrows point at excretory bladder.

Table 1. Relative organ mass after single-dose introduction of *C. cibarius* extract on the first day after infecting mice with *O. felinus*

Organ, mg/g	Control		<i>O. felinus</i>	
	solvent	<i>C. cibarius</i>	solvent	<i>C. cibarius</i>
Liver	46.2 ± 3.33	49.5 ± 1.02	49.6 ± 1.22	51.3 ± 1.29
$F_{1,36}^{OfCc} = 2.34, p = 0.136; F_{1,36}^{Cc} = 2.10, p = 0.156; F_{1,36}^{OfCc} = 0.20, p = 0.655$				
Spleen	3.8 ± 0.60	3.1 ± 0.23	3.5 ± 0.17	3.7 ± 0.14
$F_{1,36}^{Of} = 0.24, p = 0.628; F_{1,36}^{Cc} = 0.43, p = 0.514; F_{1,36}^{OfCc} = 2.70, p = 0.109$				
Thymus	1.2 ± 0.04	1.2 ± 0.06	1.2 ± 0.06	1.4 ± 0.05#
$F_{1,36}^{Of} = 3.48, p = 0.070; F_{1,36}^{Cc} = 0.25, p = 0.618; F_{1,36}^{OfCc} = 2.53, p = 0.120$				

Note: # $p < 0.05$ in comparison with the control group. Of – the “infection” factor; Cc – the “agent” factor.

infected animals with administration of *C. cibarius* extract, in comparison with mice from the control group.

No impact of *C. cibarius* extract upon animal body weight gain in the 6 weeks of the experiment was discovered: mice in each subgroup were equally gaining weight that increased on average by 2.5–3 g.

In the second series of experiments introduction of *C. cibarius* extract for 7 days in 5 weeks after infecting did not cause any statistically significant change in the number of parasites (26.2 ± 0.89 – *C. cibarius*, 28.4 ± 1.85 – solvent; $F_{1,29} = 1.16, p = 0.292$). At the same time, the worms separated from biliary ducts after introducing solvent and *C. cibarius* extract were different: the latter had considerably enlarged excretory bladder (Fig. 2, a, b). All worms were immature, had properly visualised two intestine branches in all mice, except two in each group that had a single mature *O. felinus* specimen (Fig. 2, c).

In 7-day introduction, no differences were found between animals that received *C. cibarius* extract and the control mice in terms of the relative liver mass ($F_{1,29} = 2.10, p = 0.159$) (51.6 ± 1.07 mg/g – control; 48.5 ± 1.75 mg/g – *C. cibarius*) and spleen mass ($F_{1,29} = 2.85, p = 0.103$) (3.1 ± 0.09 mg/g – control; 3.4 ± 0.15 mg/g – *C. cibarius*). There were no intergroup differences in body mass at the beginning ($F_{1,29} = 0.036, p = 0.851$) and at the end ($F_{1,29} = 1.24, p = 0.275$) of the experiment. At the same time, the body mass of mice reduced in both groups during the period of agent introduction (7 days): from 28.0 ± 0.50 g to 26.6 ± 0.46 g in the control

group of mice ($p = 0.000$) and from 28.2 ± 0.58 g to 27.2 ± 0.42 g in mice with *C. cibarius* extract ($p = 0.015$).

Biochemical blood analysis

Table 2 shows the results of biochemical studies of infected and non-infected mice after single-dose introduction of *C. cibarius* or the solvent. ANOVA did not find any influence of the “agent” factor on activity of ALT ($F_{1,35}^{Cc} = 0.01, p = 0.915$), AST ($F_{1,35}^{Cc} = 0.02, p = 0.898$) and LDH ($F_{1,35}^{Cc} = 0.29, p = 0.591$). The effect of the “infection” factor was significant with introducing both *C. cibarius* extract and the solvent (ALT: $F_{1,35}^{Of} = 13.16, p = 0.001$; ACT: $F_{1,35}^{Of} = 14.06, p = 0.001$; LDH: $F_{1,35}^{Of} = 12.68, p = 0.001$). No factor interaction was noticed (ALT: $F_{1,35}^{CcxOf} = 0.06, p = 0.805$; AST: $F_{1,35}^{CcxOf} = 0.25, p = 0.623$; LDH: $F_{1,35}^{CcxOf} = 0.05, p = 0.821$). Statistically significant influence of both the “infection” factor ($F_{1,35}^{Of} = 7.35, p = 0.010$), and the “agent” factor ($F_{1,35}^{Cc} = 4.51, p = 0.041$) was shown for ALP with no factor interaction ($F_{1,35}^{Cc+Of} = 1.06, p = 0.311$). As demonstrated with further *post hoc* comparison, ALP activity in the group of non-infected animals was decreasing with introducing *C. cibarius* extract, which was not observed in the infected mice. Moreover, the infected mice displayed much higher ALP activity with administration of *C. cibarius* extract in comparison with the non-infected animals. The effects of the “agent” and “infection” factors on the glucose level were statistically significant ($F_{1,35}^{Cc} = 4.54, p = 0.040$; $F_{1,35}^{Of} = 4.23, p = 0.047$,

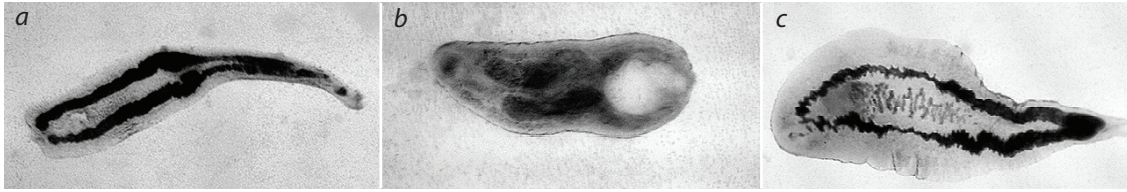


Fig. 2. Appearance of *O. felineus* specimens separated from mice livers. *a* – the control group; *b* – the group received *C. cibarius* extract; *c* – a mature *O. felineus* specimen.

Table 2. Blood biochemical values of the control and *O. felineus*-infected mice after a single-dose introduction of *C. cibarius*

Index	Control		<i>O. felineus</i>	
	Solvent	<i>C. cibarius</i>	Solvent	<i>C. cibarius</i>
ALT, unit/l	47.3 ± 6.15	45.6 ± 5.67	88.0 ± 9.40 [#]	92.3 ± 13.87 ^{##}
AST, unit/l	199.0 ± 26.17	184.4 ± 13.68	275.2 ± 33.15 [#]	283.8 ± 19.14 ^{##}
ALP, unit/l	256.8 ± 19.91	209.2 ± 11.38 [*]	282.3 ± 14.96	265.7 ± 13.22 ^{##}
LDH, unit/l	2873.3 ± 223.36	3140.9 ± 264.29	4190.0 ± 475.53 [#]	4299.3 ± 290.81 ^{##}
GLU, mmol/l	11.0 ± 0.78	10.0 ± 0.47	10.0 ± 0.36	9.1 ± 0.32
TP, g/l	88.3 ± 1.99	83.3 ± 5.63	90.8 ± 1.54	86.7 ± 2.02
CHOL, mmol/l	3.5 ± 0.23	3.0 ± 0.43	2.8 ± 0.26	2.9 ± 0.19
TG, mmol/l	0.3 ± 0.04	0.2 ± 0.03	0.2 ± 0.02 ^(#)	0.2 ± 0.01

* $p < 0.05$ – in comparison with the solvent; [#] $p < 0.05$; ^{##} $p < 0.01$; ^(#) $0.05 < p < 0.1$ – in comparison with the control.

respectively], with no factor interaction ($F_{1;35}^{CcxOf} = 0.01$, $p = 0.937$) *Post hoc* comparison, however, did not reveal any differences between the groups. There were no effects of the “agent” factor on the content of total protein (TP) ($F_{1;35}^{Cc} = 1.45$, $p = 0.237$), triglycerides (TG) ($F_{1;35}^{Cc} = 0.10$, $p = 0.757$) and cholesterol (CHOL) ($F_{1;35}^{Cc} = 0.34$, $p = 0.565$). The “infection” factor ($F_{1;35}^{Of} = 4.22$, $p = 0.047$) determined noticeable, though statistically insignificant, reduction of TG level against solvent introduction ($p = 0.072$). No effect of infection upon the content of protein and cholesterol in blood was found ($F_{1;35}^{Of} = 0.61$, $p = 0.441$; $F_{1;35}^{Of} = 1.65$, $p = 0.207$). По всем трем показателям взаимодействие $p = 0.207$). There was no factor interaction for all three parameters (TP: $F_{1;35}^{CcxOf} = 0.01$, $p = 0.914$; TG: $F_{1;35}^{CcxOf} = 0.85$, $p = 0.362$; CHOL: $F_{1;35}^{CcxOf} = 0.87$, $p = 0.357$).

Table 3 gives the results of the biochemical studies of the infected mice administered the solvent or *C. cibarius* extract daily during 7 days, 5 week after infection. There was no difference between the mice of the compared groups in all measured parameters, except glucose (ALT: $F_{1;27} = 0.01$, $p = 0.936$; AST: $F_{1;27} = 1.14$, $p = 0.295$; ALP: $F_{1;27} = 0.04$, $p = 0.838$; LDH: $F_{1;27} = 2.64$, $p = 0.116$; TP: $F_{1;27} = 1.28$, $p = 0.268$; CHOL: $F_{1;27} = 0.004$, $p = 0.951$; TG: $F_{1;27} = 0.004$, $p = 0.949$). *C. cibarius* extract increased the glucose level ($F_{1;37} = 5.35$, $p = 0.029$).

C. cibarius extract composition

Composition of different *C. cibarius* extracts was analysed. It was found that metanolic extraction is better for separating

Table 3. Biochemical blood values in *O. felineus*-infected mice after 7-day introduction of *C. cibarius*

Index	Solvent	<i>C. cibarius</i>
ALT, unit/l	61.0 ± 7.74	60.2 ± 5.02
AST, unit/l	232.1 ± 22.94	206.0 ± 10.04
ALP, unit/l	274.5 ± 13.58	270.8 ± 11.77
LDH, unit/l	3933.6 ± 308.83	3418.7 ± 103.66
GLU, mmol/l	8.3 ± 0.30	9.1 ± 0.21 [*]
TP, g/l	84.5 ± 0.83	83.0 ± 1.10
CHOL, mmol/l	2.9 ± 0.10	2.8 ± 0.27
TG, mmol/l	0.2 ± 0.01	0.2 ± 0.01

* $p < 0.05$ in comparison with solvent.

phenol compounds, and ethanol extraction – for carotinoids (Table 4). Reextraction by ethanol after methanolic extraction helps increase the number of polysaccharides and phenol compounds, but is accompanied by considerable protein loss. In all three types of extraction the equal and insignificant content of flavonoids was obtained.

Discussion

In the present work, the first data on anthelmintic activity of *C. cibarius* extracts on a model of *O. felineus*-induced opisthorchiasis in mice was obtained. Earlier, anti-inflammatory effects (Vamanu, Nita, 2014), insecticide

Table 4. Composition of *C. cibarius* extracts in three extraction techniques

<i>C. cibarius</i> extract	The content of substances in the sample (in terms of dry weight of the extract)				
	Polysaccharides mg/g	Total protein	Phenolic compounds	Flavonoids	Carotenoids, mkg/g
Ethanol	89.4±6.2	13.7±1.3	1.95±0.10	≤ 5	22.3
Methanol	82.3±3.5	12.0±0.8	2.47±0.10	≤ 5	3.2
Ethanol-methanol	148.0±4.7	≤ 5	4.42±0.12	≤ 5	6.0

activity (Cieniecka-Rosłonkiewicz et al., 2007), antioxidant and hepatoprotective properties – decreased fibrotic liver changes associated with induced inflammation (Aina et al., 2012; Khalili et al., 2014, 2015), as well as cytotoxic effects on cancer cells *in vitro* (Sari et al., 2017) for *C. cibarius* extracts were demonstrated. In view of the above, one might expect a possible inhibitory action of *C. cibarius* directly on helminths.

Our *in vitro* experiments proved that survival and mobility of juvenile *O. felineus* specimens reduces in accordance with increasing the dose of *C. cibarius* extract in the incubational medium. In 120 h after starting the experiments, actively moving worms were found only in the control group. Enlarged excretory bladder in parasites also indicate anthelmintic activity of *C. cibarius*: similar morphological changes were observed as an effect of praziquantel (Pakharukova et al., 2015). It is believed that praziquantel damages parasite’s metabolism affecting membrane transport proteins, particularly, proteins of the parasite’s excretory system (Greenberg, 2014). It is possible that *C. cibarius* also affects performance of the excretory system of *O. felineus*.

IC₅₀ of *C. cibarius* extract (1.58 mg/ml) is much higher than IC₅₀ of other clinically used agents such as praziquantel (IC₅₀ = 0.33 µg/ml for juvenile specimens and 0.14 µg/ml for adult specimens) (Pakharukova et al., 2015) or tribendimidin which is used to treat clonorchiasis (IC₅₀ = 0.05 µg/ml for adult specimens) (Keiser et al., 2013). It can be explained by low content of potentially anthelmintic compounds in used *C. cibarius* extract. Such substances in *C. cibarius* could be specific beta-glucans as immunomodulators with cytotoxic effect on tumor cells (El Enshasy, Hatti-Kaul, 2013; Valverde et al., 2015; Sari et al., 2017), ergosterol – fungal steroid with antioxidant properties, as well as phenol compounds (myricetin and catechine) involved in antioxidant processes (Ebrahimzadeh et al., 2015; Valverde et al., 2015; Muszyńska et al., 2016). Several authors emphasize high activity of flavonoids (Kozarski et al., 2015). The obtained composition of *C. cibarius* extract shows presence of a considerable number of polysaccharides, with prevalence of beta-glucans (Muszyńska et al., 2016). Flavonoids and phenol compounds are present in smaller quantities. Ergosterol was discovered earlier in *C. cibarius* extracts by other researchers (Muszyńska et al., 2016).

The *in vivo* model demonstrated a prolonged juvenile stage of parasite development in mice in comparison with hamsters (Avgustinovich et al., 2017a, 2018). Almost all parasites were immature: only two intestine branches were visible, and there were no gonads. In natural hosts (humans, cats), parasites mature in a month after infection (Beyer, 2005). It can indicate that mice have special resistance mechanisms to *O. felineus*

infection. Mice groups with solvent and *C. cibarius* extract under both schemes of administering had only single mature worm specimen. Therefore, it can be assumed that the extract did not influence maturation of *O. felineus* specimens in case of natural worm development. Enlargement of worm excretory bladder after administering *C. cibarius* extract to mice, similar to those found in the *in vitro* study, corroborates the anthelmintic effect of *C. cibarius* extract on parasites.

It should be emphasized that single-dose administration of *C. cibarius* extract on the first day after infecting caused a statistically significant reduction of the number of worms in the animals’ biliary ducts. Therefore, possible preventative anthelmintic effect of *C. cibarius* extract on the model of *O. felineus*-induced opisthorchiasis is shown for the first time. There were no statistically significant changes in the number of parasites in the animals’ biliary ducts when *C. cibarius* extract was administered for 7 days, 5 weeks after infection. Probably, some time after *O. felineus* migrate to biliary ducts, they become resistant to the effects of *C. cibarius* extract even under longer administration conditions. Thus, *C. cibarius* extract prevents worm attachment to the walls of biliary ducts and helps wash them out, but is not efficient against already attached parasites. However, we do not exclude that a higher concentration of one of the extract components could have the anthelmintic effect at the later stages of infection.

C. cibarius extract did not effect changes in the relative mass of liver, spleen and thymus in intact as well as infected animals under both schemes of introduction of *C. cibarius* extract. It means that the extract does not have any apparent toxic effect upon host organisms. Since the infected animals that received *C. cibarius* extracts had increased relative thymus weight in comparison with non-infected animals that were administered the extract, it is possible to assume that *C. cibarius* extract can stimulate T-cell immunity of host in parasitic infections.

Single-dose introduction of *C. cibarius* extract did not prevent normal body weight gain in animals, which implies absence of any negative effects upon the organism of hosts. The observed decreased mice body weight when *C. cibarius* extract was administered for 7 days is most likely related to an impact of daily stress from the substance administering procedure, which other authors also pointed out (Charmandari et al., 2005).

O. felineus infection increased activity of ALT, AST, and LDH. Such changes are noted in opisthorchiasis as well as other inflammatory processes in liver (Wonkchalee et al., 2012). Administering of *C. cibarius* extract did not normalize but also did not worsen these parameters. One can assume that single-dose introduction of the extract does not have

any adverse effect immediately after infection start as in the control group of mice. Reduced ALP can be considered a positive effect, indicating possible anti-cholestasis effect of the extract, because increased ALP activity is observed in chronic opisthorchiasis and other diseases that cause cholestasis (Dechakhampfu et al., 2010). It is possible that reduced ALP activity in the non-infected animals caused by the extract is due to N-glucans, which are considered as potential immunomodulators (Sari et al., 2017).

Based on the biochemical blood values, the 7-day administering of *C. cibarius* extract did not have a noticeable therapeutic effect upon the infected animals; at the same time, no adverse impact of the extract was found too. The observed increased level of glucose in the animal blood can be explained by the stress from the agent administering procedure.

Thus, the study has revealed an anthelmintic effect of *C. cibarius* extract administered on the first day after infecting of animals with *O. felineus* metacercariae. Damaged vital activities of parasites were shown *in vitro* and *in vivo*, visually demonstrated as enlarged excretory bladder. The observed anthelmintic effect means a higher extract efficacy at the state of parasite excystation. Absence of impaired body and organ weight in animals as well as their biochemical blood parameters prove that the fungi extract has no adverse effects. Further studying of certain components of *C. cibarius* extract is necessary to evaluate their anthelmintic properties.

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Conflict of interest

The authors declare no conflict of interest.

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