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*Article*

# Antimicrobial and anti-inflammatory potential of the edible macrofungus *Fistulina hepatica*: Inhibition of bacterial growth and COX enzymes

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**A B S T R A C T**

Edible macrofungi are valuable sources of nutraceutical and pharmacologically active metabolites. This study evaluated four extracts of *Fistulina hepatica*, a polysaccharide-rich fraction (PSH) and extracts prepared with 70% ethanol (70% EtOH), 80% methanol (80% MeOH), and chloroform ($CHCl_3$), for their antimicrobial and anti-inflammatory activities. Anti-inflammatory potential was assessed by cyclooxygenase (COX-1 and COX-2) inhibition using a colorimetric *in vitro* assay, while antimicrobial activity was evaluated by microdilution method against *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. The $CHCl_3$ extract showed the strongest bioactivity, inhibiting COX-1 by 37.5% and COX-2 by 70.8%, comparable to celecoxib (66%). It also displayed antimicrobial effects, with MICs of 0.0125 mg/mL against *B. cereus* and *P. aeruginosa* and 0.03125 mg/mL against *S. aureus*, while no MBC values were detected. Other extracts exhibited weak or no activity. These findings highlight *F. hepatica* as a promising dual-function natural source of COX-2–selective and antimicrobial agents for potential nutraceutical applications.

**Keywords:** *Fistulina hepatica*, anti-inflammatory activity, COX-1/COX-2, antimicrobial activity, macrofungal extracts, nutraceutical potential

**И З В О Д**

Јестиве макрогљиве представљају значајне изворе нутрацеутика и фармаколошки активних метаболита. У овом раду испитана су четири екстракта врсте *Fistulina hepatica*, полисахаридна фракција (PSH), као и екстракти припремљени са 70% етанолом (70% EtOH), 80% метанолом (80% MeOH) и хлороформом ($CHCl_3$), у погледу њихове антимикробне и антиинфламаторне активности. Антиинфламаторни потенцијал процењиван је инхибицијом ензима циклооксигеназа (COX-1 и COX-2) применом колориметријског *in vitro* теста, док је антимикробна активност испитивана методом микродилуције на *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa* и *Escherichia coli*. $CHCl_3$ екстракт показао је најизраженију биоактивност, инхибирајући COX-1 за 37,5% и COX-2 за 70,8%, што је упоредиво са целекоксибом (66%). Такође је исти екстракт испољио антимикробну активност, са MIC вредностима од 0,0125 mg/mL на *B. cereus* и *P. aeruginosa* и 0,03125 mg/mL на *S. aureus*, док MBC вредности нису детектоване. Остали екстракти су показали слабу активност или уопште није забележена активност. Ови резултати указују да *F. hepatica* може бити перспективан природни извор са двоструком функцијом, селективном COX-2 инхибиторном и антимикробном активношћу, са потенцијалном применом у нутрацеутицима.

**Кључне речи**: *Fistulina hepatica*, антиинфламаторна активност, COX-1/COX-2, антимикробна активност, екстракти макрогљива, нутритивни потенцијал

## 1. Introduction

Edible macrofungi have long been recognized not only as nutritious food sources but also as reservoirs of bioactive compounds with diverse pharmacological effects, including antioxidant, antimicrobial, and anti-inflammatory activities (Kozarski et al., 2015; Mayirnao et al., 2025; Rašeta et al., 2025; Singh et al., 2025). Among these, *Fistulina hepatica* (Schaeff.) With. 1801, commonly known as the beefsteak fungus, is an edible species traditionally consumed and valued for its unique chemical constituents (Rašeta et al., 2025). Recent research has increasingly focused on exploring macrofungi like *F. hepatica* as sources of natural agents capable of modulating inflammatory processes and combating microbial infections, which are critical

targets for developing alternative therapies and functional food ingredients (Bell et al., 2022; Lu et al., 2025).

Inflammation is a key underlying mechanism in many chronic diseases, and cyclooxygenase (COX) enzymes, particularly COX-1 and COX-2, play pivotal roles in the synthesis of pro-inflammatory mediators such as prostaglandins (Rouzer and Marrnet, 2009; Rašeta et al., 2026). The selective inhibition of COX-2 has gained attention for its potential to reduce inflammation with fewer gastrointestinal side effects compared to non-selective COX inhibitors (El-Mallah et al., 2022). Concurrently, the emergence of antibiotic-resistant bacterial strains has highlighted the need for new antimicrobial compounds from natural sources (Pešaković et al., 2022; Golijan Pantović et al., 2025). Basidiomycete-derived secondary metabolites are generally regarded as a promising source of antibacterial compounds, particularly those active against Gram-positive bacteria (Vallavan et al., 2020). Among them, edible macrofungi represent a promising source of bioactive compounds due to their rich content of phenolics, terpenoids, and polysaccharides, which may act synergistically against various pathogens (Kumar et al., 2021; Abdelkader et al., 2025; Rijia et al., 2025; Yu et al., 2025; Rašeta et al., 2026). Among these, polyphenolic compounds play a central role through multiple mechanisms, including disruption of microbial cell structures, modulation of host immune responses, and scavenging of free radicals across different macrofungal species (Kozarski et al., 2015; Sun et al., 2024; De Rossi et al., 2025; Rašeta et al., 2026).

Given the global challenge of escalating antibiotic resistance, the antibacterial properties of macrofungi-derived polyphenolics offer innovative prospects for the development of new therapeutic agents (Sun et al., 2024). Their antimicrobial activity arises from specific molecular and physicochemical characteristics, particularly the presence of hydroxyl groups and electron delocalization, which enable interactions with microbial cell membranes, proteins, and intracellular organelles (De Rossi et al., 2025). Moreover, their ability to form complexes with metal ions further enhances their antimicrobial efficacy (De Rossi et al., 2025). In our previous study, we also proposed that possible mechanisms of antimicrobial action include the capacity of polyphenols to complex with extracellular and soluble proteins as well as with bacterial cell walls, while more lipophilic flavonoids or other hydrophobic compounds such as terpenoids may contribute to microbial membrane disruption (Shevelev et al., 2018; Rašeta et al., 2023). Consequently, these interactions can impair vital metabolic pathways, compromise membrane integrity, and damage proteins and nucleic acids in foodborne bacteria (De Rossi et al., 2025). Beyond their antimicrobial potential, the anti-inflammatory properties of polyphenolics hold significant promise for the treatment of inflammatory disorders and the attenuation of aging-related processes (Sun et al., 2024).

This study investigates the antimicrobial and anti-inflammatory potential of *Fistulina hepatica* by evaluating four different extracts, a polysaccharide (PSH)-rich fraction and solvent extracts prepared with 70% ethanol (70% EtOH), 80% methanol (80% MeOH), and chloroform ($CHCl_3$), based on their ability to inhibit bacterial growth. In addition, the $CHCl_3$ extract was further examined for its potential to inhibit COX-1 and COX-2 enzymes. By characterizing their bioactivity against clinically relevant bacterial strains and assessing enzyme inhibition, this research aims to substantiate the dual functional properties of *F. hepatica*, offering valuable insights into its potential application in nutraceuticals and functional foods designed to alleviate inflammation and combat microbial infections.

## 2. Materials and methods

### *2.1. Fungal material*

The fruiting bodies of *F. hepatica* were collected from Liman 1 (Novi Sad, North Serbia) in November 2020. Identification and taxonomic determination of the species were carried out based on both macroscopic and microscopic characteristics, following the appropriate identification keys. The identification process was conducted under the supervision of Prof. Dr. Maja Karaman at the ProFungi Laboratory, Department of Biology and Ecology, University of Novi Sad. A voucher specimen was deposited under accession number 12-00764 in the ProFungi Laboratory's Fungarium, which is a separate depot within the internationally recognized Herbarium Collection of the University of Novi Sad, Faculty of Natural Sciences, Department of Biology and Ecology (Herbarium BUNS).

### *2.2. Extract preparation*

Extract preparation procedures were performed as described in Rašeta et al. (2025). Fruiting bodies were thoroughly cleaned, dried at 45 °C using a Memmert UF55 drying oven (Memmert GmbH + Co. KG, Schwabach, Germany), and ground to a fine powder with a Sekljanik S400 W grinder (Gorenje d.o.o., Velenje, Slovenia). For $CHCl_3$, 80% MeOH, and 70% EtOH extracts, 10 g of powdered fungal material were separately macerated in a 1:10 (w/v) solvent ratio, followed by centrifugation, filtration, and solvent evaporation. The resulting residues were dissolved in DMSO (100 mg/mL) and stored at −20 °C until further use. Polysaccharide-rich fractions (Rašeta et al., 2025) were obtained from dried fruiting bodies. A 15 g portion of the powdered material was first defatted with 80% EtOH and then extracted with 300 mL of water ($H_2O$). The extract was centrifuged (Sigma 3-30K, Burlington, Massachusetts, USA) and filtered. The filtrate was concentrated at 50 °C, precipitated with EtOH overnight, centrifuged again, and the resulting precipitate was dissolved in distilled $H_2O$, dialyzed, frozen, and lyophilized (Christ Alpha 2-4 LD plus, Osterode am Harz, Germany). The final extracts, prepared at concentrations ranging from 20 mg/mL to 100 mg/mL, were stored at −20 °C for subsequent analyses.

### *2.3. Determination of antimicrobial activity of F. hepatica*

The antimicrobial activity of prepared extracts ($CHCl_3$, 80% MeOH, 70% EtOH, and PSH-fraction) was evaluated against reference bacterial strains – two Gram-positive (*Staphylococcus aureus* ATCC 6538 and

*Bacillus cereus* ATCC 11778) and two Gram-negative (*Escherichia coli* ATCC 11775 and *Pseudomonas aeruginosa* ATCC 9027) species. Additionally, a stock solution of an antibiotic (1 mg/mL) was prepared and diluted to obtain a starting concentration of 0.5 mg/mL. Bacterial cultures (stored in deep-freeze at −80 °C) were subcultured on Mueller-Hinton agar (MHA) and incubated at 37 °C for 24 h to ensure culture vitality. Subsequently, bacterial suspensions were prepared in sterile physiological saline solution and their turbidity adjusted to 0.5 McFarland standard, corresponding to approximately $1.5 \times 10^8$ CFU/mL.

For the microdilution assay, 50 µL of the bacterial suspension was used to inoculate 49.95 mL of Mueller-Hinton broth (MHB) to obtain a bacterial density of approximately 1.5 x $10^5$ CFU/mL. Serial twofold dilutions of each extract were then prepared directly in microtiter plates, by mixing sterile distilled $H_2O$ and extract (or antibiotic solution). Each working well contained 50 µL of inoculated broth and 50 µL of tested extract/antibiotic dilution, which resulted in the final bacterial number of 0.75 x $10^5$ CFU/mL. This antimicrobial test was repeated for each bacterial strain in triplicate. In addition to working, i.e., test wells, three types of controls were included: a growth control (uninoculated MHB with sterile distilled $H_2O$) to verify medium sterility and bacterial viability, an extract control (uninoculated MHB with tested extract dilutions) to examine the effect of the extracts on the medium, and a solvent control (inoculated MHB and solvent) to assess solvent effect on bacterial growth. After preparing working wells with dilution series and control wells, microplates were incubated for 24 h at 37 °C, followed by the addition of 10 µL of 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution and further 2 h incubation. TTC staining was used to aid in the visualization of bacterial growth, and the minimum inhibitory concentration (MIC) was defined as the lowest extract/antibiotic concentration showing no turbidity or color change compared to the growth control. To determine the minimum bactericidal concentration (MBC), the contents of the wells showing the MIC and all higher concentrations were plated onto Mueller–Hinton agar. After 24h incubation at 37 °C, MBC value was defined as the lowest extract/antibiotic concentration that resulted in a 99.9% reduction in the viability of the initial microbial inoculum.

### *2.4. Determination of COX anti-inflammatory activity of F. hepatica*

The anti-inflammatory potential was evaluated using a commercial COX inhibitor screening kit (Enzo Life Sciences), which quantifies prostaglandin $E_2$ ($PGE_2$) production *in vitro* from arachidonic acid (1 mM). The reaction mixture contained COX-1 (ovine; Cayman Chemical, 60100) and COX-2 (human recombinant; Cayman Chemical, 60122) enzymes (0.2 U per well), following the protocol described by Katanić Stanković et al. (2023). All assays were performed in 96-well microplates. The reaction mixture, in addition to the tested $CHCl_3$ extract at a concentration of 1 mg/mL, contained 1 mM EDTA-$Na_2$ (Titriplex® III) prepared in 0.1 M TRIS/HCl buffer, 18 mM adrenaline bitartrate, and 100 µM hematin. The reaction was terminated by adding 10% (v/v) formic acid. Indomethacin (25 µM) and celecoxib (176 µM) were used as reference inhibitors specific to COX-1 and COX-2, respectively. Quantification of $PGE_2$ was performed following the manufacturer's protocol using the Enzo $PGE_2$ ELISA kit. After incubation and reaction termination, absorbance was measured at 405 nm with a Hidex microplate reader. The results were expressed as the percentage inhibition of COX-1 and COX-2 activities, while indomethacin and celecoxib served as positive controls. In addition, a selectivity index (SI) was calculated as the ratio of COX-2 inhibition to COX-1 inhibition to estimate the relative preference of the tested extract toward COX-2.

### *2.5. Statistical analysis*

Data are presented as mean ± standard deviation (SD) of three replicates. Statistical differences between experimental groups were evaluated using one-way analysis of variance (ANOVA). Differences were considered statistically significant at $p < 0.05$. Figure was generated using OriginPro 8 software (OriginLab Corporation, Northampton, MA, USA).

## 3. Results and discussion

### *3.1. Antimicrobial activity*

Besides plants, which are widely recognized as a source of antimicrobial compounds, fungi have also emerged as promising natural reservoirs of novel antimicrobial agents (Hamers et al., 2020; Jakubczyk and Dussart, 2020; Rašeta et al., 2023). Due to their ecological adaptability, many fungal species produce diverse secondary metabolites, including terpenes, sesquiterpenes, anthraquinones, benzoic acid derivatives, quinolines, peptides, proteins, and oxalic acids, which serve as chemical defenses against microorganisms (Alves et al., 2012; Novaković, 2015; Bhambri et al., 2022; Karaman et al., 2022). In the present study, the antimicrobial potential of *F. hepatica* extracts was evaluated against two Gram-positive bacteria (*Bacillus cereus* ATCC 11778 and *Staphylococcus aureus* ATCC 6538) and two Gram-negative strains (*Escherichia coli* ATCC 11775 and *Pseudomonas aeruginosa* ATCC 9027) using $CHCl_3$, 80% MeOH, and 70% EtOH extracts, as well as PSH-fraction. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values are presented in Table 1.

Among all tested *F. hepatica* extracts, only the $CHCl_3$ extract demonstrated measurable antimicrobial activity, whereas the other extracts and the PSH-fraction showed no inhibitory effects at the tested concentrations. The $CHCl_3$ extract was more effective against Gram-positive bacteria, inhibiting the growth of *B. cereus* at 0.0125 mg/mL and *S. aureus* at 0.03125 mg/mL. Among the Gram-negative bacteria, inhibitory activity was observed only against *P. aeruginosa* (MIC 0.0125 mg/mL), while no activity was detected against *E. coli.* No MBC values were obtained, indicating that the $CHCl_3$ extract exerted a bacteriostatic rather than bactericidal effect. Similar observations have been reported for $CHCl_3$ extracts of other macrofungi. For example, Yamaç and Bilgili (2006) demonstrated that the $CHCl_3$ extract of *Hygrophorus agathosmus* exhibited the strongest antibacterial activity among several tested solvents ($CHCl_3$, ethyl acetate, acetone, dichloromethane, EtOH), with Gram-positive bacteria being more susceptible than Gram-negative species.

Likewise, Hirasawa et al. (1999) reported that the $CHCl_3$ extract of *Lentinula edodes* showed stronger antibacterial activity compared to ethylacetate and $H_2O$ extracts.

**Table 1.**
Results of antimicrobial activity of tested *F. hepatica* extracts

| Tested bacterial strain | Tested *F. hepatica* extracts | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | PSH-fraction | | 80% MeOH | | 70% EtOH | | $CHCl_3$ | |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| | mg/mL | | mg/mL | | mg/mL | | mg/mL | |
| *B. cereus* | – | – | – | – | – | – | 0.0125 | – |
| *S. aureus* | – | – | – | – | – | – | 0.03125 | – |
| *E. coli* | – | – | – | – | – | – | – | – |
| *P. aeruginosa* | – | – | – | – | – | – | 0.0125 | – |

Abbreviations: PSH-fraction – polysaccharide fraction; 80% MeOH – hydromethanolic extract prepared with 80% methanol; 70% EtOH – hydroethanolic extract prepared with 70% ethanol; $CHCl_3$ – chloroform extract; MIC – minimum inhibitory concentration; MBC – minimum bactericidal concentration.

Direct comparison with previous studies on *F. hepatica* is limited due to differences in extraction solvents and experimental conditions. Alves et al. (2012) reported that 80% MeOH extracts inhibited both Gram-positive (*S. aureus*, *Enterococcus faecalis*, *Streptococcus pyogenes*) and Gram-negative (*E. coli*, *Morganella morganii*, *Pasteurella multocida*) bacteria, whereas Giri et al. (2012) observed the activity of MeOH extracts mainly against *Proteus vulgaris* and *E. coli*. On the contrary, Novaković (2015) reported that 70% MeOH extracts of Serbian isolates showed pronounced antibacterial activity, particularly against Gram-positive bacteria. Furthermore, Whaley et al. (2023) recently isolated several polyacetylenic fatty acid derivatives, including cinnatriacetins A and C, ethylcinnatriacetin A, and isocinnatriacetins A and B, which exhibited notable antimicrobial activity.

To date, the antimicrobial properties of *F. hepatica* have often been attributed to bioactive triterpenoid derivatives such as triaceltin, cinnatriacetin A, and cinnatriacetin B, isolated from its fruiting bodies (Tsuge et al., 1999). These compounds are known to disrupt bacterial membranes and interfere with essential metabolic pathways. Based on the results obtained in the present study (Table 1), only the $CHCl_3$ extract exhibited antibacterial activity, which may be associated with the polyphenols previously quantified in the same extract by LC–MS/MS (Table S1) (Rašeta et al., 2025). In particular, phenolic acids, primarily cinnamic acid (740.93 ± 1.85 ng/mL d.w.) and *p*-coumaric acid (82.81 ± 0.13 ng/mL d.w.), were the predominant compounds detected. These compounds have been reported to exhibit antimicrobial activity against several bacterial pathogens, including *S. aureus* and *E. coli* (MIC 10–80 μg/mL) (Lou et al., 2012). It disrupts the outer and plasma membranes, causing loss of barrier function and cytoplasmic leakage, as confirmed by electron microscopy. Furthermore, it binds to phosphate anions and intercalates into the DNA double helix, thereby interfering with replication and transcription (Lou et al., 2012). Their activity has been associated with disruption of bacterial membranes and interference with essential cellular processes. The antibacterial activity of cinnamic acid may additionally be related to the presence of its phenyl ring and carboxyl group, which are important structural features influencing biological activity (Annuur et al., 2024). The effectiveness of the $CHCl_3$ extract may also be explained by the intermediate polarity of this solvent, which facilitates the extraction of lipophilic metabolites as well as moderately polar phenolic compounds from fungal tissues (Effiong et al., 2024; Lestari et al., 2025). To the best of our knowledge, this study provides the first indication that phenolic compounds present in the $CHCl_3$ extract of *F. hepatica* may contribute to its antibacterial activity. Nevertheless, further studies involving isolation and characterization of individual compounds are required to confirm their specific roles in the observed antimicrobial effects. The antibiotic sensitivity test (Table 2) showed that Gram-negative strains (*E. coli* and *P. aeruginosa*) exhibited resistance to several commonly used antibiotics (chloramphenicol, ampicillin, and streptomycin), whereas Gram-positive bacteria remained sensitive to most tested antibiotics.

**Table 2.**
Sensitivity of four tested bacterial strains to commonly used antibiotics

| Tested bacterial strain | Tested antibiotic | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Kanamycin | | Chloramphenicol | | Ampicillin | | Streptomycin | |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| | mg/mL | | mg/mL | | mg/mL | | mg/mL | |
| *B. cereus* | 0.0625 | 0.125 | 0.25 | 0.25 | 0.125 | 0.125 | 0.03 | 0.03 |
| *S. aureus* | 0.125 | 0.50 | 0.25 | 0.50 | 0.125 | 0.50 | 0.25 | 0.50 |
| *E. coli* | 0.25 | 0.25 | R | R | 0.25 | 0.50 | 0.50 | - |
| *P. aeruginosa* | 0.50 | 0.50 | R | R | R | R | R | R |

Abbreviations: MIC – minimum inhibitory concentration; MBC – minimum bactericidal concentration; R – resistant.

These findings highlight the growing challenge of antibiotic resistance and emphasize the importance of exploring fungal metabolites as a potential source of new antimicrobial agents. In particular, multidrug-resistant pathogens such as *P. aeruginosa* represent a major clinical concern due to their ability to cause severe infections and their numerous resistance mechanisms (Kunz Coyne et al., 2022; Montero and Horcajada, 2023; Schwartz et al., 2024). Overall, the antimicrobial results suggest that *F. hepatica*, although primarily known as an edible species, also possesses promising medicinal potential. The activity of its $CHCl_3$ extract against both Gram-positive and Gram-negative bacteria supports further mycochemical and mechanistic studies aimed at identifying the active constituents responsible for the observed effects. The antimicrobial potential of phenolic compounds is often associated with the number and position of hydroxyl groups on the aromatic ring, as increased hydroxylation generally enhances their antimicrobial activity. This effect is frequently related to enzyme inhibition by oxidized derivatives through interactions with sulfhydryl groups or nonspecific binding to proteins (Rašeta et al., 2023). In fungal cells, phenolic compounds may also exert toxicity by interacting with membrane sterols such as ergosterol or by inhibiting enzymes involved in its biosynthesis (Vishwakarma et al., 2024). In addition, terpenoid metabolites are known to contribute to antimicrobial activity, with monoterpenes primarily disrupting microbial membranes and diterpenes interfering with cellular respiration and oxidative phosphorylation (Mahizan et al., 2019). Considering that only the non-polar extract exhibited notable antibacterial activity, the observed effects may result not only from the quantified phenolic compounds (Rašeta et al., 2025) but also from potential synergistic interactions with other lipophilic constituents present in the $CHCl_3$ extract, such as sterols, glycosides, and triterpenoids, which remain insufficiently characterized in *F. hepatica*. Additionally, some phenolic compounds may occur in insoluble-bound forms that can reach the lower intestine, where microbial fermentation may enhance their bioaccessibility and contribute to beneficial interactions with the intestinal environment (De Rossi et al., 2025).

### *3.2. Anti-inflammatory activity*

Inflammation, whether acute or chronic, plays a crucial role in the onset and progression of numerous pathological conditions (Chen et al., 2017). The search for safer and more effective alternatives to synthetic anti-inflammatory agents, particularly nonsteroidal anti-inflammatory drugs (NSAIDs), has encouraged increasing interest in natural sources rich in bioactive compounds. Medicinal macrofungi, owing to their complex secondary metabolite profiles, have shown promising potential in modulating inflammatory responses while minimizing the side effects commonly associated with conventional therapies (Zhao et al., 2020; Rašeta et al., 2021, 2026; Pigoń-Zając et al., 2025). In this study, the $CHCl_3$ extract of *F. hepatica* demonstrated significant inhibitory effects on both COX isoforms, COX-1 and COX-2 (Figure 1).

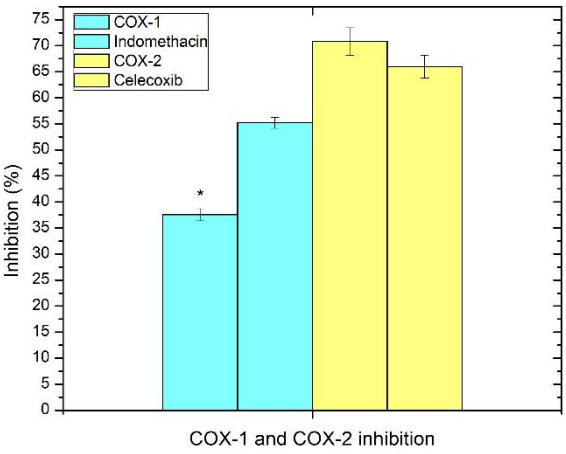


**Figure 1.** Inhibitory effects of the chloroform extract of *Fistulina hepatica* on COX-1 and COX-2 enzymes compared with the positive controls indomethacin and celecoxib. Values represent mean ± standard deviation (SD) (n = 3). $p < 0.05$ compared with the corresponding positive control (one-way ANOVA).

The COX-1 inhibition rate of the extract reached 37.54 ± 1.16%, whereas COX-2 inhibition was notably higher, at 70.83 ± 2.63%, indicating a pronounced selectivity toward COX-2. To further evaluate the relative preference toward COX isoforms, a SI was calculated as the ratio of COX-2 to COX-1 inhibition. The extract exhibited an SI value of 1.89, indicating a moderate preference for COX-2 inhibition. For comparison, the reference inhibitor indomethacin showed an SI of 1.20, reflecting a lower degree of COX-2 preference under the same experimental conditions. Notably, COX-2 inhibition achieved by the *F. hepatica* extract slightly exceeded that of the reference selective inhibitor, celecoxib (66.00 ± 2.21%), while COX-1 inhibition remained lower than that of indomethacin (55.18 ± 1.04%). Although direct comparison with synthetic drugs should be interpreted cautiously due to differences in concentration and chemical composition, these results suggest that the $CHCl_3$ extract contains metabolites capable of effectively interfering with prostaglandin biosynthesis. Statistical analysis using one-way ANOVA indicated a significant difference in COX-1 inhibition between the extract and indomethacin ($p < 0.05$), whereas the difference in COX-2 inhibition between the extract and celecoxib was not statistically significant (Figure 1). The preferential inhibition of COX-2 may also indicate a lower risk of gastrointestinal side effects typically associated with nonselective NSAIDs. The LC-MS/MS analysis of the extract revealed cinnamic acid as the most abundant compound (740.93 ± 1.85 ng/mL d.w.), followed by *p*-coumaric acid (82.81 ± 0.13 ng/mL d.w.), quinic acid (20.26 ± 0.83 ng/mL d.w.), caffeic acid (15.08 ± 0.01 ng/mL d.w.), and amentoflavone (2.81 ± 0.02 ng/mL d.w.) (Table S1) (Rašeta et al., 2025). Previous studies indicate that these compounds can modulate inflammatory pathways, suggesting their possible contribution to the observed activity (Afnan et al., 2022; Ekowati et al., 2023). For instance, cinnamic acid has been reported to inhibit COX-2 expression and reduce pro-inflammatory mediators in macrophage models (Liao et al., 2012), while *p*-coumaric acid suppresses COX-2, iNOS, and inflammatory cytokines through inhibition of NF-κB

and MAPK signaling pathways (Zhao et al., 2016). Similarly, amentoflavone has demonstrated *in vitro* anti-inflammatory activity by down-regulating COX-2 and iNOS expression (Banerjee et al., 2002). Given the limited data available for this species, these findings highlight the potential of *F. hepatica* as a source of anti-inflammatory metabolites and support further studies aimed at identifying the compounds responsible for COX inhibition and clarifying their mechanisms of action.

## Conclusion

The present study demonstrates that *Fistulina hepatica,* although primarily recognized as an edible species, also represents a promising source of bioactive metabolites with both antimicrobial and anti-inflammatory potential. Among the tested extracts, the $CHCl_3$ extract exhibited the most notable biological activity, showing pronounced inhibition of COX enzymes, with higher inhibition observed for COX-2 than COX-1 under the tested conditions, together with antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The observed bacteriostatic effects and COX inhibitory activity may be associated with phenolic constituents previously identified in this extract, particularly cinnamic acid as the most abundant compound, followed by *p*-coumaric and quinic acids. However, the contribution of other metabolites, including lipophilic components such as terpenes, cannot be excluded and warrants further investigation to clarify their role in the biological activity of this non-polar macrofungal extract.

Overall, these findings support the potential of *F. hepatica* as a source of natural bioactive compounds with combined antimicrobial and anti-inflammatory properties. Future studies should focus on isolating and structurally characterizing the active metabolites, determining more precise pharmacological parameters (e.g., $IC_{50}$ values and selectivity indices), elucidating their molecular mechanisms of action, and evaluating their safety and efficacy in relevant biological models. Such investigations may contribute to the development of *F. hepatica*-derived bioactives for potential applications in nutraceuticals and functional foods.

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## Declaration of competing interests

The authors have declared that no competing interests exist.

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## Supplementary material

### *LC-MS/MS analysis of polyphenolics*

Quantification of polyphenolic compounds and one cyclohexanecarboxylic acid, quinic acid, in the polysaccharide (PSH) fraction and in the 80% methanolic (80% MeOH), 70% ethanolic (70% EtOH), and chloroform ($CHCl_3$) extracts of *F. hepatica* determined by LC–MS/MS analysis, as previously described by Rašeta et al. (2025).

**Table S1.**
LC–MS/MS analysis of selected polyphenolics and quinic acid in the PSH-fraction, 80% MeOH, 70% EtOH, and $CHCl_3$ extracts of *Fistulina hepatica* (ng/mL dry weight)[1]

| Class of phenolic compounds and quinic acid | Concentrations in tested *F. hepatica* extracts | | | |
|---|---|---|---|---|
| | PSH-fraction | 80% MeOH | 70% EtOH | $CHCl_3$ |
| **Hydroxybenzoic acids** | | | | |
| *p*-Hydroxybenzoic acid | <3.05 | <3.05 | <3.05 | <3.05 |
| Cinnamic acid | <48.85 | 123.15±0.51 | 94.45±0.69 | 740.93±1.85 |
| Protocatechuic acid | <1.53 | 2.28±0.05 | 1.73±0.01 | <3.05 |
| **Cyclohexanecarboxylic acid** | | | | |
| Quinic acid | 814.08±3.45 | 531.80±6.01 | 500.77±3.02 | 20.26±0.83 |
| **Hydroxycinammic acids** | | | | |
| *p*-Coumaric acid | <3.05 | 18.82±0.02 | 23.54±0.04 | 82.81±0.13 |
| Caffeic acid | <3.05 | 55.31±0.09 | 40.52±0.08 | 15.08±0.01 |
| **5-Caffeoylquinic acid** | | | | |
| Chlorogenic acid | 3.39±0.01 | <3.05 | <3.05 | <3.05 |
| **Biflavonoid** | | | | |
| Amentoflavone | 4.55±0.03 | 3.22±0.02 | 2.09±0.01 | 2.81±0.02 |

[1] All these results are published in Rašeta et al. (2025). Data represent the mean ± standard deviation (SD). Values marked with "<" indicate concentrations below the limit of quantification (LOQ) but above the limit of detection (LOD); the compound was detected based on the presence of a measurable peak. Abbreviations: PSH-fraction – polysaccharide fraction; 80% MeOH – hydromethanolic extract prepared with 80% methanol; 70% EtOH – hydroethanolic extract prepared with 70% ethanol; $CHCl_3$ – chloroform extract.

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