



RESEARCH ARTICLE

Dual anthelmintic and hepatoprotective potential of *Populus tremula* bark polysaccharide complex *in vitro*

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ABSTRACT

Opisthorchiasis, caused by the liver fluke *Opisthorchis felinus*, remains a significant public health problem in Western Siberia and other parts of Eurasia. The present study investigated the anthelmintic and hepatoprotective properties of a polysaccharide complex (PC) extracted from *Populus tremula* bark. Anthelmintic activity was assessed *in vitro* against *O. felinus* worms using motility scores, MTS viability assay, and propidium iodide (PI) staining. Hepatoprotective potential was evaluated in HepG2 cells using lipopolysaccharide/interferon- γ (LPS/IFN- γ) induced inflammation and free fatty acid (FFA) induced lipotoxicity models; reactive oxygen species (ROS), reduced glutathione (GSH), and intracellular lipid content were measured fluorimetrically. Antioxidant activity was further characterised by DPPH radical scavenging and ferrous ion chelating assays. PC significantly reduced worm motility and viability (calculated motility index decreased by approximately 45%, from 3.80 to 2.10), and induced focal tegumental PI accumulation, consistent with surface damage. In the LPS/IFN- γ model, PC at 125 $\mu\text{g/mL}$ decreased ROS by 14% ($p=0.001$) and partially restored GSH content by 25% ($p=0.016$). In FFA treated cells, the same concentration attenuated ROS by 15.5% ($p=0.001$) and reduced lipid accumulation by 22.8% ($p=0.008$). PC did not affect HepG2 viability. The complex demonstrated modest DPPH radical-scavenging activity (33.6% at 200 $\mu\text{g/mL}$) and a pronounced Fe^{2+} chelating capacity (24.70 $\pm$ 2.63 mg EDTA equivalents/g). These first initial *in vitro* findings identify *P. tremula* bark PC as a candidate dual-action agent for treating of opisthorchiasis, combining direct anthelmintic activity with multi-targeted hepatoprotective effects that warrant confirmation in further mechanistic and *in vivo* studies.

Keywords: *Opisthorchis felinus*; polysaccharide complex; anthelmintic potential; hepatoprotective potential; antioxidants.

INTRODUCTION

Parasitic liver diseases caused by trematodes, cestodes, and nematodes remain a significant global health burden (Peters *et al.*, 2021). Among these, trematode infections pose a particular epidemiological threat (Khalil *et al.*, 2022). In recent years, the greatest clinical significance has been attributed to members of the families *Schistosomatidae*, *Opisthorchiidae*, *Fasciolidae*, and *Paragonimidae* (Chai & Jung, 2022; Liu *et al.*, 2024b).

Opisthorchis felinus (*O. felinus*) is a key causative agent of opisthorchiasis. Infection can manifest as asymptomatic carriage or progress to severe hepatobiliary pathology, including cholecystitis, cholangitis, and periductal fibrosis. Chronic invasion is also associated with an elevated risk of developing cholangiocarcinoma (Fedorova *et al.*, 2020; Probst *et al.*, 2022). Evidence suggests that chronic *O. felinus* invasion modulates the host immune response, potentially exacerbating comorbidities (Fedorova *et al.*, 2017). This

parasite is the dominant etiological agent of opisthorchiasis across a vast geographical area encompassing Russia, Kazakhstan, and several European countries (Fedorova *et al.*, 2020; Qian *et al.*, 2024). A particularly high prevalence of *O. felinus* infection is recorded in Western Siberia (Fedorova *et al.*, 2020).

Pharmacotherapy is the primary strategy for reducing the prevalence of opisthorchiasis. Praziquantel (PZQ), an isoquinoline derivative, remains the sole drug of choice for trematode infections. WHO-recommended regimens include 25 mg/kg administered three times daily for 2–3 days or a single 40 mg/kg dose; a regimen of a single 50 mg/kg dose followed by 25 mg/kg the next day is also common (Qian *et al.*, 2024). The PZQ mechanism of action involves disrupting Ca^{2+} homeostasis in the parasite by modulating interactions among slow calcium channel subunits, leading to muscle contraction and sustained paralysis (Chan *et al.*, 2013; Lee *et al.*, 2024). Additional mechanisms include tegumental damage that exposes parasitic antigens to host immune effector mechanisms

(Ribeiro-dos-Santos *et al.*, 2006) and inhibition of nucleoside transporters, resulting in ATP depletion and impaired nucleic acid synthesis (Angelucci *et al.*, 2007).

Despite its efficacy against *O. felineus*, PZQ therapy has significant limitations. These include the cytotoxicity of its S-enantiomer (Sun *et al.*, 2016) and the potential for the emergence of trematode resistance (Harder, 2020; Lvova *et al.*, 2023). A critical concern is the debated association between repeated PZQ administration and an increased risk of cholangiocarcinoma. This association may stem from the massive release of parasitic antigens that triggers systemic inflammation and oxidative/nitrosative stress (Kamsa-Ard *et al.*, 2015; Lvova *et al.*, 2023), although some *in vivo* studies suggest a protective effect of PZQ treatment (Hanpanich *et al.*, 2017). Furthermore, PZQ lacks preventive activity and is ineffective against larval stages (Spangenberg, 2020). These drawbacks (cytotoxicity, emerging resistance, and potential carcinogenic risk) underscore the urgent need for novel therapeutic strategies.

In this context, plant-derived compounds from regions endemic for opisthorchiasis are of considerable interest. A polysaccharide complex extracted from the bark of *Populus tremula* L. (PC) shows particular promise; related *Populus* preparations have shown anti-inflammatory, antioxidant and hepatoprotective properties (Debbache-Benaida *et al.*, 2013; Dudonné *et al.*, 2011). Given the reported success of complex plant extracts against other trematodes, this PC warrants investigation, either as a standalone agent or in combination with PZQ, to enhance antiparasitic effects and mitigate pathophysiological consequences. A commonly used approach for the primary evaluation of anthelmintic potential is *in vitro* screening based on assessments of parasite motility and morphology (Pechdee *et al.*, 2025; Sangkam *et al.*, 2024).

Therefore, the aim of the present study was to investigate the anti-opisthorchiasis and hepatoprotective potential of *P. tremula* bark PC. The anthelmintic efficacy was evaluated *in vitro* by determining worm viability, motility, and tegumental morphology in adult *O. felineus*, while hepatoprotective potential was examined using the HepG2 cell line and antioxidant potential was measured in DPPH radical scavenging and iron (II) chelating assays.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Preclinical Research Center, Central Research Laboratory, Siberian State Medical University, Ministry of Health of the Russian Federation (Protocol No. 1, September 02, 2023).

Preparation of the PC from *Populus tremula* Bark

The PC was obtained from the bark of *Populus tremula* L. The raw material was harvested in April 2025 in the Tomsk district of the Tomsk region (Russia) during the sap flow phase, before bud break. Botanical identification was performed using the “Flora of Siberia” atlas (Krasnoborov & Malyshev, 2003). The plant material was dried under natural conditions to a residual moisture content of 8-10%.

In a 2000 L industrial extractor (Hubei weihua machinery co., Ltd., China), a liquid extract with a 1:1 ratio of raw material to extractant (water) was obtained by repercolation. The resulting liquid extract was concentrated in an SX-250 vacuum evaporation system (Hubei weihua machinery co., Ltd., China) to a residual moisture content of 25-40%. Concentrated bark extract (350 g) was placed in a 5 L flask, mixed with 70% ethanol at a 1:12 (w/v) ratio (4200 mL), and sonicated (28 kHz) for 5 min with stirring. The supernatant was decanted, and the residue was centrifuged at 3 000 rpm for 10 min. The resulting pellet was resuspended in an equal volume of 70% ethanol, sonicated, and centrifuged twice more under the same conditions. The final pellet was transferred to a

round-bottom flask and evaporated to constant weight on a rotary evaporator at 40°C and 50 mbar.

Physicochemical characterization of the PC

Total carbohydrate content was determined by the phenol-sulfuric acid method (Gulina *et al.*, 2023), and uronic acids were quantified by the carbazole-sulfuric acid method (Rovkina *et al.*, 2018). Protein and nucleic acid contents were assessed by the Lowry method (Rovkina *et al.*, 2018) and the Spirin method (Gulina *et al.*, 2023), respectively. Total flavonoids were measured spectrophotometrically by complexation with aluminum chloride and expressed as luteolin equivalents (Pisareva *et al.*, 2008).

Monosaccharide composition was determined by gas chromatography with flame ionization detection after derivatization, as previously described (Reshetov *et al.*, 2019). Chromatography was performed on an Ultimate 3000 HPLC system (Dionex, Germany) coupled to an RI-101 refractive index detector (Shodex, Japan). An Ultrahydrogel 250 column (300×7.8 mm, 6 µm) was used with 0.9% aqueous NaCl as the mobile phase at a flow rate of 0.5 mL/min and a detector cell temperature of 40°C. Molecular weight distribution was evaluated by size-exclusion chromatography with refractive-index detection, using dextran standards for calibration (Zykova *et al.*, 2024). Calibration was performed using dextran standards ranging from 180 to 322 000 Da (PSS-DXTKIT-07, Agilent, USA).

Isolation and treatment of adult *O. felineus* worms with the PC and PZQ

The adult *O. felineus* worms were obtained from experimentally infected golden hamsters (*Mesocricetus auratus*) as previously described (Wong *et al.*, 2023). 40 female hamsters weighing 130-150 g and maintained under specific pathogen-free (SPF) conditions were used. Animals were sourced from the Federal Research Center, Institute of Cytology and Genetics (Novosibirsk, Russia). Three months post-infection, hamsters were euthanized in a CO₂ atmosphere. Adult flukes were carefully extracted from the hepatobiliary tract, washed with sterile 0.9% NaCl solution (Grotex LLC, Russia), and incubated in RPMI 1640 medium containing 2× penicillin-streptomycin (PanEco Ltd., Russia) for 2 hours at 37°C in a 5% CO₂ atmosphere. Worms were pooled across hamsters and randomly allocated to the experimental groups, so that each well or group contained worms of mixed origin. For the motility assessment, worms were transferred to six-well culture plates (SPL Life Sciences Co., Republic of Korea) at ten worms per well, with two wells per treatment group. All procedures were conducted in three independent biological replicates using worms extracted from separate hamsters. The wells contained RPMI 1640 medium (PanEco Ltd., Russia) supplemented with 1× penicillin-streptomycin, 10 g/L glucose, and 2 g/L sodium bicarbonate (all from PanEco Ltd., Russia). For the MTS assay, ten worms per group were analysed individually. For propidium iodide staining, five worms per group were examined in two independent replicates.

For anthelmintic activity assessment, the PC was dissolved directly in culture medium to a final concentration of 1 000 µg/mL. Praziquantel (PZQ) was initially dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in culture medium to a final concentration of 600 µg/mL, ensuring that the final DMSO concentration did not exceed 0.5%. Adult worms were incubated with PC or PZQ for 24 hours at 37°C in a 5% CO₂ incubator, after which motility, viability, and surface integrity were evaluated as described in the respective sections below.

In vitro motility assessment of *O. felineus* adult worms

The motility of adult *O. felineus* worms was assessed by microscopic examination (Ningbo Yongxin Optics Co Ltd, China) and scored visually using a 4-point scale as previously described (Pechdee *et al.*, 2025). The scoring criteria were as follows: 4 = very active

movements, comparable to those of control worms; 3 = regularly active movements, less vigorous than control but involving the entire body; 2 = low activity, with movement restricted; 1 = immobile worms. The motility index (MI) for each experimental group was calculated using the formula:

$$RM (\%) = \frac{MI_{\text{experiment}}}{MI_{\text{control}}} \times 100\% \quad (1)$$

$$MI = \frac{\sum n \times Nn}{\sum Nn}$$

MI – the index of motility of the worm group, n – motility score (points), Nn – the number of trematodes given n points.

Worm viability study by MTS assay

The viability of adult *O. felineus* worms was assessed using the MTS reagent according to a previously established protocol (Perina et al., 2024). Individual parasites were placed in 1.5 mL tubes containing 200 µL of 1× PBS and 40 µL of MTS reagent (Promega, USA). Following incubation for 2 hours at 37°C, the medium was collected and centrifuged at 5 000 rpm for 10 minutes to remove eggs and excretory-secretory products. The supernatant was transferred to a 96-well plate, and optical density (OD) was measured at 490 nm using an INNO Microplate Spectrophotometer (LTK Co., Ltd., Republic of Korea). As a positive control, worms were heat-inactivated at 65°C for 30 minutes prior to the assay (Perina et al., 2024).

To account for variations in worm size and cellularity, OD values were normalized to total protein content. Protein concentration was determined using a bicinchoninic acid assay (BCA Protein Assay Kit, Sigma Aldrich, USA) following the manufacturer's instructions (Goldring, 2019). Worms were homogenized in lysis buffer (0.1% SDS, 0.1 M NaOH) and sonicated (Bandelin Sonoplus 2070, Germany). The homogenate was centrifuged at 3 000 g for 10 minutes, and the supernatant was used for protein quantification. Final viability was expressed as the OD value normalized to the protein content of each individual fluke. A PC-only blank (PC in medium in the absence of worms) was measured in parallel and subtracted to correct for the intrinsic absorbance of PC.

O. felineus tegumental damage evaluation

Surface damage to adult *O. felineus* worms was assessed by staining with the fluorescent dye propidium iodide (PI), which intercalates into nucleic acids and serves as a marker of compromised membrane integrity. Worms were incubated with PI (2 µg/mL) for 20 minutes, followed by two washes with physiological saline. For microscopic visualization, specimens were mounted on glass slides in a drop of Prolong Gold Antifade Reagent (Invitrogen, USA) and covered with a coverslip. Images were acquired using a Leica DM6 fluorescence microscope (Leica Microsystems GmbH, Germany) (Pakharukova et al., 2015).

Cytotoxicity study

The cytotoxicity of the PC and the reference compound praziquantel (PZQ; TCI, Japan) toward HepG2 cells was evaluated using the neutral red uptake assay (Repetto et al., 2008). Prior to the assay, cells were maintained under standard culture conditions (5% CO₂, DMEM/F12 medium (BioinnLabs, Russia) supplemented with 10% fetal bovine serum (Global Kang Biopharmaceuticals, China), 292 mg/L L-glutamine (GlutaMAX, Gibco, Thermo Fisher Scientific Inc., USA), 40 µg/mL gentamicin, and 0.25 µg/mL amphotericin B (AAS Solution, Sigma-Aldrich, USA). The test substances were added to the cells at concentrations ranging from 3.9 to 1 000 µg/mL. Following 24 hours of incubation, cell viability was assessed spectrophotometrically by measuring absorbance at 540 nm (reference wavelength 650 nm) using a Tecan Infinite 200 PRO M Plex multifunctional microplate reader (Tecan Austria GmbH, Austria). Viability was expressed as

a percentage relative to untreated control cells. Untreated cells (without PC) served as the baseline control, and their signal was subtracted from the readings.

Induction of lipotoxicity in HepG2 cells

The hepatoprotective properties of the PC were evaluated using a lipotoxicity model in the HepG2 cell line. Culture medium enriched with free fatty acids (FFA) was prepared according to standard protocol (Yao et al., 2011). The FFA-rich DMEM/F12 medium (BioinnLabs, Russia) contained 292 mg/L L-glutamine (GlutaMAX, Gibco, Thermo Fisher Scientific Inc., USA), 40 µg/mL gentamicin, 0.25 µg/mL amphotericin B (AAS Solution, Sigma-Aldrich, USA), 2% fatty acid-free BSA (Sigma-Aldrich, USA), 1 mM sodium oleate (Sigma-Aldrich, USA) from a 30 mM stock solution in 0.9% NaCl, 0.5 mM sodium palmitate (Sigma-Aldrich, USA) from a 30 mM stock solution in 0.9% NaCl, and 2% 1M HEPES (Gibco, Thermo Fisher Scientific Inc., USA). Control medium contained identical components with the exception of free fatty acids.

HepG2 cells were seeded into 96-well culture plates (SPL Life Sciences Co., Republic of Korea) at a density of 1×10⁴ cells per well. After 24 hours, the cells were pre-incubated for 2 hours with the PC (62.5 and 125 µg/mL) or the reference compound gemfibrozil at a concentration of 100 µM (Sigma-Aldrich, USA). Gemfibrozil concentration was selected based on previously published data demonstrating its *in vitro* lipid-lowering efficacy (Zhang et al., 2019). Following pre-incubation, the cells were washed, and the medium was replaced with either FFA-rich or control medium (without FFA). The test substances were then re-added, and the cells were incubated for an additional 24 hours. After incubation, cells were washed to remove the test compounds prior to analyses of intracellular lipid accumulation and reactive oxygen species (ROS).

Induction of inflammation in HepG2 cells with LPS/IFN-γ

To induce an acute inflammatory response, HepG2 cells were stimulated with 10 µg/mL lipopolysaccharide (LPS) and 50 ng/mL interferon-gamma (IFN-γ) for 48 h. The LPS concentration was chosen based on the reported low sensitivity of HepG2 cells to this endotoxin (Cubit et al., 2024), whereas the IFN-γ dose reflects concentrations that have been shown to effectively activate hepatocyte-derived cell lines (Chen et al., 2018). Combined LPS/IFN-γ treatment is a widely adopted strategy for generating a robust pro-inflammatory microenvironment *in vitro* (Kiyofuji et al., 2015). As with the lipotoxicity model, the PC was added to the cells at final concentrations of 62.5 and 125 µg/mL two hours prior to the addition of pro-inflammatory agents and maintained throughout the entire 48-hour incubation period.

Quantification of intracellular lipid accumulation

Intracellular lipid levels were assessed in FFA-treated cells using the lipophilic fluorescent dye Nile Red as previously described (Yao et al., 2011). Briefly, following the treatment period, cells were washed to remove unbound test compounds, stained with a working solution of Nile Red (1 µg/mL in 1× PBS) and incubated for 10 minutes at room temperature. After washing to remove excess dye, fluorescence intensity was measured using a Tecan Infinite 200 PRO M Plex multifunctional microplate reader (Tecan Austria GmbH, Austria) at excitation/emission wavelengths of 488 nm and 550 nm, respectively, with values background-corrected against cells maintained in parallel without PC or FFA. Nile Red fluorescence was additionally visualized using a Leica Dmi8 biological microscope (Leica Microsystems GmbH, Germany).

Measurement of intracellular reactive oxygen species (ROS)

The level of intracellular ROS in FFA or LPS/IFN-γ induced cells was determined fluorimetrically using the probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Zhang et al.,

2023). Following the treatment period (induction of lipotoxicity or LPS/IFN- γ treatment and incubation with PC), cells were washed and loaded with DCFH-DA at a final concentration of 10 μ M. After 20 minutes of incubation at 37°C in the dark, the cells were washed again to remove excess probe. Fluorescence intensity, reflecting intracellular ROS levels, was recorded using a Tecan Infinite 200 PRO M Plex multifunctional microplate reader (Tecan Austria GmbH, Austria) at excitation/emission wavelengths of 485 nm and 530 nm, respectively. For background correction, parallel wells received neither PC nor the corresponding inducer (FFA or LPS/IFN- γ , as applicable), and their DCF signal was subtracted from all treated samples. Representative fluorescence microscopy images were additionally acquired using a Leica Dmi8 biological microscope (Leica Microsystems GmbH, Germany) at $\times$ 40 magnification.

Measurement of intracellular reduced glutathione (GSH)

Intracellular levels of reduced glutathione (GSH) in LPS/IFN- γ or LPS/IFN- γ /PC-treated cells were assessed using the thiol specific fluorescent probe dibromobimane, adapted from a previously described protocol (Cox & Cardozo, 2007). Following the 48 hour treatment period, HepG2 cells were washed once with pre warmed PBS. A freshly prepared solution of 40 μ M dibromobimane (Sigma Aldrich, USA) in PBS was added to each well, and the plate was incubated at 37°C for 30 minutes in the dark. Fluorescence intensity was then measured directly using a Tecan Infinite 200 PRO M Plex microplate reader (Tecan Austria GmbH, Austria) at excitation/emission wavelengths of 393 nm and 477 nm, respectively. Fluorescence from cells without PC and LPS/IFN- γ served as the background and was subtracted from treated samples. Dibromobimane fluorescence was additionally visualized using a Leica Dmi8 biological microscope (Leica Microsystems GmbH, Germany).

Evaluation of DPPH radical scavenging activity

The 2,2-diphenyl 1-picrylhydrazyl (DPPH) radical scavenging activity of the fractions was assessed using the stable free radical DPPH as previously described (Lee *et al.*, 2001). Briefly, the reaction mixture consisted of 900 μ L of 96% ethanol and 100 μ L of 1 mM ethanolic DPPH solution, to which the PC was added at final concentrations of 25, 50, 100, 150, and 200 μ g/mL. After incubation for 30 min at room temperature, the absorbance was measured at 517 nm against ethanol using a Tecan Infinite 200 PRO M Plex multifunctional microplate reader (Tecan Austria GmbH, Austria). The percentage of DPPH radical scavenging was calculated as (D is the absorbance):

$$\text{DPPH scavenging (\%)} = \frac{D_{\text{control}} - D_{\text{sample}}}{D_{\text{control}}} \times 100\% \quad (2)$$

PC-only blank containing PC in the reaction mixture without the DPPH radical was measured in parallel and subtracted to correct for the intrinsic absorbance of PC.

Quantification of iron (II) chelating ability

The ferrous ion chelating ability was determined according to a standardized protocol (Santos *et al.*, 2017). A calibration curve was prepared using disodium ethylenediaminetetraacetic acid (Na₂EDTA) at concentrations ranging from 6.25 mg/L to 40 mg/L. Aliquots of 50 μ L of EDTA solution were mixed with 150 μ L of distilled water and 20 μ L of 0.3 mM FeSO₄ in a 96 well plate. After 5 min incubation at room temperature, 30 μ L of 0.8 mM ferrozine was added, and the absorbance was measured at 562 nm after 15 min using an INNO microplate reader (LTK Co., Ltd., Republic of Korea). Control samples contained 200 μ L of distilled water and 20 μ L of 0.3 mM FeSO₄. Test samples of PC were prepared by mixing 50 μ L of PC (1 mg/mL), 150 μ L of distilled water, and 20 μ L of 0.3 mM FeSO₄, and then following the same procedure as described. The chelating

ability was expressed as mg of EDTA equivalents per gram of the substance, calculated using the EDTA calibration curve. PC-only blank containing PC in the reaction mixture without ferrozine was measured in parallel and subtracted to correct for the intrinsic absorbance of PC.

Statistical analysis

Statistical data analysis was performed in GraphPad Prism 8.0 (GraphPad Software, USA). The number of worms and of independent biological replicates differed between assays: motility was assessed at ten worms per well, two wells per treatment group, in three independent replicates; the MTS viability assay was performed on ten worms per group in a single experiment; and propidium iodide staining was performed on five worms per group in two independent replicates. Worm motility was recorded on a four-level ordinal scale (scores 1–4) and was analysed as a categorical variable. Differences in the distribution of the four categories between experimental groups were evaluated using Pearson's chi-square test of independence, based on absolute frequencies. The motility index (MI) and relative motility index (RM) were each calculated as a single aggregate value across all replicates, from the pooled category counts. Because worms extracted from different hamsters were pooled and randomly allocated across wells and treatment groups, no group was disproportionately represented by worms from a single hamster. All cell-culture experiments (cytotoxicity, FFA-induced lipotoxicity, LPS/IFN- γ stimulation, and the associated ROS, reduced glutathione and Nile Red measurements) were performed in three independent biological replicates. The normality of the quantitative variables was assessed using the Shapiro-Wilk test. Quantitative data are presented as mean $\pm$ standard deviation (SD). The mean values of several independent groups were compared using one-way analysis of variance (ANOVA). For pairwise group comparisons, we applied the Bonferroni correction. The level of statistical significance was $p < 0.05$.

RESULTS

Chemical composition and molecular properties of the PC

The isolated PC accounted for approximately one-third of the initial air-dried raw material (Table 1). Carbohydrates represented the major component ($\approx$ 96%), with uronic acids comprising 12.6%. Protein and nucleic acid contamination was negligible, and flavonoid content was minimal.

Table 1. General chemical composition of the *Populus tremula* L. polysaccharide complex. Data are presented as mean $\pm$ SD

Parameter	Value, %
Yield (% of air-dried raw material)	37.3 $\pm$ 4.7
Content (%)	
Carbohydrates	96.20 $\pm$ 2.10
Uronic acids	12.6 $\pm$ 0.4
Proteins	0.44 $\pm$ 0.05
Nucleic acids	0.0014 $\pm$ 0.0002
Flavonoids (as luteolin)	0.0086 $\pm$ 0.0007

Monosaccharide analysis revealed that the fraction consisted predominantly of arabinose (44.0 $\pm$ 3.9%), galactose (39.8 $\pm$ 2.8%), and galacturonic acid (10.2 $\pm$ 1.9%); glucose and mannose were detected only in trace amounts.

Size-exclusion chromatography yielded an asymmetric, partially resolved peak (Figure 1). The calculated molecular weight parameters are presented in Table 2.

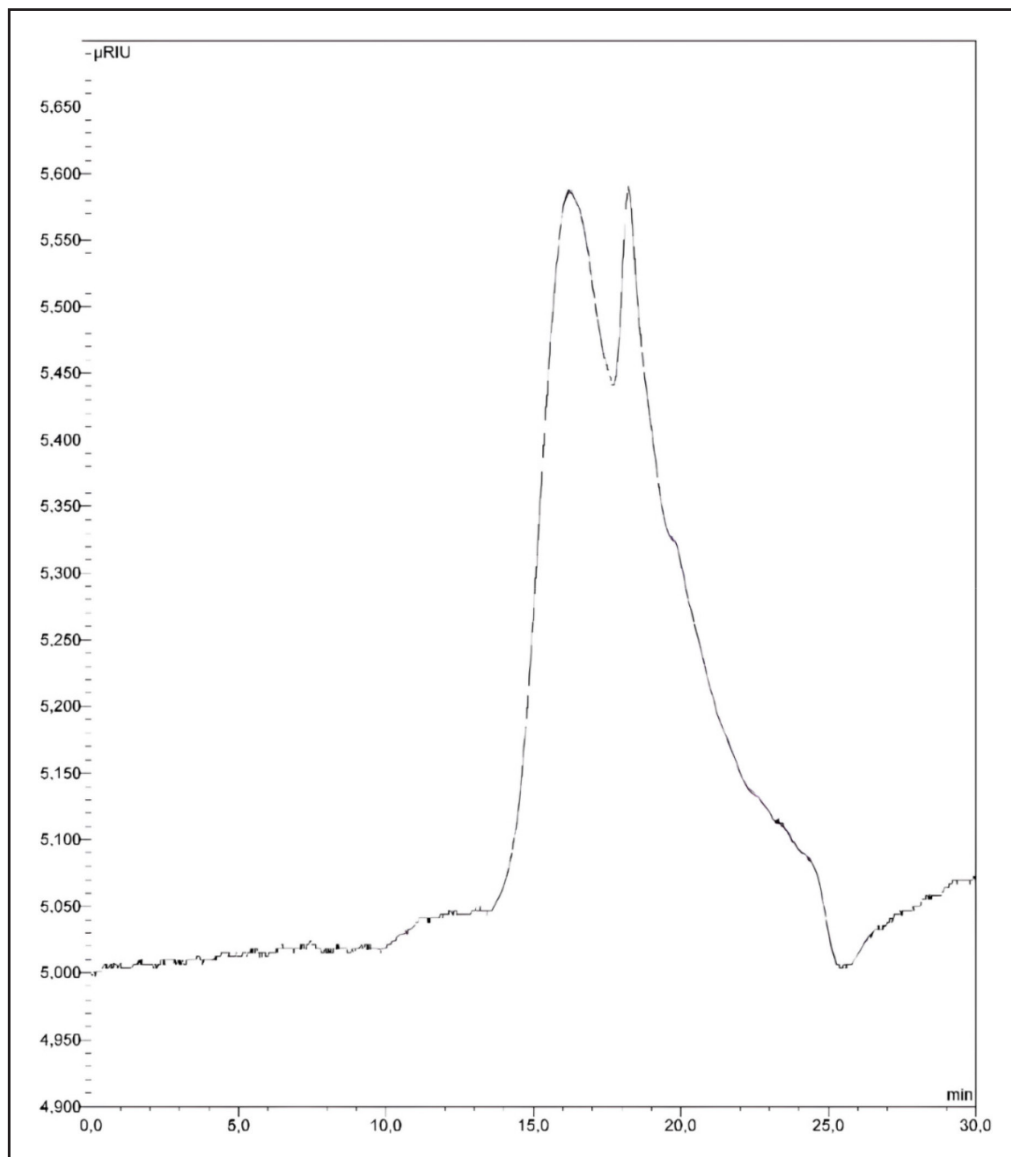


Figure 1. Size-exclusion chromatogram of the polysaccharide complex from *P. tremula* bark.

Table 2. Molecular weight distribution of the *Populus tremula* L. polysaccharide complex

Parameter	Mean	RSD (%)
Mw (Da)	28 921	12
Mn (Da)	609	77
Mz (Da)	201 828	15
Polydispersity (Mw/Mn)	61	35

Mw – weight-average molecular weight; Mn – number-average molecular weight; Mz – z-average molecular weight, RSD – relative standard deviation.

The weight-average molecular weight (Mw) was approximately 29 kDa, and the polydispersity index (Mw/Mn) was 61, indicating a broad molecular weight distribution. Because these values were estimated against linear dextran standards, they represent apparent (relative) molecular weights; given the branched, multi-component composition of the preparation, the PC is best regarded as a heterogeneous polysaccharide mixture rather than a uniform polysaccharide.

Anthelmintic activity of PC and PZQ against adult *O. felineus* in vitro

Assessment of parasite motility and morphology is the standard *in vitro* approach for initial screening of anthelmintic activity, even though reduced motility alone is not an absolute indicator of parasite death (Sangkam *et al.*, 2024).

Motility assays employed two separate control groups: a vehicle control for PC and a solvent control (0.5% DMSO) for PZQ. In both controls, adult *O. felineus* worms maintained normal motor activity throughout the observation period, confirming their viability under the experimental conditions (Figure 2A).

Praziquantel induced complete immobility in 73.3% of parasites, while the remaining worms exhibited severely suppressed motility. The relative motility index fell to 33.9% of the control value (Figure 2A), consistent with the well characterised paralytic action of this drug (Park *et al.*, 2021). Light microscopy revealed extensive epithelial sloughing and tegumental oedema in PZQ treated worms (Figure 2C).

Incubation with PC (1 000 µg/mL, 24 h) produced a distinct pattern of motor suppression. Approximately 30% of adult flukes displayed profound motility impairment (score 2), and a further 30% were fully paralysed (score 1; $p < 0.001$ vs. control). The motility

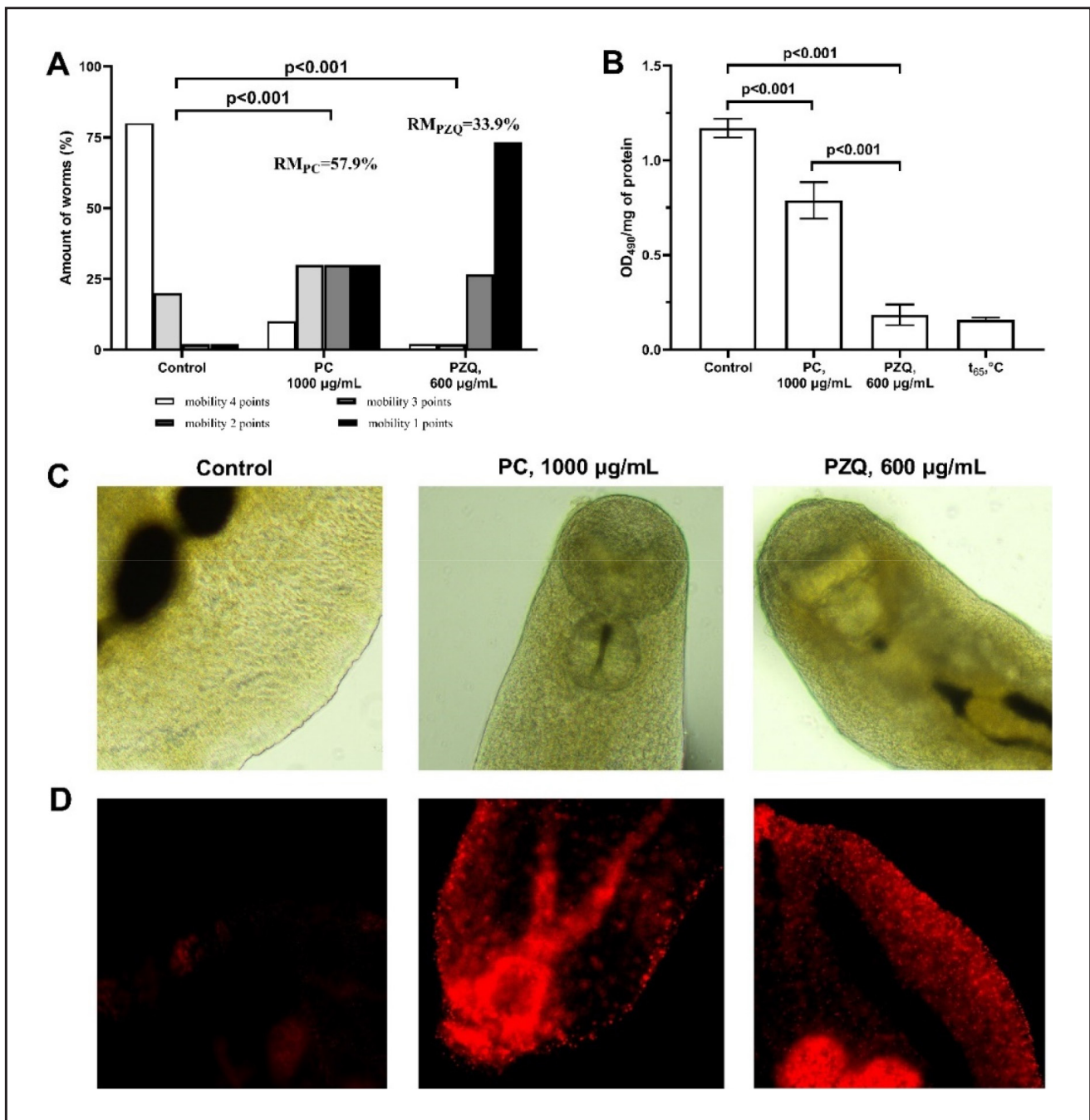


Figure 2. Effect of polysaccharide complex (PC) from *Populus tremula* bark and the reference drug praziquantel (PZQ) on adult *O. felinus* worms *in vitro*. A – Motility scores and relative motility index; B – Viability assessed by the MTS assay and expressed as OD₄₉₀ normalized to protein content; C – Representative light microscopy images of worm surface morphology (×10 magnification); D – Fluorescence microscopy images after propidium iodide staining, showing nuclei of membrane damaged cells (×10 magnification). PC was applied at 1 000 µg/mL and PZQ at 600 µg/mL for 24 h. 10 worms per well with 2 wells per group in 3 independent experiments (A). Motility category distributions were compared between groups by Pearson's chi-square test based on absolute frequencies; data in A are shown as relative frequencies. Significance versus the corresponding control is indicated (p-values as shown). Viability assessed by the MTS assay from a single experiment with ten worms per group and is presented as mean±SD across individual worms (B). 5 worms in each group in 2 different experiments were used for propidium iodide staining (D).

index in the PC treated group fell by approximately 45%, from 3.80 to 2.10 (Figure 2A). Light microscopy confirmed visible damage to the surface structures of PC exposed worms (Figure 2C).

These observations were corroborated by the MTS assay. The mean optical density (OD₄₉₀) of water soluble formazan - reflecting mitochondrial metabolic activity - was 1.17±0.05 per mg protein in untreated viable worms. Heat inactivated (positive control) parasites yielded a baseline OD of 0.16±0.01 (Figure 2B). PZQ (600 µg/mL) reduced worm viability by 84.6% (p<0.05). PC decreased viability by

32.6% (p<0.05). This reduction in the MTS signal reflects impaired metabolic activity rather than confirmed cell death; together with the reduced motility and focal propidium iodide labelling, it is consistent with an *in vitro* anthelmintic effect of the fraction on adult *O. felinus* worms (Figure 2B).

Further validation was obtained by fluorescence microscopy using propidium iodide (PI), a membrane impermeant intercalating dye that selectively labels DNA in cells with compromised plasma membrane integrity.

Intact (negative control) worms excluded PI, whereas heat inactivated (positive control) parasites were almost entirely PI positive (Figure 2D). PZQ treatment resulted in intense PI accumulation, accompanied by pronounced phenotypic alterations of the worms. These findings are consistent with data reported for the closely related species *O. viverrini*, in which PZQ induces extensive tegumental destruction and sloughing (Apinhasmit & Sobhon, 1996).

PC induced focal PI fluorescence, localised predominantly within the tegumental syncytium (Figure 2D). As noted above, complete immobilisation was observed in only 30% of worms exposed to PC. The partial nature of the effect suggests that impaired motility represents an early, visible consequence of tegumental surface damage. Under *in vivo* conditions, such disruption of the worm surface is likely to facilitate antibody dependent cellular cytotoxicity, thereby promoting effective immune mediated parasite clearance.

Cytotoxicity of PC and PZQ in HepG2 cells

Incubation of HepG2 cells with PC across the concentration range of 3.9-1 000 µg/mL for 24 h had no effect on cell viability at any tested dose ($p>0.05$; Figure 3A). Even at the highest concentration tested (1 000 µg/mL), viable cells accounted for $87.4\pm7.0\%$ of the untreated control, confirming the absence of cytotoxic activity under the conditions employed.

In contrast, PZQ caused a dose dependent loss of cell viability (Figure 3B). The decline became significant at 31.3 µg/mL ($76.6\pm5.3\%$ of control) and progressed markedly with increasing concentration. At 125 µg/mL, viability fell to just $22.6\pm2.8\%$ of control, and at the highest concentration (1 000 µg/mL), it dropped to a minimum of

$7.2\pm0.3\%$. These findings indicate that PC, unlike the reference drug PZQ, did not show detectable cytotoxicity toward HepG2 cells at the concentrations tested.

PC suppresses LPS/IFN- γ induced oxidative stress and preserves GSH stores in HepG2 cells

Stimulation of HepG2 cells with LPS (10 µg/mL) and IFN- γ (50 ng/mL) for 48 h resulted in a pronounced oxidative stress: DCF fluorescence intensity increased by 50%, from 720 ± 47 a.u. in untreated controls to 1080 ± 81 a.u. in the LPS/IFN- γ group ($p<0.001$; Figure 4A). This rise in ROS was paralleled by a marked depletion of intracellular reduced glutathione, with dibromobimane fluorescence declining by almost 35%, from $3,135\pm251$ a.u. to $2,026\pm340$ a.u. ($p<0.001$; Figure 4B).

Co treatment with PC at 125 µg/mL significantly attenuated both parameters. The LPS/IFN- γ induced ROS elevation was reduced by 14%, with DCF fluorescence falling to 926 ± 61 a.u. ($p=0.001$ vs. LPS/IFN- γ alone), while GSH levels were partially restored by about 25%, reaching $2\,531\pm270$ a.u. ($p=0.016$ vs. LPS/IFN- γ alone). The lower PC concentration (62.5 µg/mL) did not produce significant changes in either parameter, indicating a concentration dependent protective effect. Representative fluorescence microscopy images (Figures 4C and 4D) corroborated the quantitative data, visually demonstrating a reduction in the LPS/IFN- γ driven oxidative burden and GSH depletion upon exposure to 125 µg/mL PC.

Taken together, these results demonstrate that PC attenuates LPS/IFN- γ induced redox imbalance in HepG2 cells, as evidenced by reduced ROS accumulation and partial restoration of intracellular GSH, indicating that PC modulates the oxidative stress and redox status of HepG2 cells under pro-inflammatory (LPS/IFN- γ) stimulation; since inflammatory mediators were not measured, these data do not directly demonstrate anti-inflammatory activity.

Hepatoprotective activity of PC in FFA treated HepG2 cells

Incubation of HepG2 cells in medium enriched with 1 mM sodium oleate and 0.5 mM sodium palmitate for 24 h led to a pronounced intracellular accumulation of reactive oxygen species (ROS). DCF fluorescence intensity rose by 65%, from 550 ± 47 a.u. in untreated control cells to 907 ± 68 a.u. in FFA exposed samples ($p<0.001$; Figure 5A). This elevation in ROS is a hallmark of lipotoxic injury and reflects the disruption of cellular redox homeostasis by excess free fatty acids.

Co treatment with PC reduced the FFA induced ROS burden in a concentration dependent manner. At 62.5 µg/mL, the DCF signal remained close to that of FFA treated cells (844 ± 71 a.u.; $p>0.05$ vs. FFA alone), whereas at 125 µg/mL, PC significantly decreased DCF fluorescence to 785 ± 61 a.u., corresponding to a 15.5% reduction relative to the FFA group ($p=0.001$ vs. FFA alone). These data demonstrate that the higher concentration of PC effectively limits lipotoxicity associated oxidative stress.

Consistent with the results on oxidative stress, FFA exposure provoked a marked increase in intracellular lipid content measured by Nile Red fluorescence, which rose by 65-85% compared with untreated controls ($p<0.001$; Figure 5B). The reference hypolipidemic agent gemfibrozil (100 µM) almost completely normalised lipid levels, reducing them to $109.9\pm13.0\%$ of the control value ($p<0.001$ vs. FFA). At 125 µg/mL, Nile Red fluorescence reached $145.5\pm4.0\%$ of the control, which represents a 22.8% decrease relative to the FFA only condition ($p=0.008$). The lower concentration of PC (62.5 µg/mL) had no significant impact on lipid accumulation. Gemfibrozil thus produced a markedly stronger reduction in lipid accumulation than PC, and PC is not equivalent to the reference treatment; under the conditions tested, PC exhibited only a moderate lipid-lowering effect.

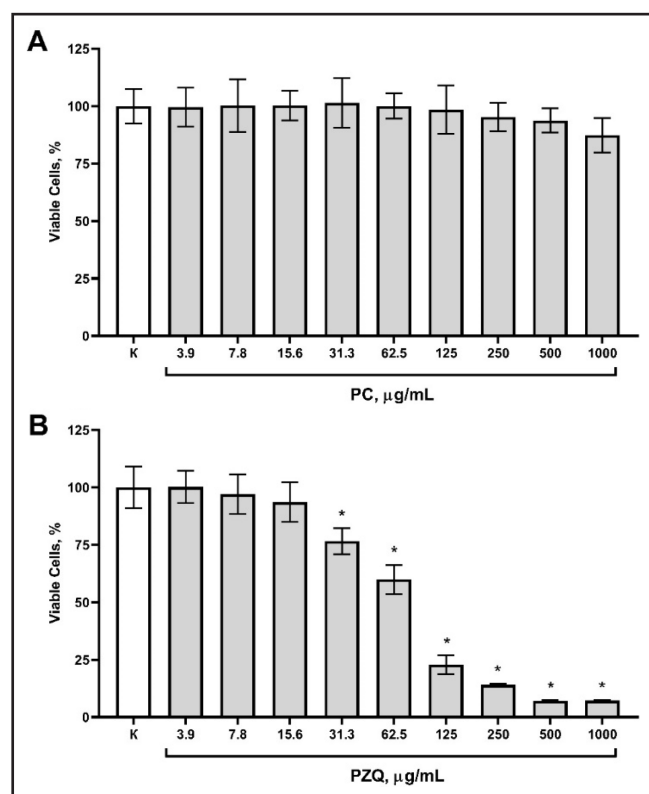


Figure 3. Viability of HepG2 cells after 24 h exposure to A – polysaccharide complex (PC) from *Populus tremula* bark and B – praziquantel (PZQ). Data are presented as mean $\pm$ SD; values are expressed as a percentage of untreated controls. * $p<0.05$ vs. control. HepG2 cells were treated for 24 h across the tested concentration range; $n = 3$ independent biological replicates; * $p<0.05$ versus untreated control (one-way ANOVA with Bonferroni correction).

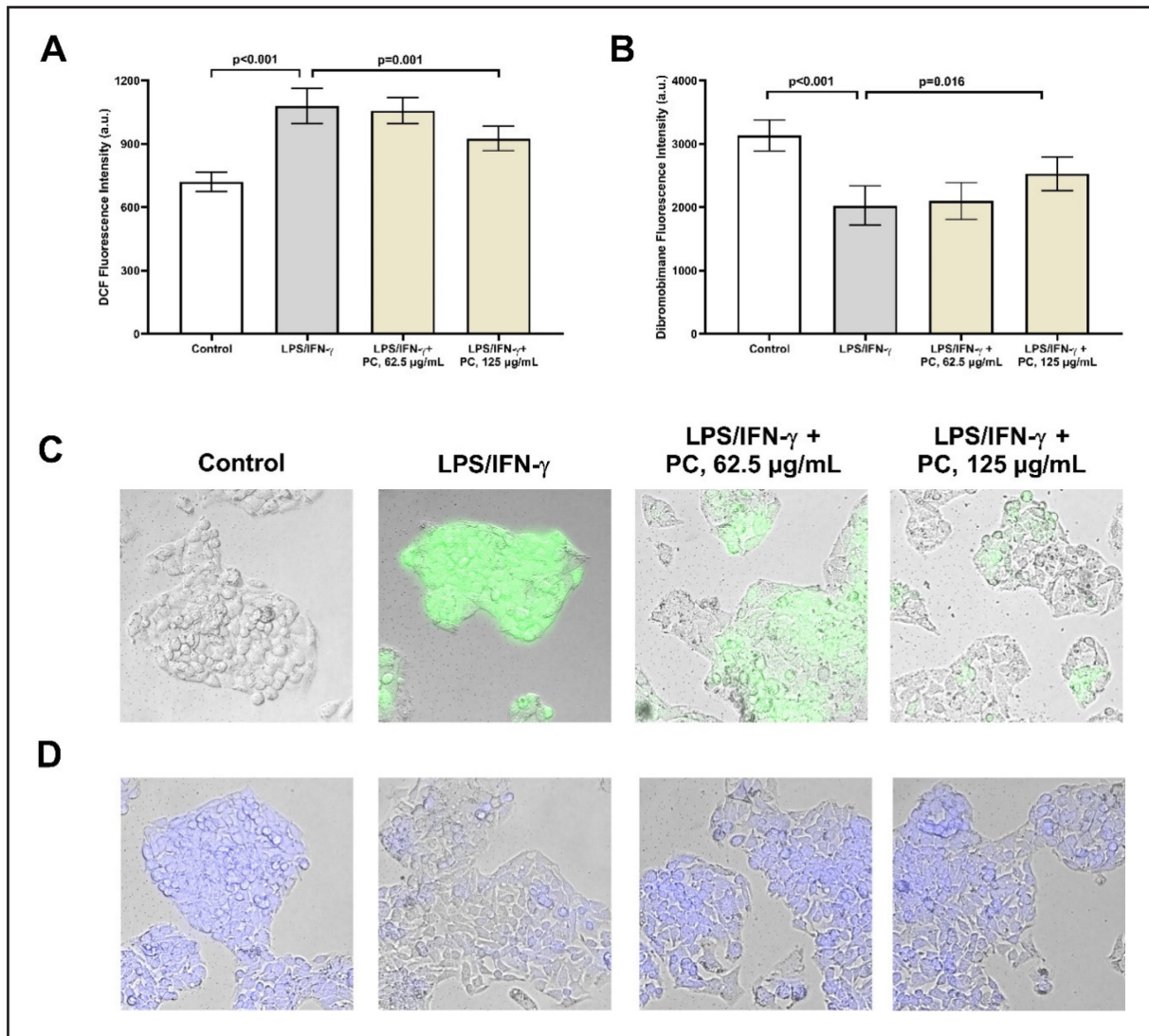


Figure 4. Effect of polysaccharide complex (PC) from *Populus tremula* bark on intracellular reactive oxygen species (ROS) and reduced glutathione (GSH) levels in HepG2 cells following 48 h incubation with lipopolysaccharide (10 μ g/mL) and interferon- γ (50 ng/mL). A – DCF fluorescence intensity reflecting ROS content. B – Dibromobimane fluorescence intensity reflecting GSH content. All bar chart data are expressed as mean $\pm$ SD. C and D – Representative fluorescence microscopy images (x40 magnification) illustrating ROS and GSH signals in the respective treatment groups. PC was applied and tested at 62.5 and 125 μ g/mL for 24; n = 3 independent biological replicates; p-values indicate significance between groups calculated by one-way ANOVA with Bonferroni correction.

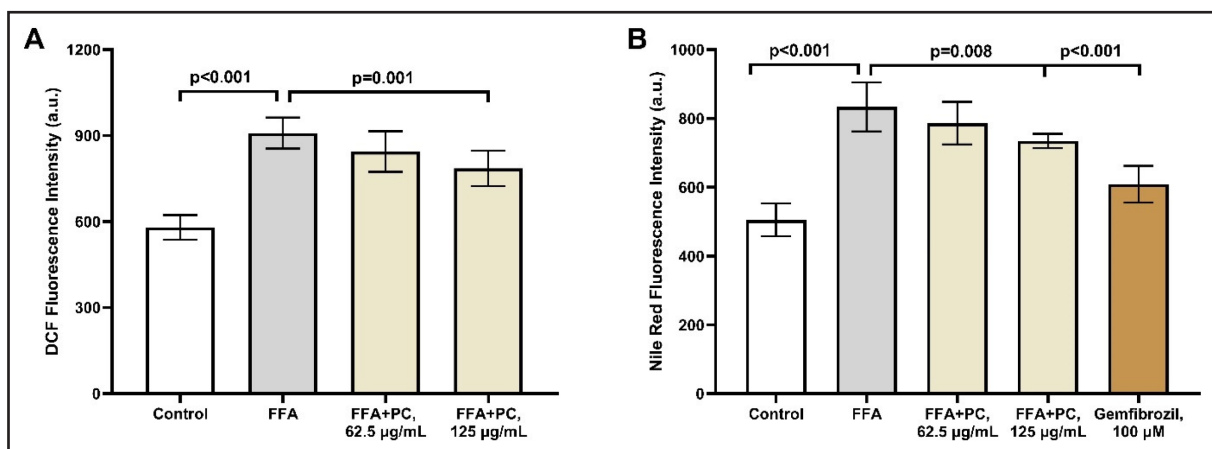


Figure 5. Effect of polysaccharide complex (PC) from *Populus tremula* bark on A – DCF fluorescence intensity (reactive oxygen species) and B – Nile Red fluorescence intensity (intracellular lipid content), compared with the reference hypolipidemic agent gemfibrozil, in HepG2 cells after 24 h incubation in medium containing sodium oleate (1 mM) and sodium palmitate (0.5 mM). PC was tested at 62.5 and 125 μ g/mL for 24 h of incubation; n = 3 independent biological replicates; data are presented as mean $\pm$ SD; p-values indicate significance between groups, calculated by one-way ANOVA with Bonferroni correction.

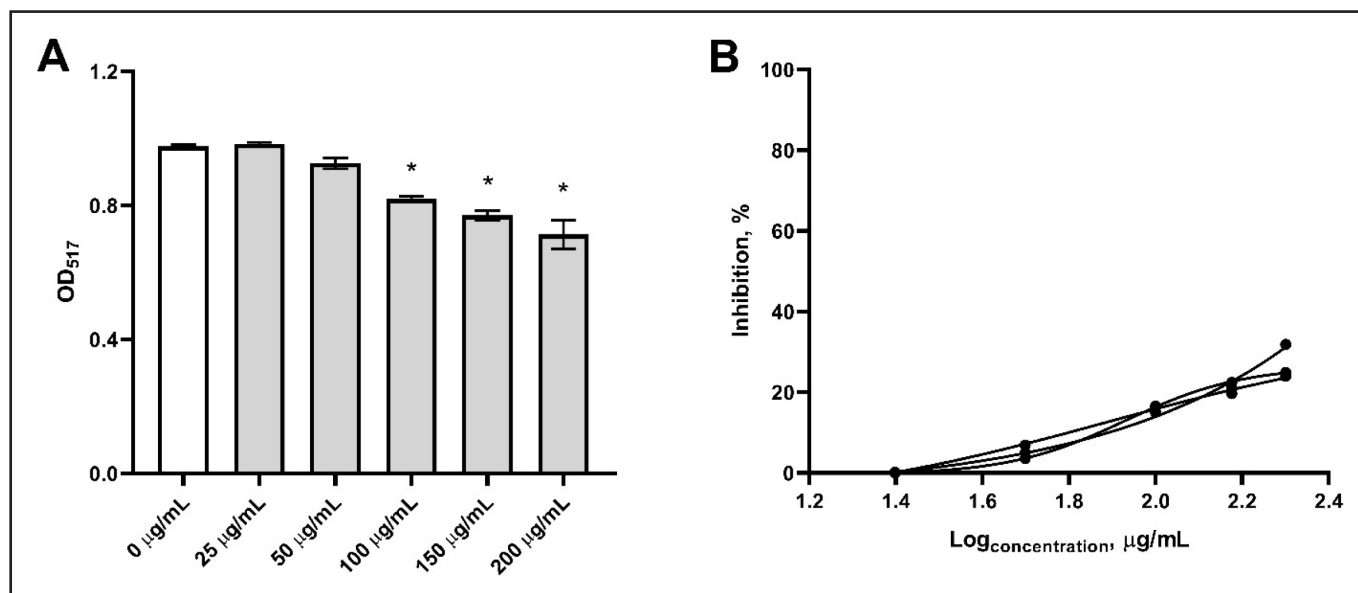


Figure 6. Effect of PC on (A) the optical density of DPPH (100 µM) solution and (B) the semi logarithmic dependence of DPPH radical scavenging efficiency on PC concentration. PC was tested in the 0-200 µg/mL range; n = 3 technical replicates, data are presented as mean±SD; *p < 0.05 compared with the optical density value at 0 µg/mL PC (control).

Taken together, these findings indicate that FFA exposure was associated with increased ROS and lipid accumulation, while PC attenuated both effects, with a more pronounced effect on oxidative stress than on intracellular lipid accumulation.

Antioxidant and iron chelating activity of PC

The DPPH (2,2 diphenyl 1 picrylhydrazyl) radical scavenging assay was employed to assess the antiradical activity of PC. The fraction, composed predominantly of arabinogalactans, exhibited antioxidant activity in the 100-200 µg/mL range (Figure 6A). At the highest tested concentration (200 µg/mL), PC neutralised 33.6% of the DPPH radicals present in the model system (Figure 6B). As DPPH is a cell-free chemical assay, this value reflects the intrinsic radical-scavenging capacity of PC and does not, by itself, establish antioxidant activity within hepatocytes or *in vivo*.

The ferrous ion chelating ability of PC was determined using the ferrozine based assay. PC at 200 µg/mL bound 65% of the Fe²⁺ in the reaction mixture, corresponding to 24.70±2.63 mg EDTA equivalents per gram of PC. This pronounced chelating activity indicates that PC can effectively sequester redox active iron, thereby limiting its participation in free radical generation.

DISCUSSION

The search for novel antiparasitic agents remains a priority in biomedical research, driven by the well-documented limitations of current chemotherapeutic options for trematode infections. These include the cytotoxicity of PZQ, the emergence of drug resistance, and the debated association between repeated PZQ courses and an elevated risk of cholangiocarcinoma (Lvova et al., 2023). In this context, plant-derived bioactive compounds have attracted considerable interest, as they offer the potential not only for direct anthelmintic action but also for modulation of the pathological processes that underlie host tissue damage (Vale et al., 2020).

The anthelmintic activity of PC against *O. felinus* was assessed using three independent approaches, all of which indicated direct anthelmintic activity *in vitro*. The fraction significantly reduced adult worm motility, decreased parasite viability as measured by the MTS assay, and induced focal accumulation of propidium iodide within the tegumental syncytium. As PC was evaluated against adult worms at a single concentration (1 000 µg/mL), no concentration-response

relationship or effective concentration could be established, and dose-ranging studies are required to characterise its anthelmintic potency. The trematode tegument is a multinucleated syncytium that performs essential barrier, nutritional, and immune-protective functions, and its structural integrity is critical for parasite survival (Sangkam et al., 2024). Tegumental damage has been reported for a range of plant-derived preparations and isolated compounds. Curcumin, for instance, caused marked tegumental disruption, spine loss, and syncytial denudation in *Fasciola gigantica* (Ullah et al., 2017), whereas artemisinin and its derivatives induced extensive tegumental sloughing and swelling in *Clonorchis sinensis* (Keiser & Vargas, 2010).

Notably, complete immobilisation was observed in only ~30% of PC-treated worms, a pattern that clearly distinguishes the effect of the fraction from the rapid calcium-dependent paralysis induced by PZQ and points toward a distinct mechanism of action. Compromised tegumental integrity *in vivo* is likely to facilitate antibody recognition of the parasite and render it susceptible to antibody-dependent cellular cytotoxicity (ADCC) - one of the key effector arms of anti-helminth immunity (Anthony et al., 2007; Motran et al., 2018). During helminth infections, ADCC operates within the context of a polarized Th2 response and involves IgE/IgG production and the activation of eosinophils, macrophages, and neutrophils. The binding of antibodies to surface-exposed parasite antigens triggers degranulation of effector cells and the release of cytotoxic proteins, ultimately leading to parasite killing (Anthony et al., 2007; Motran et al., 2018).

However, helminths have evolved sophisticated immune evasion strategies including molecular mimicry, antigen shedding, and secretion of immunomodulatory molecules, that allow them to persist within the host for decades (Wiedemann & Voehringer, 2020). In this context, PC's ability to disrupt tegumental integrity is a therapeutically significant property, as it may expose concealed antigens to immune recognition. Tegumental damage could increase exposure of parasite antigens to host immune components, but this possibility requires validation in appropriate host-parasite models.

A substantial body of evidence now documents the anti-trematode activity of plant-derived compounds spanning diverse chemical classes. Screening studies have identified promising alkaloids (epiisopilosine, pipartine), terpenoids (nerolidol, parthenolide, artemisinin and its derivatives), flavonoids (genistein,

quercetin), and polyphenols (curcumin, resveratrol, chlorogenic acid) (Eze *et al.*, 2022; Liu *et al.*, 2024a). A noteworthy feature of many of these agents, including PC, is that they combine direct anthelmintic effects with pronounced antioxidant activity – a dual profile that is particularly relevant to the pathogenesis of opisthorchiasis (Perina *et al.*, 2025).

Chronic *O. felineus* infection is characterised by persistent inflammation of the bile ducts which, together with mechanical injury caused by the parasite, serves as a major source of reactive oxygen species (ROS) and reactive nitrogen species. This inflammatory is a key driver of long-term complications, including fibrosis and carcinogenesis (Pakharukova *et al.*, 2019; Smout *et al.*, 2024). When ROS production exceeds the capacity of endogenous antioxidant systems, the resulting oxidative stress becomes a universal mechanism of hepatocellular injury and fibrogenesis (Schulz *et al.*, 2000). Experimentally, chronic opisthorchiasis has been shown to cause a significant decline in hepatic reduced GSH levels as early as eight weeks post-infection in hamsters, providing direct evidence of oxidative and carbonyl stress in the liver (Saltykova *et al.*, 2017).

GSH is the central component of non-enzymatic antioxidant defence. Beyond directly scavenging ROS, GSH participates in the detoxification of dicarbonyl compounds and the maintenance of intracellular redox homeostasis (Boysen, 2017; Schulz *et al.*, 2000). Depletion of the GSH pool not only reflects the severity of oxidative stress but also directly contributes to the activation of pro-inflammatory signalling cascades – most notably RAGE-dependent pathways – and to hepatocellular apoptosis (Čapek & Roušar, 2021; Saltykova *et al.*, 2017), thereby creating a microenvironment that favours fibrosis progression and malignant transformation of cholangiocytes (Smout *et al.*, 2024).

In this context, our finding that PC not only reduced ROS levels but also significantly elevated intracellular GSH content in the LPS/IFN- γ -stimulated HepG2 model (most prominently at 125 μ g/mL) is of particular importance. This dual capacity to suppress ROS and restore the GSH pool has previously been documented *in vivo* using plant extracts in experimental opisthorchiasis (Aukkanimart *et al.*, 2024), indicating that natural compounds can modulate the glutathione system under helminthic infection.

Chronic liver fluke infection is known to induce abnormal lipid profiles in both humans and experimental animals, characterised by hepatic cholesterol accumulation that contributes to the progression of hepatobiliary pathology (Chaidee *et al.*, 2019). In the lipotoxicity model, which reproduces the metabolic stress characteristic of opisthorchiasis-associated disturbances of lipid metabolism, PC likewise attenuated ROS production, and the decrease in oxidative stress correlated with reduced intracellular lipid accumulation. This effect is pathogenetically significant because an excess of free fatty acids and their metabolites not only exacerbates oxidative stress but also directly activates pro-inflammatory signaling cascades, thereby promoting hepatic fibrosis (Yao *et al.*, 2011).

An additional facet of the antioxidant activity of PC is its ability to efficiently chelate ferrous ions. This property, which derives from the abundance of hydroxyl and carbonyl groups in the polysaccharide structure (Iddrisu *et al.*, 2024), restricts the participation of Fe²⁺ in the generation of highly aggressive ROS, particularly the hydroxyl radical, via the Fenton reaction (Duo *et al.*, 2025; Sousa *et al.*, 2020). In the present study we did not measure ferroptosis, an iron-dependent form of regulated cell death, however, in light of recent studies implicating ferroptosis in the hepatic pathology associated with trematode infections (Xu *et al.*, 2026; Zhang *et al.*, 2025), it is conceivable that the iron-chelating properties of PC may additionally limit the substrate pool available for the initiation of ferroptosis in the chronic opisthorchiasis microenvironment.

Taken together, PC exhibits a favourable pharmacological profile that integrates two complementary modes of action. On the one hand, the fraction exerts direct anthelmintic activity against adult *O. felineus*, manifested by a significant reduction in worm motility and viability, and accompanied by damage to the tegument – the key structure that shields the parasite from the host immune system. On the other hand, PC produces hepatoprotective effects *in vitro* in a HepG2 cell model, manifested as attenuation of oxidative stress and lipid accumulation in hepatocytes under both lipotoxic and pro-inflammatory conditions, an activity reinforced by the fraction's ability to restore the intracellular glutathione pool and to chelate pro-oxidant Fe²⁺ ions.

Several limitations of this study should be noted. PC was tested against adult worms at a single concentration, precluding a concentration-response analysis; in addition, the worm served as the statistical unit for the motility and MTS analyses, and motility and morphology were scored without formal blinding. Accordingly, because worms sharing a well and a replicate are not fully independent, the chi-square comparison of pooled motility-category frequencies should be regarded as a screening-level analysis, and the motility and relative-motility indices are presented as descriptive summaries only. The reported molecular weights are apparent values derived from linear dextran standards and are appropriate for describing this heterogeneous, multi-component preparation. HepG2 experiments represent an *in vitro* model that did not assess direct hepatocellular injury, enzyme release, apoptosis, mitochondrial function, or histopathology. Given the broad molecular weight distribution of aspen bark polysaccharides, indicating a heterogeneous mixture, further research could focus on fractionating the samples by molecular weight and identifying the fractions responsible for the biological activity.

Given that PC belongs to the class of polysaccharides, which, unlike certain flavonoids, are generally characterised by a more predictable safety profile upon prolonged administration (Mordvinov *et al.*, 2021), the present findings provide first initial *in vitro* evidence supporting further investigation of this complex as a candidate for the development of a combined anti-opisthorchiasis agent, aimed both at parasite elimination and at protecting the liver from infection-driven injury. These consistent effects across three independent lines of evidence (anthelmintic, redox, and lipid-metabolic) now warrant confirmation through mechanistic studies in cells and *in vivo*.

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Conflict of Interest

The authors declare that there are no conflicts of interest

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