



Article

Fomentariol, a *Fomes fomentarius* Compound, Exhibits Anti-Diabetic Effects in Fungal Material: An In Vitro Analysis

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Abstract: The present study screened various fungal species for inhibitors of alpha-glucosidase, alpha-amylase, and DPP-4, enzymes that are crucial in carbohydrate metabolism. Ethanolic extracts exhibited superior inhibitory activity compared to water extracts, suggesting their potential as sources of anti-diabetic agents. Further fractionation revealed fomentariol from *Fomes fomentarius* as a potent inhibitor of alpha-glucosidase and DPP-4, with higher activity against alpha-glucosidase than acarbose. Fomentariol presents a novel avenue for diabetes management, demonstrating the simultaneous inhibition of key enzymes in glucose metabolism. However, comprehensive clinical studies are needed to evaluate its safety and efficacy in humans.

Keywords: diabetes; anti-diabetics; fungi; *Fomes fomentarius*; fomentariol



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1. Introduction

Diabetes mellitus (DM) remains a significant global health challenge. According to the International Diabetes Federation, approximately 10.5% of global adults were living with diabetes in 2021, a figure projected to rise to 783 million by 2045 [1]. The primary objective of anti-diabetic medications is achieving and maintaining optimal glycaemic control to delay the onset of DM-related complications, including neuropathy, nephropathy, retinopathy, and cardiovascular diseases. Despite advances in pharmacotherapy, a substantial proportion of individuals with diabetes fail to achieve their glycemic targets [2,3]. This inadequacy is attributed to various factors, including the progressive nature of the disease, the limited efficacy of existing drugs, adverse effects, patient adherence issues, and accessibility to medication [3]. Several classes of anti-diabetic medications are currently available, including biguanides, sulfonylureas, thiazolidinediones, dipeptidyl peptidase-4 (DPP-4) inhibitors, sodium-glucose cotransporter 2 inhibitors (SGLT2i), and insulin therapies [4]. While these medications have effectively managed blood glucose levels, they often fail to provide sustained glycemic control [5]. Additionally, adverse effects such as hypoglycemia, weight gain, and gastrointestinal disturbances can impede their long-term use [6]. In this context, there is an increasing interest in exploring alternative therapeutic agents, particularly those derived from natural sources.

Several studies have reported the potential of fungal isolates as a source of bioactive compounds with anti-diabetic properties. Previous studies have identified potential anti-diabetic compounds from various fungal sources, including *Ganoderma lucidum* [7], *Inonotus obliquus* [8], and *Trametes versicolor* [9]. Additionally, a recent study by Nurjannah et al. identified several potential anti-diabetic compounds from eight fungal isolates, highlighting the diversity of bioactive compounds that can be derived from fungi [10].

Fomes fomentarius (*F. fomentarius*), a species of fungus, has long been recognized in various cultures for its medicinal properties. *F. fomentarius* belongs to a group known as wood-decay fungi. These organisms exhibit a unique nutrition method: they digest their

food externally and absorb nutrients [11]. This external digestion is of particular interest in the context of diabetes research. Some wood-decay fungi are believed to possess inhibitory molecules that prevent other organisms from utilizing the carbohydrates digested from wood. This ability suggests the potential for these fungi to influence carbohydrate metabolism, a key aspect in managing diabetes [12–14]. Furthermore, *F. fomentarius* produces a broad range of secondary metabolites, which makes them an interesting source in the search for new active molecules [15]. Exploring *F. fomentarius* and its extracts could lead to the identification of novel molecules that might aid in controlling blood glucose levels or address other aspects of diabetes management [16].

In this work, we screened various fungal species for potential inhibitors of three enzymes involved in carbohydrate metabolism: alpha-glucosidase, alpha-amylase, and DPP-4. We identified an active molecule, fomentariol, a compound of *F. fomentarius*, which inhibited both alpha-glucosidase and DPP-4 at the same time. We found that the inhibitory activity of fomentariol for alpha-glucosidase was higher than the established inhibitor acarbose. However, the inhibitory activity of fomentariol for DPP-4 was lower than that of the clinically used sitagliptin.

2. Materials and Methods

2.1. Fungal Sample Collection and Identification

Fungal material was characterized and kindly provided by Professor Franc Pohleven from the Department of Wood Science and Technology at the Biotechnical Faculty, University of Ljubljana. We prepared water and ethanolic extracts from the following fungal species: *Armillariella mellea*, *Fomes fomentarius*, *Trametes versicolor*, *Trametes hirsute*, *Fomitopsis pinicola*, *Piptoporus betulinus*, *Clitocybe inversa*, *Bjerkandera adusta*, *Hypholoma capnoides*, *Lactarius deliciosus*, *Oudemansiella mucida*, *Gleophyllum odoratum*, *Psathyrells hydrophila*, *Pleurotus ostreatus*, *Pholiota nameko*, and *Lentinula edodes*.

2.2. Preparation of Fungal Extracts

We took 1 g of the fungal material for each sample and mixed it with 15 mL of either purified water or absolute ethanol. Ethanol (99.5%) used for the extraction of fomentariol was obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Purified water was obtained from a Simplicity 185 purification system (Millipore, Billerica, MA, USA). Before use, the sample was filtered through 0.22 µm nylon membranes (Agilent Technologies, Santa Clara, CA, USA). All reagents used were of analytical grade except water, which was of HPLC grade. Mixtures of fungal material with water or ethanol were then homogenized and shaken continuously for 12 h at room temperature. After this period, we collected the supernatant by performing two consecutive rounds of centrifugation, each lasting 15 min at a speed of 10,000 × *g*. We applied a lyophilization process for the water extracts to obtain dry extracts, which were then stored in a frozen state until needed for the tests. Similarly, the ethanolic extracts were dried using a rotary evaporator and then frozen. Before conducting the experiments, we prepared the extracts as follows: 2 mg of the dry water extracts were dissolved in 1 mL of purified water. In comparison, 2 mg of the dry ethanol extracts were dissolved in 1 mL of purified water that contained 25% (*v/v*) dimethyl sulfoxide (DMSO).

2.3. α-Glucosidase Purification Methods

The methods of mammalian α-glucosidase purification were combined and slightly modified from Yoshikawa et al. [17], Shihabudeen et al. [18] and Oki et al. [19]. We prepared a homogenate using rat-intestinal acetone powder (sourced from Sigma-Aldrich), which we dissolved in a phosphate buffer solution enriched with EDTA (Ethylenediaminetetraacetic acid) and DTT (Dithiothreitol). This mixture was then subjected to sonication and vortex mixing, after which Triton X-100 was added. After these steps, the resulting suspension was centrifuged to separate the solid and liquid components. We collected the supernatant (the clear liquid above the settled solids) for further processing. This

supernatant underwent sequential ammonium sulphate precipitation steps, a method used to selectively precipitate and isolate proteins. The resulting pellet, containing the concentrated proteins, was then resuspended in a phosphate buffer at pH 7.0. To remove any small-molecule contaminants and further refine our sample, we dialyzed this suspension overnight against the same buffer. This step ensures that the final product is suitable for subsequent experimental analyses.

2.4. Alpha-Glucosidase and Alpha-Amylase Inhibition Assays

Alpha-glucosidase inhibitory activity was measured as described previously [20]. In our laboratory experiments, we conducted two distinct enzyme assays: alpha-glucosidase and alpha-amylase, each with its specific protocol. For the alpha-glucosidase inhibition assay, we diluted the enzyme solution in a phosphate buffer at pH 6.8 and then pre-mixed it with aqueous sponge extracts to achieve a final concentration of 0.08 mg/mL. A substrate, 4-methylumbelliferyl- α -D-glucopyranoside (MUG), was added to this mixture at a concentration of 0.03 mM. This mixture was incubated at 37 °C for 120 min. To stop the reaction, we added 0.2 M Na₂CO₃. The alpha-glucosidase activity was assessed by measuring the release of 4-methylumbelliferone anion at an excitation wavelength of 365 nm and an emission wavelength of 445 nm. We calculated the remaining enzyme activity by comparing the fluorescence intensity of the sample with that of a control enzyme solution without a sponge extract. This assay was performed in triplicate, and the results were presented as the mean \pm standard deviation.

The alpha-amylase inhibition assay followed a modified version of a previously described method [21], adapted for a 96-well plate format. We dissolved porcine pancreatic alpha-amylase (type IV, Sigma-Aldrich) in ice-cold distilled water to prepare a 1.75 unit/mL enzyme solution. This solution was pre-incubated with sponge ethanolic extracts (0.1 mg/mL concentration dissolved in 25% *v/v* DMSO and aqueous extracts (0.1 mg/mL concentration in water). The reaction was initiated by adding a 0.5% potato starch solution (Sigma-Aldrich) and incubated at room temperature for three minutes. The mixture was then transferred to a sealable plate containing the DNS color reagent solution (as described by Bernfeld in 1955) and heated in a 95 °C thermo block for 15 min.

Following dilution with distilled water, the mixture's absorbance was measured at 540 nm in a new 96-well plate. This assay was also performed in triplicate, with results expressed as the mean \pm standard deviation. We conducted a 100% enzyme activity assay for control by replacing the sponge extract with 25% *v/v* DMSO. Additionally, in the blank incubation, the DNS reagent was added first to the mixture of inhibitor and substrate, followed by the enzyme solution, to account for any absorbance due to the sponge extracts and lactose in the enzyme reagent.

The absorbance due to maltose generated was calculated as follows:

$$A_{540 \text{ nm}} (100\% \text{ control or sponge extract}) = A_{540 \text{ nm}} \text{ Test} - A_{540 \text{ nm}} \text{ Blank}$$

Remaining alpha-amylase activity was calculated as $A_{540 \text{ nm}} (\text{extract}) / A_{540 \text{ nm}} (100\% \text{ control})$.

2.5. Dpp-4 Inhibition Assay

The DPP-4 inhibition assay was meticulously conducted in our study following the manufacturer's guidelines. The process began in a black 96-well plate, where 30 μ L of human recombinant DPP4 solution (0.1 μ g/mL, supplied by Abnova, Taipei City, Taiwan) in 50 mM Tris buffer (pH 8.3) was preincubated at 37 °C for 10 min with 30 μ L of the extracts. Following this preincubation, 50 μ L of a 0.5 mM substrate solution, specifically glycyl-prolyl-7-amino-4-methylcoumarin (procured from Bachem, Bubendorf, Switzerland), was added to the Tris buffer. The DPP4 activity was kinetically measured over 30 min at 37 °C. This measurement assessed the release velocities of amino-4-methyl coumarin from the substrate at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. To calculate the residual DPP4 enzymatic activity in samples with different

inhibitor concentrations, we divided the slope of the sample solutions by the slope of the control solution, where the tested extracts were replaced with the solvent. All these measurements were conducted in triplicate.

2.6. Analytical Techniques for Active Molecule Identification

Samples that showed significant activity were then further processed using ultrafiltration, dialysis, and chromatographic techniques. For ultrafiltration, we used 3 kDa Amicon® falcons (Sigma-Aldrich Chemie, Taufkirchen, Germany), while 2 kDa and 10 kDa Cassettes (Thermo Fisher Scientific Inc., Waltham, MA, USA) were used for dialysis. Column chromatography used two distinct stationary phases: Dow styrene-DVB and Diaion HP-20 (Sigma-Aldrich). We adhered to the manufacturer's instructions for the separation parameters. High-performance liquid chromatography (HPLC) separation was performed using a Shimadzu Prominence system with a C18 Kinetex 2.6 µm column. A gradient elution was applied using mobile phases A (water with 5% acetonitrile and 0.1% TFA) and B (acetonitrile with 5% water and 0.1% TFA). The compounds were analyzed using a fluorescence detector (with an excitation wavelength of 330 nm and emission wavelength of 590 nm) and a UV-VIS detector (absorbance at 288 nm and 350 nm). The separated fractions were then retested for their inhibitory activity.

To identify the active molecules, we employed liquid chromatography–mass spectrometry (LC–MS) and nuclear magnetic resonance (NMR) as described in Maljuric et al.'s method [22]. The LC–MS analysis was performed on a Q-TOF mass spectrometer coupled with a UPLC chromatographic system from Waters, Milford. The quantitative NMR determination was performed using a Bruker Ascend 400 (400 MHz) spectrometer based in Billerica, MA, USA.

3. Results

All the extracts were tested for their inhibitory activity against alpha-glucosidase, alpha-amylase, and DPP-4. Extracts and exhibiting activity were further separated using the earlier methods and retested for inhibitory activity. Finally, we purified a single molecule that potently inhibited alpha-glucosidase and DPP-4 from the ethanolic extract of *Fomes fomentarius* and identified this molecule as fomentariol [23].

Alpha-glucosidase. Extracts that exhibited inhibitory activity against alpha-glucosidase were further examined by testing inhibition using serial dilutions of the extracts (Figure 1).

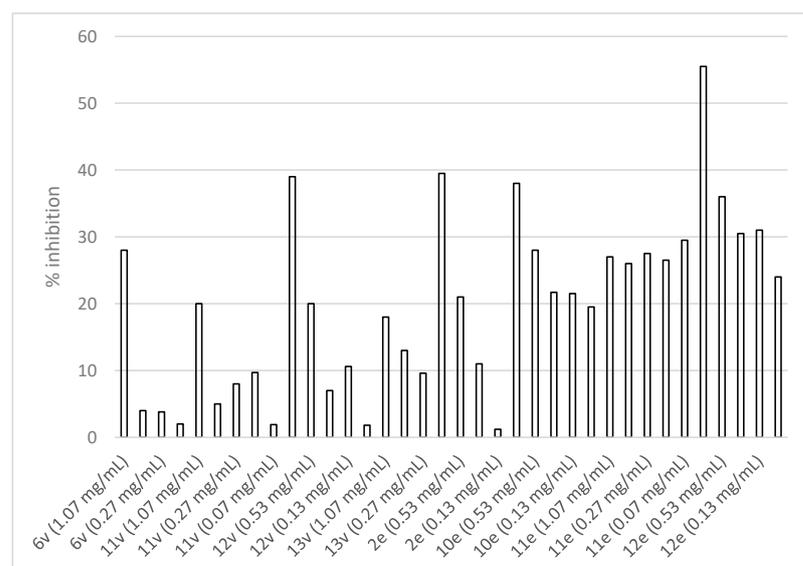


Figure 1. Percentage of inhibition of serial dilutions of water (v) and ethanol (e) extracts. Included samples: 2 = *Fomes fomentarius*, 6 = *Piptoporus betulinus*, 10 = *Lactarius deliciosus*, 11 = *Oudemansiella mucida*, 12 = *Gleophyllum odoratum*, 13 = *Psathyrells hydrophila*.

Next, HPLC-purified and NMR-quantified fomentariol from the ethanolic extract of *F. fomentarius* was tested for alpha-glucosidase inhibitory activity and compared to the established inhibitor acarbose (Figure 2).

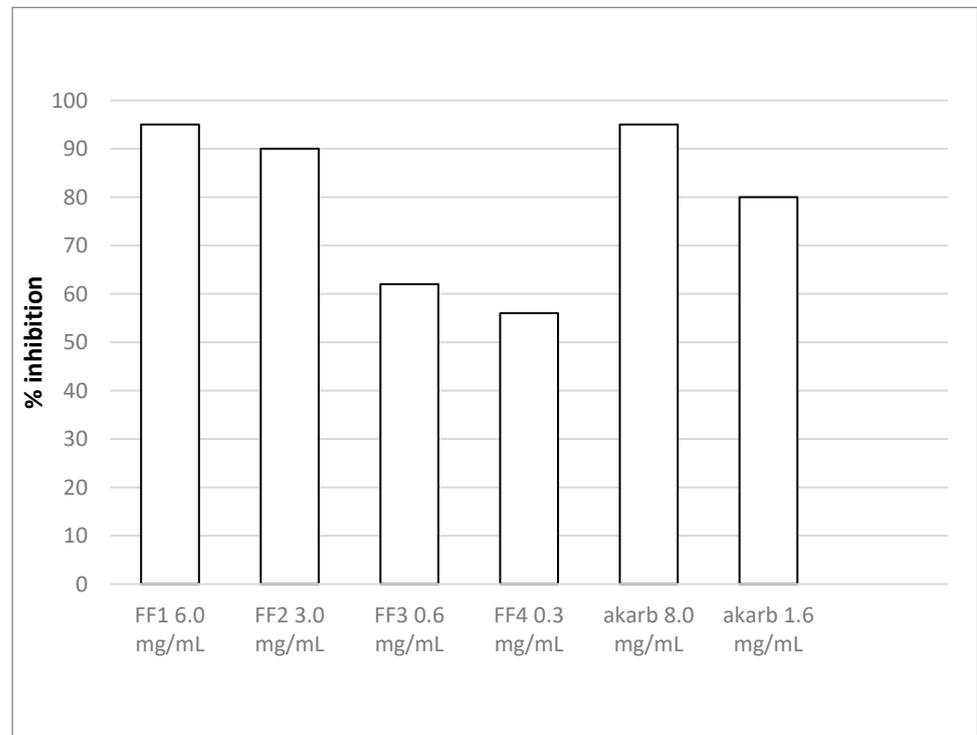


Figure 2. Average percentage of inhibition of fomentariol and acarbose at different concentrations. FF, fomentariol; akarb, acarbose.

4. Alpha-Amylase

Extracts that exhibited inhibitory activity against alpha-amylase were further examined by testing the inhibition using different concentrations of the extracts (Figure 3).

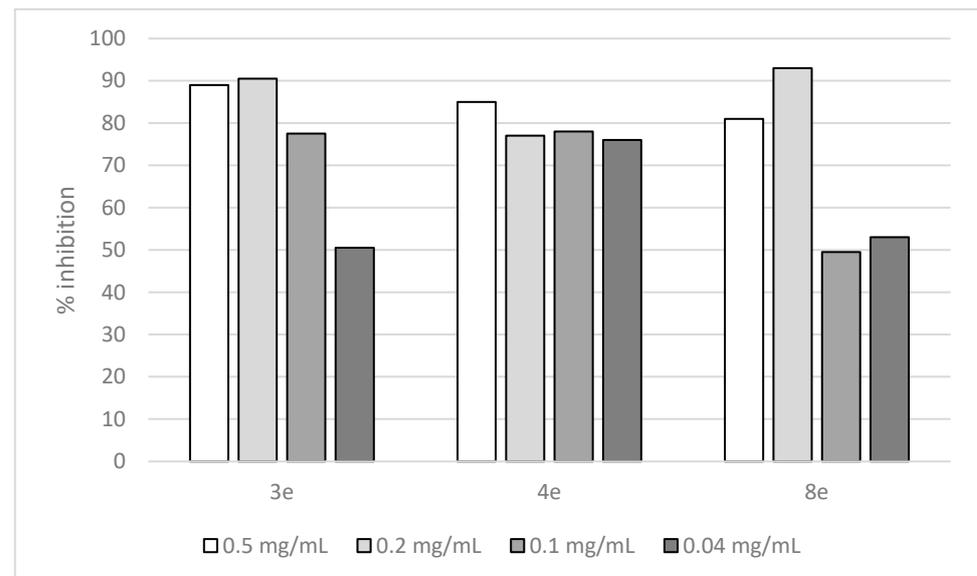


Figure 3. Average percentage of inhibition of amylase for ethanolic extracts 3 (*Trametes versicolor*), 4 (*Trametes hirsute*) and 8 (*Bjerkandera adusta*) in concentrations: 0.5 mg/mL, 0.2 mg/mL, 0.1 mg/mL, and 0.04 mg/mL.

After further separation and testing, the ethanolic extract of sample 8 showed the highest inhibition against alpha-amylase. However, we could not isolate and determine a single compound responsible for inhibitory activity.

DPP-4. The ethanolic extracts of samples 2 and 12 exhibited significant inhibitory activity against DPP-4 (Figure 4). Both samples were further separated and retested.

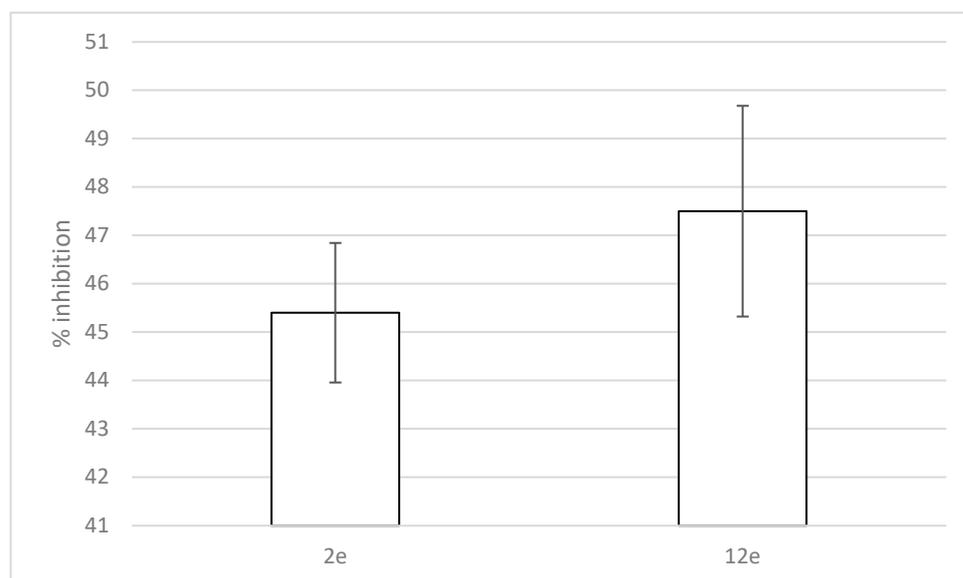


Figure 4. Percentage of inhibition of DPP-4 for ethanolic extracts (e) of samples 2 (*Fomes fomentarius*) and 12 (*Gleophyllum odoratum*) with standard deviations.

Using separation methods, we purified and identified an active molecule in sample 2 but not in sample 12. The molecule exhibiting activity in *Fomes fomentarius* turned out to be fomentariol. Fomentariol, purified using HPLC and quantified using NMR, was retested for inhibitory activity against DPP-4 (Figure 5).

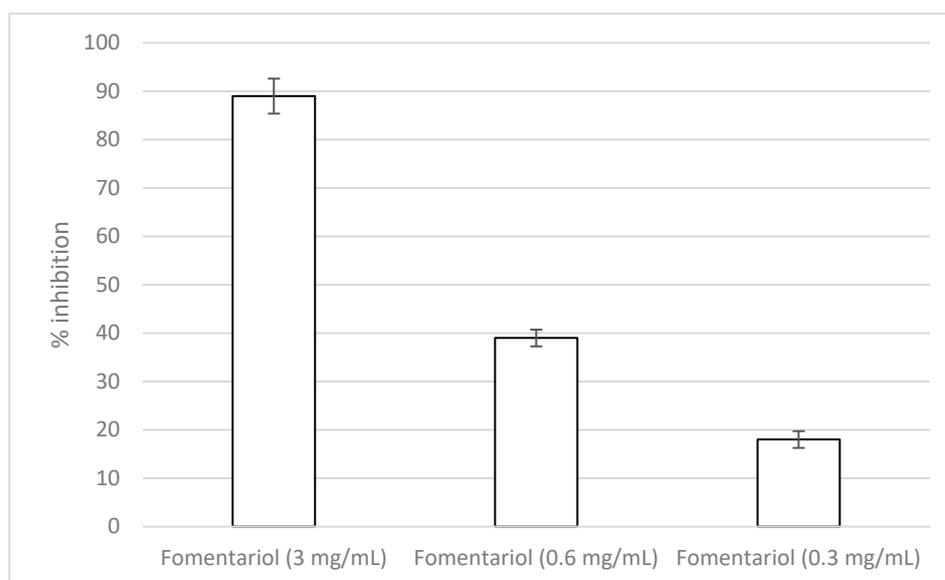


Figure 5. Average percentage of inhibition of fomentariol at different concentrations.

An active fraction of *F. fomentarius* was analyzed with HPLC (Figure 6). The main peak was isolated and later identified as fomentariol. The identity of fomentariol was proposed using the LC-MS method and NMR as described in Maljuric et al. [22].

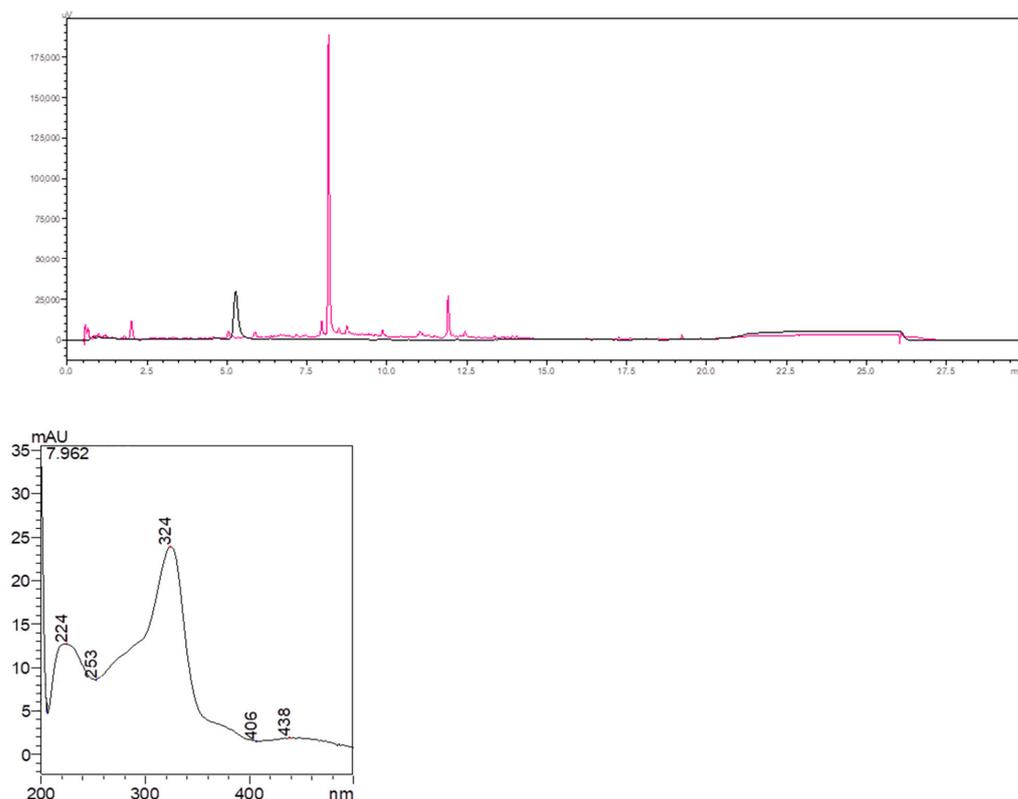


Figure 6. HPLC profile of the active fraction of *Fomes fomentarius* and the absorption spectra of the main peak containing fomentariol. mAU, milli absorbance unit.

5. Discussion

The present study screened various fungal species for potential inhibitors of three enzymes involved in carbohydrate metabolism: alpha-glucosidase, alpha-amylase, and DPP-4. We found that the tested fungal extracts were a potent source of inhibitors for these enzymes involved in glucose homeostasis. Ethanol extracts showed superior activity to water extracts. Surprisingly, a large part of the extract had at least small inhibitory effects towards one of the tested enzymes. However, during further separation steps, some of these effects diminished, probably due to the presence of more than one active molecule in the sample acting in a complementary way. Furthermore, we focused on the fractions containing smaller molecules that are easier to identify structurally. We purified and identified an active molecule, fomentariol, which inhibited alpha-glucosidase and DPP-4. We found that the inhibitory activity of fomentariol for alpha-glucosidase was higher than the established inhibitor acarbose. However, the inhibitory activity of fomentariol on DPP-4 was lower than that of the clinically used sitagliptin.

F. fomentarius, a bracket fungus known as horse's hoof fungus, has shown promising potential in various therapeutic applications. This fungus has been used in traditional medicine to treat various conditions [24]. A growing number of studies have further solidified the medicinal value of *F. fomentarius*, revealing that water and methanol extracts from the fungus exhibit anti-diabetic and anti-inflammatory/anti-nociceptive activities, respectively [25,26]. Fomentariol is abundant in *F. fomentarius*, allowing isolation in larger quantities [27]. The discovery of fomentariol adds a new dimension to our understanding of the bioactive compounds in *F. fomentarius*. Fomentariol has exhibited anti-inflammatory activity by suppressing the activation of the signal transducer and activator of transcription 3 (STAT3) [28]. A recent experiment by Maljurić et al. [22] isolated and determined the amount of fomentariol from fungal material using the semipreparative HPLC method. In this investigation, we established anti-diabetic effects as additional clinical values of fomentariol. The IC_{50} of fomentariol for alpha-glucosidase was calculated to be 1.3 μM ,

while the established inhibitor acarbose has a literature IC_{50} value of 0.5 μ M [29]. The IC_{50} of fomentariol for DPP-4 was calculated to be 2.4 μ M, while the clinically used sitagliptin has an IC_{50} of 18 nM [30]. While fomentariol exhibited moderate inhibitory activities against both enzymes, its ability to inhibit both enzymes simultaneously may have potential therapeutic advantages. Dual inhibition of α -amylase and α -glucosidase has been shown to be a promising strategy for the management of postprandial hyperglycemia [31]. Comprehensive in vivo studies and clinical trials are necessary to fully understand its safety, efficacy, and potential side effects in humans.

The anti-diabetic potential of fomentariol has not been previously reported. However, the bioactivity of fomentariol may be related to the ecological role of the fungus in its natural habitat. *F. fomentarius* is a saprophytic fungus that plays an important role in the decomposition of wood, and it has been suggested that the production of bioactive compounds may be a strategy used by the fungus to compete with other microorganisms in its environment [32]. Further research is needed to fully understand the potential bioactivity of fomentariol and its relationship to the ecological role of *F. fomentarius*.

In the present study, we focused on the inhibitory effects of fomentariol on three enzymes due to their physiological role in regulating glycaemic hemostasis. The enzymes alpha-glucosidase and alpha-amylase play a crucial role in carbohydrate digestion by cleaving complex carbohydrates into monosaccharides, which are then absorbed in the gastrointestinal tract [33,34]. The inhibition of these enzymes is a key strategy in managing type 2 diabetes, primarily by preventing postprandial hyperglycemia [35]. On the other hand, DPP-4 is another enzyme integral to glucose metabolism, although its role is more indirect than alpha-glucosidase and alpha-amylase. DPP-4 inactivates incretin hormones such as glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which are secreted into the blood in response to glucose intake, and play a significant role in regulating blood glucose levels. Incretins enhance insulin secretion and suppress glucagon release, thereby aiding in glucose homeostasis [36–38]. Therefore, inhibiting DPP-4 enhances the bioavailability of these incretin hormones, contributing to the reduction in postprandial hyperglycemia.

In conclusion, this in vitro analysis has successfully identified the anti-diabetic properties of fomentariol, a compound derived from *F. fomentarius*. Our investigation purified and identified fomentariol and demonstrated its ability to inhibit two key enzymes, alpha-glucosidase, and DPP-4, which play crucial roles in the metabolism of carbohydrates and glucose regulation. This discovery opens a new avenue in the exploration of natural compounds for diabetes management, suggesting that fomentariol could potentially offer a novel therapeutic approach. Further studies are needed to understand the pharmacokinetics, pharmacodynamics, and potential therapeutic value of fomentariol in a living organism.

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