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Antidiabetic effect of two different *Ganoderma* species tested in alloxan diabetic rats†

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This study was designed to define total protein, phenol and flavonoid content as well as LC-MS/MS phenolic profile related to antioxidant and antidiabetic activity of ethanolic (EtOH) and water extracts of *G. pfeifferi* and *G. resinaceum*. *G. resinaceum* water extract possessed the highest ability to scavenge DPPH[•] and O₂^{•-}, while the EtOH extract of the same species showed better activity on NO[•] related to other extracts. The highest level of bioactive compounds was determined generally in EtOH extracts. Antidiabetic action was evaluated by the oral glucose tolerance test (OGTT) and histological examination of pancreas and liver in normoglycemic and alloxan-induced diabetic animals. Histological examination of pancreatic tissue demonstrated that *G. pfeifferi* extracts have protective effects. To conclude, analysed extracts could be considered as a promising candidate for further research with the aim to promote antidiabetic activity, which is for the first time reported for *G. pfeifferi*.

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1. Introduction

An imbalance between formation of reactive oxygen species (ROS) metabolites and the rate at which they are scavenged by enzymatic and non-enzymatic antioxidants is referred to as oxidative stress which can further lead to a range of health disorders in humans.^{1–3} A series of protective antioxidant mechanisms exist in human organisms, such as catalase (CAT), glutathione-*S*-transferase (GST) and many others for both prevention of free radical production and reparation of oxidative damage.^{3–7} Oxidative stress plays an important role in some physiological conditions and in many diseases and disease states, including diabetes mellitus (DM), cancer or neurodegenerative disorders.⁸ The restriction of the use of cancerogenic synthetic phenolic antioxidants (BHA, BHT, PG and others) in recent years has caused a rapidly increased interest towards naturally occurring antioxidants which could effectively inhibit oxidation in living systems.^{1,2}

To date, a large number of bioactive compounds including polysaccharides and their protein complexes, dietary fibres, and other compounds extracted from fungal fruiting bodies,

cultured mycelium, or cultured broth in submerged cultivation have been reported to express anti-hyperglycaemic activity.^{9,10} Natural extracts of *G. lucidum* have been recognized and used as an alternative therapy for DM since ancient time in China¹¹ in which ganoderan A, B and C have been characterized recently to be useful for treatment of type 1 DM and for type 2 DM.¹²

Ganoderma species are saprotrophic lignicolous basidiomycete with large, leathery, perennial, woody brackets called “conks”, growing on deciduous trees either with or without a stem. They represent valuable sources of nutrients and different types of bio remedies, which have been used for centuries in Asian countries to promote health and longevity.^{1,8} *Ganoderma* is a genus of polypore mushrooms that include about 80 species, many from tropical regions. While the medicinal use of mushrooms has a very long tradition in Asian countries, it has been largely forgotten in Europe; thus, it is not surprising that *Ganoderma* species that occur only in Europe have been poorly investigated.¹³ The species *G. lucidum*, crown fungi, is used for the preparation of infusions and sold as functional supplements worldwide.¹⁴ Likewise, there are evidences of potential benefit from using various *Ganoderma* extracts with respect to antioxidant,^{1,14–16} antibacterial,^{13,14} antiviral,⁹ antidiabetic,^{8,12} anticancer activity,^{16–18} cardioprotective and nephroprotective effect.¹⁹ They can grow wild or can be cultivated to be used as an important natural source against various diseases caused by oxidative stress.^{1,2,20} In traditional Chinese ethnomedicine, *G. resinaceum* Boud. in Pat. 1890 has been also proved to exert many pharmacological effects, for hyperglycaemia, immunoregulation and liver disease including improvement of function of lungs, liver, kidney, spleen and stomach.²¹ It contains various types of

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bioactive compounds, namely ganoderic acids A and B,²² β -glucans,¹⁰ lanostanoid terpenoides (ganoderesin B, ganoderol B, lucidone A)¹⁸ and phenolic compounds.¹⁴ A few compounds have been reported for *G. resinaceum* until nowadays while fifteen nortriterpenoids including six new nortriterpenoids (1–6) and nine known analogs (7–15) have been determined recently supporting results that the side chain of *Ganoderma* triterpenoids played an important role in α -glucosidase inhibitory activity.²² Structure analysis of a potential bioactive compounds indicated that most powerful compounds have a pentatonic 20(24)- γ -lactone ring, also ganoderlactone D has a 3-OH and 12-OH that correspond to the structure–activity relationships.²³ Study reported by Zhao *et al.*¹⁹ suggests that ganoderic acids such as ganoderolactone B, D, E and ganodernoid A may play important roles in the antidiabetic effects of *G. lucidum*. Therefore, α -glucosidase inhibitors have been proposed as a treatment for type 2 DM by preventing the digestion of carbohydrates.¹⁹

Phylogenetic analysis grouped *G. pfeifferi* together with *G. resinaceum*, *G. subamboinense* and three strains of *G. lucidum* from the United States and Taiwan into one monophyletic group that is characterized by the production of chlamydozoospores in culture.²⁴ In contrast to *G. applanatum* and *G. lucidum* and lately *G. resinaceum*, from which a great number of biologically and pharmacologically important lanostane triterpenes and polysaccharides have been isolated, *G. pfeifferi* (Bres 1889) a typical European species is one of the chemically less examined *Ganoderma* species.^{9,13,24} *G. pfeifferi* manifests antimicrobial^{13,24} activity related to sterols (ergosta-7,22-diene-3-one, ergosta-4,6,8(14),22-tetraene-3-one, 5 α ,8 α -epidioxyergosta-6,22-diene-3 β -ol) and triterpenes (lucialdehyde D, ganoderone B and ganoderone C) as well as lanostanoid triterpenes (ganodermadiol, lucidiol and applanoxic acid G)⁹ *etc.* Among previously described substances, triterpenoids, polysaccharides and glycoproteins could have antidiabetic effects,¹² while phenols are significant as antioxidant compounds.^{14,16} *G. pfeifferi* contains unique sesquiterpenoids and other small molecular weight compounds among which some exhibits remarkable antimicrobial activities.¹³ Stimulating effects of *G. pfeifferi* on the viability of skin cells, the UV protection properties and the antibacterial and anti-aging activities suggest a possible use of this fungal species in the form of cosmetics, perhaps in combination with special minerals. For this purpose, EtOH and water extracts are already registered with the INCI numbers (International Nomenclature of Cosmetic Ingredients).¹³

To the best of our knowledge, no detailed study has been carried out on the *in vivo* effect of *G. pfeifferi* and *G. resinaceum* extracts on antidiabetic and antioxidant activities, lipid peroxidation and enzyme antioxidants in alloxan-induced DM. Hence, the aim of this study was to determine the *in vitro* and *in vivo* antioxidative as well as antidiabetic activities of extracts of both species in rat model of alloxan-induced DM.

2. Material and methods

2.1. Fungal material and extracts preparation

Fruiting bodies of two *Ganoderma* fungal species, *G. pfeifferi* and *G. resinaceum* were collected in September 2010, at the location

of Nature park Begečka Jama and Campus of University of Novi Sad (Serbia), respectively. Determination and identification of the collected material was carried out at Department of Biology and Ecology, University of Novi Sad. Voucher specimens are deposited in the Herbarium of the University of Novi Sad (BUNS), under number (12-00723, 12-00722).

All experiments were performed using ethanol (EtOH) and water extracts which were prepared as described previously.¹⁶ Both suspensions were prepared in distilled water to reach the final concentration at 100 mg mL⁻¹ dry weight (d.w.) and stored at +4 °C and –20 °C prior to analysis.

2.2. Laboratory animals and experimental procedures

Experiments were carried out on white Wistar rats of both sexes (weighing 210–340 g and ages up to four months) were purchased from Military Medical Academy of Belgrade (Republic of Serbia). Animals were housed in UniProtect airflow cabinet (Ehret GmbH, Emmendingen, Germany) and standard plexiglass cages at a constant 22 \pm 1 °C room temperature, 55% \pm 1.5% humidity and with standard circadian rhythm (12 h day/night cycle). They were allowed free access to tap water and standard pelleted laboratory rodent feed (Veterinary Institute Subotica, Serbia) during the whole experiment. The experimental procedures were conducted in accordance with the European Directive (2010/63/EU) for animal experiments and they were reviewed and approved by Ethics Committee for Protection and Welfare of Experimental Animals at University of Novi Sad, Serbia.

Animals were randomly divided into ten groups, each group consisting of six animals. Five groups were without alloxan pre-treatment, and five groups were with alloxan-induced diabetes (AID). Normoglycemic and diabetic animals were treated in the same manner with an oral aqueous suspension of the EtOH and water extracts of two analysed fungal species at dose of 1 mg mL⁻¹, whereas the control animals received the same volume of physiological solution. The extract suspensions were administered for 5 days and on day 5 (2 h after administration of the last dose of fungal extracts) the rats were narcotized with a 25% solution of urethane in a dose of 5 mL kg⁻¹ intraperitoneal (i.p.). After losing of the righting reflex, the animals were exsanguinated with intracardial puncture in order to take samples of blood and other tissues for further examination. After sacrifice, samples of the rat's pancreas and liver were fixed in 10% buffered formalin for histological examination. One part of liver was quickly frozen with liquid nitrogen and stored in freezer at –80 °C for the antioxidant activity analysis. All animals were weighed before the experiment and after the 5 day treatment period.

2.3. Antioxidant activity

2.3.1. *In vitro* assays. Antioxidant activities were evaluated by standard *in vitro* assays described earlier.¹⁶

The method of Espin *et al.*²⁵ was used for evaluation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, where reaction mixture consisted of 10 μ L of extract, 60 μ L of DPPH work solution (90 μ mol L⁻¹) and 180 μ L of methanol.

Absorbance was measured after 30 min at 540 nm (Multiskan Ex Thermo Fisher Scientific).

Super oxide anion, SOA scavenging capacity was evaluated according to the method described by Nishikimi *et al.*²⁶ Reaction mixture consisted of 10 μL of extract, 100 μL of NADH (677 $\mu\text{mol L}^{-1}$), 100 μL of phenazine methosulfate (PMS) (60 $\mu\text{mol L}^{-1}$), 200 μL nitro blue tetrazolium (144 $\mu\text{mol L}^{-1}$) and 1.1 mL of phosphate buffer (pH 8.3). After 5 min, 250 μL aliquots were transferred to a plate and their absorbance was measured at 540 nm using a plate reader (Multiskan Ex Thermo Fisher Scientific).

NO radical scavenging activity of the extracts was determined according to the method reported by Green *et al.*²⁷ The reaction mixture contained 30 μL of extracts, 500 μL of sodium nitroprusside dihydrate (10 mmol L^{-1}) and 500 μL of phosphate buffer (pH 7.4). Test tubes were incubated at room temperature for 90 min, exposed to light. After incubation time, 1 mL of Griess reagent (0.2% solution of naphthylethylenediamine dihydrochloride and 2% solution of sulfanilamide in 4% phosphoric acid in a ratio of 1 : 1) was added to test tubes. 250 μL aliquots were transferred to a plate and their absorbance was measured at 546 nm using a plate reader (Multiskan Ex Thermo Fisher Scientific).

FRAP assay was performed according to modified procedure by Benzie and Strain.²⁸ The reaction mixture consisted of 10 μL of extracts, 225 μL of FRAP reagent (acetate buffer, pH 3.6, 2,4,6-tris(2-pyridyl)-s-triazine (10 mmol L^{-1}) and FeCl_3 (20 mmol L^{-1} in the ratio 10 : 1 : 1) and 22.5 μL of distilled water. After 6 min of incubation, the absorbance was measured at 593 nm using a plate reader (Multiskan Ex Thermo Fisher Scientific).

The radical scavenging capacity (RSC) in used assays was expressed as IC_{50} ($\mu\text{g mL}^{-1}$) for DPPH, SOA and NO, whereas reducing power (FRAP) of analysed fungal species was expressed as mg of ascorbic acid equivalents per g dry weight (mg AAE per g d.w.). Synthetic antioxidant propyl gallate (PG) was used as a positive control for RSC assays.

2.3.2. *In vivo* assays. Liver homogenate was prepared from liver tissue (1 g) which was homogenized in a Potter homogenizer with Tris-HCl : sucrose in a ratio 1 : 3 at +4 °C. The obtained homogenates were filtered and the biochemical parameters were determined. The following biochemical parameters for oxidative stress were analysed in liver homogenate: extent of lipid peroxidation (LPx), activity of peroxidase (Px), catalase (CAT), glutathione peroxidase (GSHPx), glutathione reductase (GSHR), xanthine oxidase (XOD) and content of reduced glutathione (GSH) as described in Kaurinovic *et al.*³

2.4. Chemical constituents in relation to antioxidative activity

2.4.1. Total protein, total phenol and total flavonoid content. Total protein content was determined by modified Bradford's method²⁹ (BrandTech® Scientific, Inc.). Absorbance of the reaction mixture was measured after 30 min at 595 nm using microplate reader (Multiskan Ex, Thermo Fisher Scientific). Absorbance values were converted to protein concentration by comparing with a standard curve prepared with

dilutions of the 2 mg mL^{-1} bovine serum albumin (BSA) and expressed as mg of protein equivalents per mL of sample (mg BSAE per mL sample).

Total phenol content was determined according to Singleton *et al.*³⁰ 125 μL of Folin-Ciocalteu reagent (0.1 M) was added to 25 μL of extracts (or standard solution) and after 10 min 100 μL of sodium carbonate (7.5%) was added and reaction mixture was incubated for 2 h. Absorbance was measured after incubation at 760 nm (Multiskan Ex, Thermo Fisher Scientific). Total phenol content was calculated on the basis of a calibration curve of standard solution of gallic acid and expressed as mg of gallic acid equivalents per g of dry weight (mg GAE per g d.w.).

Total flavonoid content was determined according to Chang *et al.*³¹ 30 μL of extracts (or standard solution) was diluted with 90 μL of methanol and 6 μL of aluminium trichloride (0.75 mol L^{-1}), 6 μL of sodium acetate (1 mol L^{-1}) and 170 μL of distilled water was added. Absorbance was measured after 30 min at 415 nm (Multiskan Ex, Thermo Fisher Scientific). From the calibration curve of quercetin solution was calculated total flavonoid content which is expressed as mg of quercetin equivalents per g of dry weight (mg QE per g d.w.).

2.4.2. LC-MS/MS determination of phenolic compounds.

The quantification of the 45 phenolic compounds in analysed extracts *via* LC-MS/MS technique was done according to Orčić *et al.*³² The Agilent 1200 series liquid chromatography unit was used for separation of extracts, using a Zorbax Eclipse XDB-C18 RR 4.6 mm \times 50 mm \times 1.8 mm (Agilent Technologies) reversed-phase column held at 40 °C. Determination was carried out by Agilent series 6410B triple-quadrupole mass spectrometer with electrospray ionization (ESI). MassHunter ver. B.03.01. software was used for instruments control and data analysis. Mobile phase solvents A (0.05% aqueous formic acid) and B (methanol), premixed in 1 : 1 ratio, were used to dilute analyzed extracts to the final concentration of 2 mg mL^{-1} . Gradient elution was performed using the following solvent gradient: starting with 70% A/30% B, reaching 30% A/70% B in 6.00 min, then 100% B at 9.00 min, holding until 12.00 min, with re-equilibration time of 3 min. The flow rate was 1 mL min^{-1} and the injection volume was 5 μL . ESI-MS was used to detect phenolic compounds by using the ion source parameters as follows: nebulization gas (N_2) pressure 276 kPa, drying gas (N_2) flow 9 L min^{-1} and temperature 350 °C, capillary voltage 4 kV, and negative polarity. The data were acquired in dynamic MRM mode by using the optimized compound specific parameters given before.³² The identification of the peaks was done by using standards and the total of identified phenolics was also calculated.

2.5. Antidiabetic activity

Alloxan (alloxan monohydrate, Sigma Chemicals Co, St Louis, MO, USA) was used for diabetes induction in the laboratory animals. Alloxan was dissolved in physiological solution and administered in a dose of 100 mg kg^{-1} i.p. Hyperglycaemia was detected 48 h after the application of alloxan. Animals with a glycemia higher than 15 mmol L^{-1} were included in further experiment. Normoglycemic animals underwent oral glucose

tolerance test. In this test for the induction of hyperglycaemia we used saturated solution of glucose at dose of 3 g kg^{-1} . Blood glucose level was measured from rat's tail vein using commercial Accu Check Active kits for human use (Roche, Switzerland). Blood glucose measurements were at the beginning of experiment, 48 h after the administration of alloxan and 2 h after the administration of the last dose of mushroom extract. For the oral glucose tolerance test blood glucose measurements were just before and 30 min after the administration of glucose solution.

2.5.1. Histopathological analysis. Liver and pancreas samples were fixed in buffered formalin (24 hours at $+4 \text{ }^\circ\text{C}$), then dehydrated in rising isopropanol concentrations and embedded in paraffin (Histowax, The Netherlands) and cut on a rotary microtome (Leica, Germany) at $5 \text{ }\mu\text{m}$. The histology slides were stained by a standard hematoxylin-eosin technique and analysed using a Leica DMLB microscope (Leica, Germany) and photographed on a Leica MC 190 HD camera (Leica, Germany) at magnification $400\times$.

2.6. Statistical analysis

Statistical analysis was performed using IBM SPSS statistical software, version 19.0 (IBM Corp., Armonk, NY, USA). Data were reported as the mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) or Kruskal–Wallis test was employed for the comparisons between experimental groups. *Post-hoc* testing for ANOVA was performed using Tukey's test. Mann–Whitney *U* test was used for *post-hoc* testing in Kruskal–Wallis analysis. Furthermore, paired-samples *t*-test was used for the evaluation of antidiabetic activity, by comparing pre-test and post-test glycaemia values. IC_{50} values (mg mL^{-1}) were determined by the linear regression analysis of RSC (OriginLab 8), while statistical data correlation was obtained by Pearson correlation coefficient to estimate the relationship between the antioxidant potential of analysed fungal extracts and total protein, total phenol and total flavonoid contents. A difference between groups was considered statistically significant for a *P* value less than 0.05 ($P < 0.05$).

3. Results and discussion

3.1. Antioxidant activity

3.1.1. *In vitro* antioxidant activity. The results of antioxidant activity of four extracts are presented in Table 1. The highest scavenging potential on DPPH \cdot and SOA was detected in water extracts of *G. resinaceum* ($\text{IC}_{50} = 14.0 \pm 0.6$ and $30.0 \pm 0.2 \text{ }\mu\text{g mL}^{-1}$, respectively) while highest scavenging potential on NO \cdot ($\text{IC}_{50} = 215.2 \pm 0.8 \text{ }\mu\text{g mL}^{-1}$), was detected in EtOH extract of the same species (Table 1). Obtained RSC activity was lower compared to the activity of the commercial antioxidant propylgallate (PG; $\text{IC}_{50} = 0.39 \pm 0.01$ to $10.0 \pm 0.5 \text{ }\mu\text{g mL}^{-1}$). In this research higher antioxidative activity was detected compared to previously published data for *G. resinaceum*.^{14,18}

For the FRAP assay, the most effective activity was obtained for water extract of *G. resinaceum* ($297 \pm 1 \text{ mg AAE per g d.w.}$), followed by EtOH extracts of *G. pfeifferi* and *G. resinaceum*, while

the lowest activity was obtained for water extract of *G. pfeifferi* ($65 \pm 1 \text{ mg AAE per g d.w.}$) (Table 1). Our results are in accordance with previously published data¹⁴ in which the activity of water *G. resinaceum* extract was more powerful compared to EtOH extract. At the same time analysed extracts of *G. resinaceum* species from Serbia showed better potential compared to extracts of *G. resinaceum* species from Turkey.¹⁴ Also, high correlation between total phenol content and FRAP activity in EtOH extract of *G. resinaceum* was detected ($R^2 = 0.99$) probably due to its richness of active ingredients which possess an antioxidant effect such as α -tocopherol, linoleic α -acid, phenolic acids *etc.*¹

In this study, *G. pfeifferi* (compared to *G. resinaceum*) proved to be a better source of phenols (with the exception of EtOH extract of *G. resinaceum*) which are regarded as one of the most powerful natural antioxidants.^{1,2,12} Among phenols, preliminary the dominant content of flavonoids was detected in EtOH extracts as well as the content of proteins which is, best to our knowledge, the first scientific report.

3.1.2. *In vivo* antioxidant activity. Small deviations from the normal activity of antioxidant enzymes may have a dramatic effect on the resistance of cells to oxidant-induced damage to the genome and cell killing through the process of oxidative stress.¹⁵ The represented type of *in vivo* antioxidant activity showed that extracts of *G. pfeifferi*, for both types of extracts, are efficient in the protection of tissues and cells from oxidative stress measured by antioxidant status in liver homogenate. *In vivo* assays are important because a numerous plant or fungal phenols are bio transformed during their active metabolism pathways.³

As shown in Table 2, in comparison with control samples intensity of lipid peroxidation (LPx) was significantly reduced in animals treated with analysed extracts, except in animals treated with *G. pfeifferi* EtOH extract where the intensity of LPx was slightly increased ($11.0 \pm 0.5 \text{ nmol MDA per mg P}$). In groups treated with *G. resinaceum* extracts both with and without alloxan pre-treatment, similar activity in comparison to control samples was detected, which points the effects of this less examined fungal species. Teng *et al.*¹¹ indicate the importance of proteoglycans as potent hypoglycaemic agents in *G. lucidum* extracts.

The amount of GSH, secondary metabolite important in detoxification pathways of organisms *via* conjugation with toxic metabolites,³ was statistically significant increased mostly in animals treated with *G. pfeifferi* EtOH and water extracts (285 ± 38 and $231 \pm 7 \text{ nmol GSH per mg P}$, respectively), as well as in animals treated with *G. resinaceum* EtOH extract (alloxan-pretreated) ($213 \pm 10 \text{ nmol GSH per mg P}$). Related to GSH content, increased GSHR and GSHPx activities were confirmed only in animals treated with *G. pfeifferi* EtOH extract (alloxan-pretreated) (6.25 ± 0.03 and $6.21 \pm 0.01 \text{ nmol per mg per min P}$, respectively) (Table 2). Chemical compounds with $-\text{SH}$ groups could express antioxidative activity in lower concentrations, while in higher concentrations predominant activity could be prooxidant.^{1,2} Reduction of alloxan to dialuric acid takes over the thiol, most often GSH which is oxidized, and final product is alloxan–GSH.³³ Administration of three extracts in

Table 1 Antioxidant activity, total proteins, total phenols and total flavonoids content and correlations between chemical constituents and antioxidant activities of EtOH and water extracts of *G. pfeifferi* and *G. resinaceum*^a

Assay	<i>G. pfeifferi</i>		<i>G. resinaceum</i>		PG
	EtOH	H ₂ O	EtOH	H ₂ O	
DPPH ($\mu\text{g mL}^{-1}$)	18.9 \pm 0.4 ^c	36.1 \pm 0.9 ^c	22 \pm 1 ^d	14.0 \pm 0.6 ^b	0.39 \pm 0.01 ^a
SOA ($\mu\text{g mL}^{-1}$)	91.9 \pm 0.2 ^c	65.7 \pm 0.1 ^d	62.7 \pm 0.4 ^c	30.0 \pm 0.2 ^b	10.0 \pm 0.5 ^a
NO ($\mu\text{g mL}^{-1}$)	370 \pm 1 ^d	273.8 \pm 0.4 ^c	215.2 \pm 0.8 ^b	830.8 \pm 0.3 ^c	6.1 \pm 0.8 ^a
FRAP (mg AAE per g d.w.)	151.5 \pm 0.2 ^b	65 \pm 1 ^d	80.2 \pm 0.5 ^c	297 \pm 1 ^a	n.a.
TPR (mg BSAE per mL)	11.6 \pm 0.3 ^a	2.2 \pm 0.3 ^d	7.2 \pm 0.7 ^b	3.4 \pm 0.2 ^c	—
TP (mg GAE per g d.w.)	43.7 \pm 0.6 ^a	23.1 \pm 0.9 ^b	44.0 \pm 0.2 ^a	11.4 \pm 0.4 ^c	—
TF (mg QE per g d.w.)	26.3 \pm 0.7 ^a	10.1 \pm 0.8 ^c	22.9 \pm 0.2 ^b	4.4 \pm 0.9 ^d	—
Correlation coefficient – R ² *					
DPPH	TPR	0.99*	0.47	0.97*	0.99*
	TP	0.72*	0.03	0.93*	0.40
	TF	0.83*	0.01	0.96*	0.04
SOA	TPR	0.72*	0.04	0.99*	0.99*
	TP	0.99*	0.51*	0.98*	0.54*
	TF	0.40	0.60*	0.99*	0.11
NO	TPR	0.96*	0.51*	0.97*	0.99*
	TP	0.44	0.97*	0.94*	0.54*
	TF	0.98*	0.99*	0.97*	0.11
FRAP	TPR	0.99*	0.86*	0.99*	0.99*
	TP	0.59*	0.96*	0.99*	0.38
	TF	0.92*	0.91*	0.99*	0.03

^a TPR, total protein content; TP, total phenol content; TF, total flavonoid content; EtOH, ethanolic extract; H₂O, water extract; n.a., non active. Data are reported as mean \pm standard deviation of triplicates. ^{a,b,c,d,e} – different letters in the same row indicate significant difference between extracts ($p < 0.01$). R²* – all values are statistically significant ($p < 0.05$).

animals pre-treated with alloxan (EtOH and water of *G. pfeifferi* and EtOH of *G. resinaceum*) caused statistically significant changes of LPx and GSH. This result certainly indicates that there exist some protective effects of these fungal species of the genus *Ganoderma* and supports the above-mentioned mechanism (Tables 2 and 3).

Treatment with all *G. pfeifferi* extracts (except water with alloxan-pre-treatment) produced increase in Px activity, which was not statistically significant (Table 2). Based on data showed in Table 3, treatment with *G. resinaceum* extracts in animals pre-treated with alloxan resulted in reduced Px activity, which may be result of the fact that phenolic compounds as hydrogen donors in Px-catalysed reaction were not present in a sufficient concentration or do not contribute to increase in Px activity.³⁴

This is in accordance with determined *in vitro* antioxidant potential in this study. *G. resinaceum* extracts expressed better *in vitro* antioxidant potential with good correlation between this potential and total phenol and total protein content, indicating a synergistic effect on antioxidant activity which is probably not based only on the presence of phenolic compounds but also on the presence of proteins.

Administration of both *G. pfeifferi* extracts caused a decrease in the values of XOD activity in experimental animals (47 \pm 1 for EtOH and 45.2 \pm 0.4 nmol per mg per min P for water, respectively) compared to control sample (55 \pm 2 nmol per mg per min P). Based on published data, flavonoids are considered as significant inhibitors or activators of certain enzymes from the group of oxidoreductase, which is in accordance with results

Table 2 *In vivo* antioxidant activity of *G. pfeifferi* extracts in liver homogenate of experimental animals

Parameter	Control	<i>G. pfeifferi</i> EtOH	<i>G. pfeifferi</i> H ₂ O	Alloxan + saline	Alloxan + saline + <i>G. pfeifferi</i> EtOH	Alloxan + saline + <i>G. pfeifferi</i> H ₂ O
LPx	10.8 \pm 0.7 ^b	11.0 \pm 0.5 ^b	8.7 \pm 0.2 ^a	9.69 \pm 0.01 ^a	7.4 \pm 0.1 ^a	6.3 \pm 0.6 ^a
GSH	124 \pm 10 ^b	285 \pm 38 ^a	231 \pm 7 ^a	99.3 \pm 0.4 ^c	104 \pm 7 ^c	133 \pm 18 ^b
GSHR	6.21 \pm 0.01 ^b	3.3 \pm 0.2 ^d	4.2 \pm 0.2 ^c	6.7 \pm 0.1 ^a	6.25 \pm 0.03 ^b	4.2 \pm 0.4 ^c
GSHPx	21 \pm 1 ^a	16 \pm 2 ^b	14.8 \pm 0.6 ^b	14.9 \pm 0.6 ^b	17 \pm 1 ^b	14 \pm 3 ^b
Px	11.7 \pm 0.1 ^c	15 \pm 1 ^a	13.0 \pm 0.1 ^b	8.1 \pm 0.8 ^d	10 \pm 1 ^c	6.6 \pm 0.1 ^e
CAT	61 \pm 10 ^d	108 \pm 12 ^b	133 \pm 4 ^a	97 \pm 2 ^c	89 \pm 7 ^c	109 \pm 3 ^b
XOD	55 \pm 2 ^c	47 \pm 1 ^d	45.2 \pm 0.4 ^d	66 \pm 2 ^a	69 \pm 1 ^a	58.7 \pm 0.4 ^b

Table 3 *In vivo* antioxidant activity of *G. resinaceum* extracts in liver homogenate of experimental animals^a

Parameter	Control	<i>G. resinaceum</i> EtOH	<i>G. resinaceum</i> H ₂ O	Alloxan + saline	Alloxan + saline + <i>G. resinaceum</i> EtOH	Alloxan + saline + <i>G. resinaceum</i> H ₂ O
LPx	10.8 ± 0.7 ^b	9.9 ± 0.2 ^b	10.1 ± 0.3 ^b	9.69 ± 0.01 ^a	10.5 ± 0.4 ^b	9.6 ± 0.3 ^a
GSH	124 ± 10 ^c	166 ± 13 ^b	153 ± 19 ^b	99.3 ± 0.4 ^d	213 ± 10 ^a	171 ± 1 ^b
GSHR	6.21 ± 0.01 ^a	2.3 ± 0.3 ^c	1.2 ± 0.1 ^d	6.7 ± 0.1 ^a	5.3 ± 0.6 ^b	5.04 ± 0.04 ^b
GSHPx	21 ± 1 ^a	14 ± 1 ^b	15 ± 1 ^b	14.9 ± 0.6 ^b	13.2 ± 0.8 ^c	15.2 ± 0.8 ^b
Px	11.7 ± 0.1 ^a	8.3 ± 0.3 ^b	8 ± 1 ^b	8.1 ± 0.8 ^b	8 ± 2 ^b	7.9 ± 0.9 ^b
CAT	61 ± 10 ^d	58 ± 4 ^c	65 ± 2 ^d	97 ± 2 ^b	110 ± 8 ^a	86 ± 3 ^c
XOD	54.6 ± 2 ^c	77 ± 1 ^a	65 ± 5 ^b	66 ± 2 ^b	63 ± 2 ^b	73 ± 2 ^a

^a EtOH, ethanolic extracts; H₂O, water extracts. GSH content is expressed as nmol GSH per mg of proteins. GSHR, GSHPx, Px, CAT and XOD activity are expressed in nmol per mg per min of proteins. Intensity of lipid peroxidation is expressed in nmol malondialdehyde per mg of proteins. Results are expressed as means ± SD. ^{a,b,c,d,e} different letters in the same column indicate significant difference between analyzed samples (Tukey's test, $p < 0.01$).

of our study, since higher total flavonoid content was noticed in *G. pfeifferi* extracts (Table 1). Treatment with alloxan and analyzed extracts (Tables 2 and 3) increased the activity of XOD compared to control group, which is characteristic of liver damage in diabetes and points to the existence of oxidative stress.³⁵

Values of CAT showed an insignificant decrease of activity in animals treated only with *G. resinaceum* EtOH extracts (58 ± 4 nmol per mg per min P) compared to control group (61 ± 10 nmol per mg per min P), while in other groups treated with *G. resinaceum* EtOH extracts, the significant increase of activity was detected (Table 3). Treatment with *G. pfeifferi* extracts resulted in statistically significant increase of CAT activity (Table 2). Increase in CAT activity in groups treated with extracts of both species, may be the result of the stronger activity of Px enzyme.²⁹

3.2. Chemical composition of analysed fungal species

Total protein content in analysed extracts ranged from 2.2 to 11.6 mg BSAE per mL (Table 1) with EtOH extracts of both species being the richest. To the best of our knowledge these results on total protein content of the analysed fungal species

are preliminary, while total protein content for the same extracts of *G. applanatum* and *G. lucidum* (39 and 27 mg BSAE per mL, respectively)¹⁵ was higher compared to obtained results (Table 1). Still, high correlation between total protein content and the antioxidant potential (Table 1) suggests sufficient antioxidant potential of protein-polysaccharide complexes in examined extracts, previously detected.¹

Similar to total protein, higher total phenol and total flavonoid contents were also detected in EtOH extracts for both species (Table 1) which is in accordance with previous results for other *Ganoderma* species.¹⁶ Compared to our study, total phenol content in MeOH and water extracts of *G. resinaceum* from Turkey¹⁴ was 37.32 and 36.39 mg GAE per g d.w., is higher only regarding water extract of the same species (11.4 ± 0.4 mg GAE per g d.w.). High correlations between total phenol and total flavonoid content and antioxidative activity (Table 1) for both *G. resinaceum* extracts might suggest that phenolic compounds play important role in the expressed antioxidative potential.^{14,16} Although similar in total phenol and total flavonoid contents, antioxidative activity of *G. pfeifferi* was weaker than for *G. resinaceum* (Table 1). Overall, total phenol and total

Table 4 LC-MS/MS detection of phenolic compounds in examined extracts^a

Phenolic compound	Fungal species and extract type			
	<i>G. pfeifferi</i> EtOH	<i>G. pfeifferi</i> H ₂ O	<i>G. resinaceum</i> EtOH	<i>G. resinaceum</i> H ₂ O
<i>p</i> -Hydroxybenzoic acid	23.00	5.10	12.20	<0.30 ^b
Protocatechuic acid	6.50	6.20	4.01	2.65
<i>p</i> -Coumaric acid	1.50	1.00	0.80	0.60
Vanillic acid	6.50	4.50	<4.00 ^b	<4.00 ^b
Gallic acid	30.50	1.50	15.85	1.20
Caffeic acid	0.80	0.60	0.40	0.25
Quinic acid	8.51	6.35	6.90	3.00
Chlorogenic acid	1.26	0.80	<0.30 ^b	<0.30 ^b

^a EtOH, ethanolic extract; H₂O, water extract. Bold number: amount of qualified phenolic compounds in examined extracts. ^b Number: detected compound – peak observed, concentration is lower than the LoQ (limit of quantification), but higher than LoD (limit of detection).

Table 5 Glycemia values in animals with alloxan-induced diabetes^a

Experimental group	Glycaemia before alloxan administration (mmol L ⁻¹)	Glycaemia 48 h after alloxan administration (mmol L ⁻¹)	Glycaemia on the last day of treatment (mmol L ⁻¹)
Alloxan + saline	6.8 ± 0.6	33.1 ± 0.2	32.8 ± 0.4
Alloxan + <i>G. pfeifferi</i> , EtOH	7.1 ± 0.5	33 ± 2	31 ± 2
Alloxan + <i>G. pfeifferi</i> , H ₂ O	7.2 ± 0.5	32 ± 3	31 ± 2
Alloxan + <i>G. resinaceum</i> , EtOH	7.1 ± 0.4	32 ± 2	28 ± 2
Alloxan + <i>G. resinaceum</i> , H ₂ O	7.0 ± 0.4	31 ± 2	27.3 ± 0.3 ^b

^a EtOH, ethanolic extract; H₂O, water extract. ^b $p < 0.05$ statistically significant results vs. control group (alloxan + saline).

flavonoid contents were higher in EtOH extracts most probably due to the polarity of the extraction solvent which strongly affects the level of phenolic compounds.¹⁶

High correlation between both total protein and total phenol contents and antioxidative potentials points out the role of these bioactive compounds in analysed extracts. Best to our knowledge, antioxidative potential of *G. pfeifferi* extracts was detected for the first time in this research, and these results indicate the importance of this poorly researched species.

Considering the antioxidant activity as a function of the phenolic constituents, beside total phenol content, we have also quantified eight phenolic compounds using LC-MS/MS technique, while concentration of other detected compounds were under the limits of quantification (LOQ) of used technique (Table 4). Only, *G. pfeifferi* EtOH contained all detected phenolic compounds and the highest content (Table 4). *p*-Hydroxybenzoic and gallic acids represented the major compounds in EtOH extracts of both examined fungal species. Among the examined extracts, the highest level of *p*-hydroxybenzoic and gallic acid was determined in *G. pfeifferi* EtOH (23.00 and 30.50 µg per g d.w., respectively). In examined extracts, *p*-coumaric and quinic acid were determined in all examined extracts in a similar concentration range (0.25–1.5 µg per g d.w.). With respect to *G. resinaceum* extracts, the highest concentration was noticed for gallic acid (15.85 µg per g d.w.) which is preliminary determined in this species as well as quinic and *p*-coumaric acid.¹⁴ Other quantified compounds in *G. resinaceum* were noticed at lower concentration in relation to literature data.¹⁴

Determined phenolic acids in this study, with the highest concentration of *p*-hydroxybenzoic and gallic acid agrees with

the fact that the most common fungal benzoic acid derivatives with antioxidant potential are *p*-hydroxybenzoic, protocatechuic, gallic, vanillic, syringic acid *etc.*²

Finally, this study reported LC-MS/MS phenolic profile of *G. pfeifferi* and EtOH extract of *G. resinaceum* for the first time.

3.3. Pharmacodynamic activity

3.3.1. Changes in rat body weight. The changes in rat body weight (BW) following administration of *G. pfeifferi* and *G. resinaceum* extracts at daily doses of 1 mL kg⁻¹ *per os* were compared with the corresponding values in control animals treated with physiological solution. After the 5 day treatment period in most of the normoglycaemic animals treated with saline, EtOH and water extracts of analysed fungal species statistically significant increase in BW was detected, except for the treatment with *G. pfeifferi* water extract (1.2 ± 0.1 g) (ESI data 1a†). Decrease in BW was detected in all animal groups with alloxan-induced diabetes but without statistical significance (ESI data 1b†).

3.3.2. Antidiabetic activity. Best to our knowledge, there is no available published data on *G. pfeifferi* and *G. resinaceum* antidiabetic activity. The control value of glycaemia in all experimental groups was around 7 mmol L⁻¹ before alloxan administration, and above 30 mmol L⁻¹ 48 h after alloxan administration. Five days oral treatment with fungal extracts resulted in slight decrease in glycaemia values, while statistically significant effect was detected only in animals treated with *G. resinaceum* water extract (27.3 ± 0.3 mmol L⁻¹, compared to the control group 32.8 ± 0.4 mmol L⁻¹; Table 5). Glycaemia values obtained in OGTT are shown in Table 6. Treatment with

Table 6 Glycemia values in normoglycemic animals after OGTT (oral glucose tolerance test)^a

Experimental group	Glycaemia before treatment (mmol L ⁻¹)	Glycaemia before glucose administration (mmol L ⁻¹)	Glycaemia 30 min after glucose administration (mmol L ⁻¹)
Control	6.7 ± 0.4	6.6 ± 0.6	9.6 ± 0.7
<i>G. pfeifferi</i> , EtOH	7.1 ± 0.6	7.1 ± 0.5	10.3 ± 0.7
<i>G. pfeifferi</i> , H ₂ O	7.1 ± 0.5	7.0 ± 0.6	10.9 ± 0.9 ^b
<i>G. resinaceum</i> , EtOH	6.9 ± 0.4	7.1 ± 0.4	8.7 ± 0.3
<i>G. resinaceum</i> , H ₂ O	6.8 ± 0.5	7.3 ± 0.5	9.1 ± 0.6

^a EtOH, ethanolic extract; H₂O, water extract. ^b $p < 0.05$ statistically significant results vs. control group (saline).

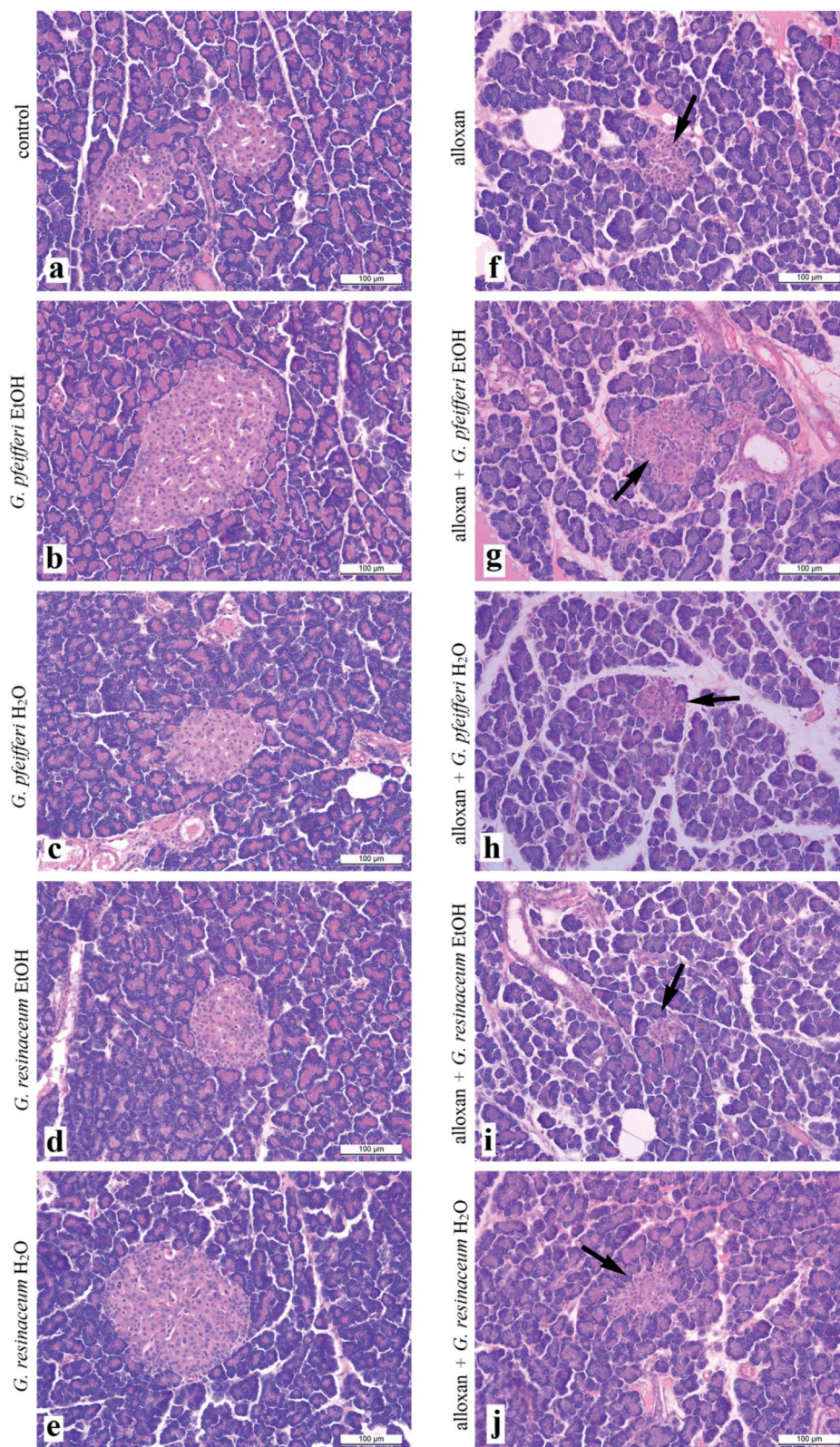


Fig. 1 Histological cross section of pancreas treated with EtOH and H₂O extracts of *G. pfeifferi* and *G. resinaceum* (arrow points to Langerhans islet).

both *G. resinaceum* extracts prevented significant increase in glycaemia values (6.9 ± 0.4 vs. 8.7 ± 0.3 for EtOH; 6.8 ± 0.5 vs. 9.1 ± 0.6 mmol L⁻¹ for water). Similar result was obtained for *G.*

pfeifferi EtOH extract (7.1 ± 0.6 vs. 10.3 ± 0.7 mmol L⁻¹) while water extract did not prevent significant increase in glycaemia (7.1 ± 0.5 vs. 10.9 ± 0.9 mmol L⁻¹).

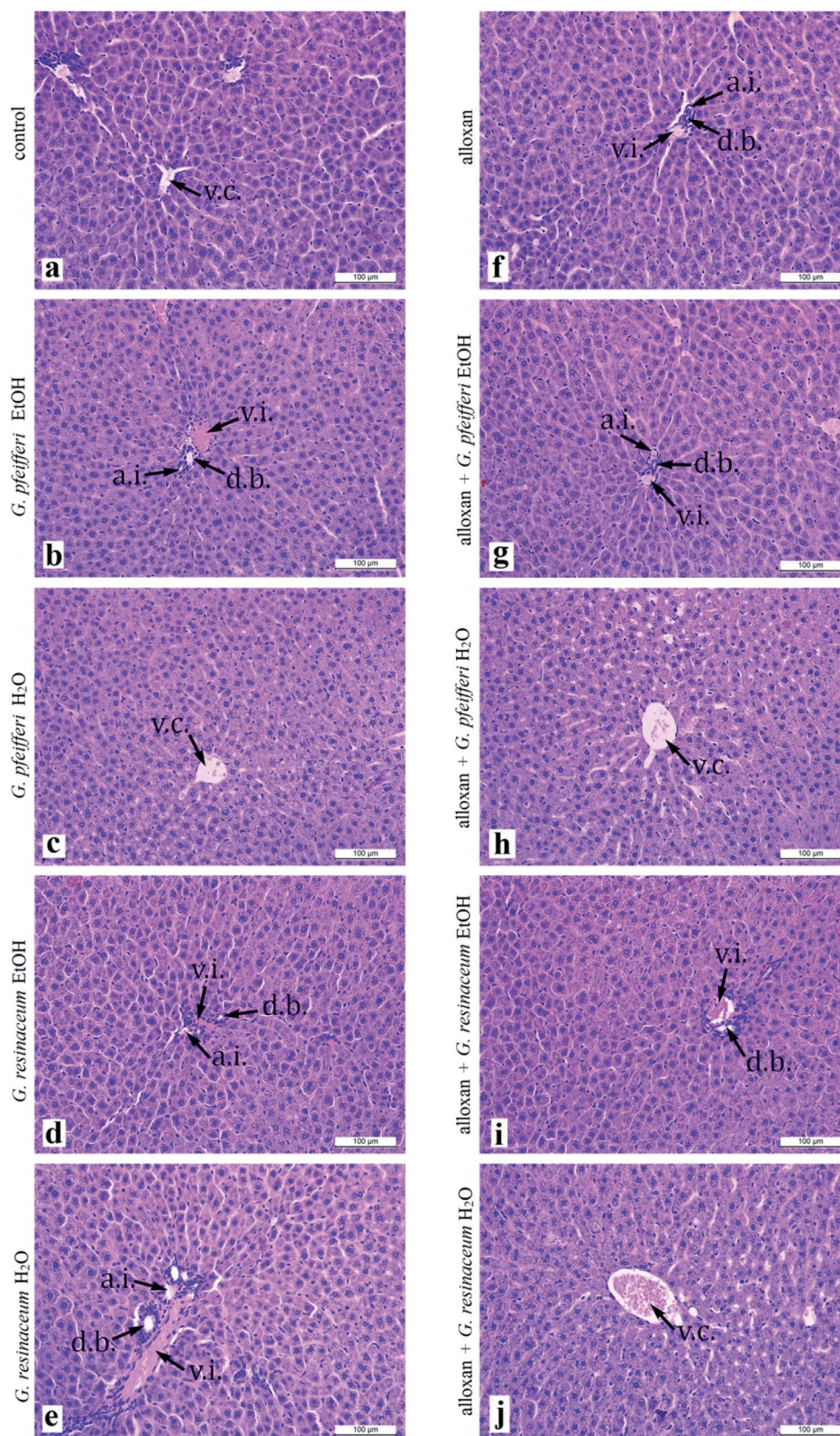


Fig. 2 Histological cross section of liver treated with EtOH and H₂O extracts of *G. pfeifferi* and *G. resinaceum* (a.i. – arteria interlobularis; d.b. – ductus biliferi; v.c. – vena centralis; v.i. – vena interlobularis).

A possible explanation of antihyperglycemic effect of examined extracts could be the activity of polysaccharides (glucans – ganoderan A and B),¹⁰ that have been recently proven to express

the antidiabetic effect in animals treated with *G. lucidum*²² which has resemblance to examined *G. resinaceum*. This effect may suggest that polysaccharides from water extract cannot

stimulate the insulin synthesis but could stimulate the insulin release from the preserved pancreatic islets directly.¹⁰ De Silva *et al.*¹⁷ explain hypoglycaemic effects of *G. lucidum* polysaccharides through the release of Ca^{2+} ions into β -pancreatic cells, which leads to the release of insulin.¹⁰ Other studies suggest that triterpenes (ganoderic acid B) could be acting as antidiabetic agents *via* inhibition of α -glucosidase.^{12,22}

Based on literature data,³⁶ phenolic compounds may affect carbohydrate metabolism at various levels: improving post-prandial and fasting blood glucose levels, insulin secretion, insulin sensitivity, helping on DM prevention and limiting the rate of glucose absorption from the intestines into the bloodstream. Among quantified phenolics of analysed extracts, gallic acid which has not yet been determined in these fungal species, is outspread by content in both EtOH extracts (Table 4). Therapeutic efficacy of this phenolic may lead to an improvement in insulin-dependent glucose transport in tissue through the translocation under signalling pathways.³⁶ The results presented in OGTT (Table 6) suggest that, beside antioxidant activity, gallic acid may be one of the compounds responsible for glycaemia reduction in 5 day treatment with fungal extracts.

3.3.3. Morphological analysis. Histological analysis of the pancreas cross-sections by light microscopy showed the presence of clearly visible undamaged Langerhans islets in the samples taken from the control groups (Fig. 1a–e). In the control groups treated with physiological solution and fungal extracts, Langerhans islets have normal characteristics constructed of centrally positioned acidified β -cells of spaced-apart capillaries and in the periphery, positioned α -cells which are characterized by lighter cytoplasm. In the alloxan-treated experimental animals, damaged Langerhans islets can be clearly seen: (Fig. 1f–j). Protective role of fungal extracts was not evident only with the exception of *G. pfeifferi* EtOH extracts, where an increase in number of slightly larger Langerhans islets with a noticeably lower density of β -cells was noticed (Fig. 1j). However, it might be supposed that the administered dose of alloxan was too high, so the protective effect of analysed fungal extracts on pancreatic tissue was not detected³⁷ or second explanation was determination of the highest total phenol content in EtOH extracts of *G. pfeifferi* which may have an effect on diabetes mellitus type 2.³⁸ Liu *et al.*³⁹ reported lymphocyte proliferation due to polysaccharides in *G. lucidum*. At this moment we can only presume that the regenerative effect on pancreas cells might be also modulated by polysaccharides present in *G. pfeifferi* EtOH extract, although this effect should be further examined.

Analysis of liver histology sections (Fig. 2a–j) in both experimental groups, control and alloxan-pre-treated did not show any pathohistological changes in liver tissue, even though the applied doses of alloxan were sufficient for diabetes induction. The hepatocytes characterised usual shape, with centrally placed nucleus, and the light, highly granulated acidophilic cytoplasm. They were arranged in linear cords of cells which radiate from the central vein to the periphery of the classic liver lobule. In each of the lobes corners, we easily identified branches of the hepatic artery proper, hepatic portal vein and bile ducts, which represent components of portal triad. These

results suggest that 5 day treatment with *G. pfeifferi* and *G. resinaceum* extracts after alloxan administration resulted in certain hepatoprotective effects. Related to hypoglycaemic activity, hepatoprotective ability of these samples to protect β -cells from the diabetogenic action of alloxan, possibly by the presence of antioxidant substances such as gallic acid.⁴⁰

4. Conclusion

This study revealed for the first-time antidiabetic activity *via* alloxan-induced diabetes of examined *Ganoderma* species. Neither antidiabetic activity of *G. pfeifferi* and *G. resinaceum* nor the antioxidant potential of *G. pfeifferi* have been reported before. Water extracts of *G. resinaceum* showed the highest effect as free radical scavengers of DPPH[•] and $\text{O}_2^{\cdot-}$ as well as on FRAP assay, except EtOH extract which showed the most powerful activity in NO assay. Five days treatment with both *G. pfeifferi* extracts had protective effects on liver biochemical parameters (GSH, LPx, GSHPx, GSHR, CAT and XOD) while *G. resinaceum* extracts had the most powerful reduction of the lipid peroxidation intensity suggesting protective role in oxidative stress. Based on correlation and antioxidant potential it was clearly indicated that the presence of phenolic compounds such as gallic acid (quantified in the highest concentration in *G. pfeifferi* EtOH extract), which is characterised as effective antioxidants due to their existence of hydroxyl groups.

In animals treated with *G. pfeifferi* EtOH extract, after alloxan pre-treatment, pancreas cross-sections revealed increase in a number of slightly larger Langerhans islets with a lower density of β -cells, which was not observed in the cross-sections from control animals. Further studies on different animal and human models are essential to verify the beneficial effects of fungal species including more studies for the isolation and characterization of the triterpenes, polysaccharides or individual phenolic acids as active principles responsible for these activities.

Conflicts of interest

Authors declared that there are no conflict of interest.

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