Bioactivity profiling of *Sanghuangporus lonicerinus*: antioxidant, hypoglycaemic, and anticancer potential via *in-vitro* and *in-silico* approaches

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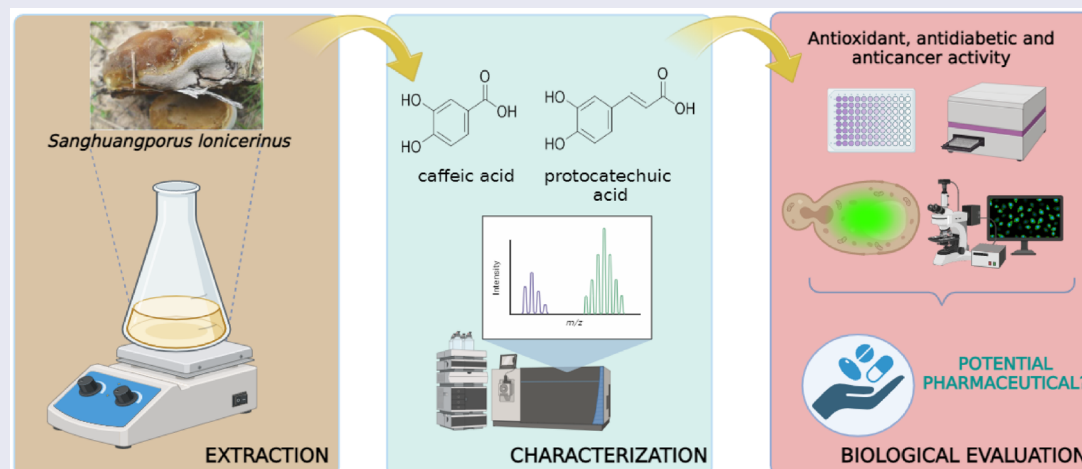
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ABSTRACT

This study investigates the mycochemical profile and biological activities of hydroethanolic (EtOH), chloroform (CHCl$_3$), and hot water (H$_2$O) extracts of *Sanghuangporus lonicerinus* from Uzbekistan. Antioxidant capacity was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), NO, and FRAP assays, and *in vitro* hypoglycaemic effects were evaluated through α-amylase and α-glucosidase inhibition. Antiproliferative potential was explored by analysing the binding affinities of EtOH and H$_2$O extracts to estrogen receptor α (ERα), ERβ, androgen receptor (AR), and glucocorticoid receptor (GR), with molecular docking providing structural insights. LC-MS/MS analysis revealed solvent-dependent phenolic profiles, with the EtOH extract containing the highest total phenolic content (143.15 ± 6.70 mg GAE/g d.w.) and the best antioxidant capacity. The EtOH extract showed significant hypoglycaemic effects, with 85.29 ± 5.58% inhibition of α-glucosidase and 41.21 ± 0.79% inhibition of α-amylase. Moderate ERβ binding suggests potential for estrogen-mediated cancer therapy, while strong AKR1C3 inhibition by the EtOH extract supports its therapeutic potential.

GRAPHICAL ABSTRACT

Sanghuangporus lonicerinus

caffeic acid   protocatechuic acid

Intensity / m/z

EXTRACTION   CHARACTERIZATION

Antioxidant, antidiabetic and anticancer activity

POTENTIAL PHARMACEUTICAL?

BIOLOGICAL EVALUATION

Introduction

Mushrooms are a significant source of essential nutrients, including carbohydrates, proteins, vitamins, and minerals[1–3]. Due to their bioactive compounds, certain mushrooms exhibit notable medicinal properties, such as anti-inflammatory, antimicrobial, anti-obesity, antitumor, and antiviral activities[2–10]. Additionally, several medicinal mushrooms have been documented in Uzbekistan, including novel fungi discovered in the study area that are new to science[11–15]. However, these fungi have not been thoroughly scientifically evaluated for their potential in ethnomycology, ethnomedicine and mycochemistry, or as sources of biological actives and bioactive compounds as a member of the Hymenochaetoid taxa[8].

Sanghuangporus species, previously classified as *Phellinus* or *Inonotus*[16,17], represent one of the most important groups in the family Hymenochaetaceae. They are highly valued for their medicinal properties and have been traditionally used in ethnomedicine across East and Southeast Asia[8,18,19]. In contemporary pharmaceutical research, *Sanghuangporus* spp. have demonstrated a range of beneficial functions. These include enhancing blood circulation, scavenging free radicals, exerting anti-inflammatory effects, combating carcinogenesis, exhibiting antioxidative properties, modulating the immune system, exerting antidiabetic effects, demonstrating antimicrobial activity, and offering anti-aging benefits[20,21]. The genus *Sanghuangporus* was formally established in 2016[22]. To date, over 14 described species of *Sanghuangporus* have been identified globally, with the majority found in Asia[12,23]. The medicinal macrofungus *Sanghuangporus lonicerinus* was first described in 1953 by Soviet mycologist Bondartsev under the name *Phellinus lonicerinus* (Bondartsev) Bondartsev & Singer. It was discovered on *Lonicera* trees in the Samarkand region of Uzbekistan[24]. However, this species has often been confused with the American *Tropicoporus linteus*[25,26], which has not been found in Eurasia. Similar species restricted to *Lonicera* trees include *S. ligneus* from Iran[27] and *S. lonicericola* from East Asia[22,28]. *S. lonicerinus* is a member of the cosmopolitan family Hymenochaetaceae[8,18,22]. This genus, including *S. lonicerinus*, represents one of the most important groups of medicinal macrofungi and has been utilised in traditional Chinese medicine for over two centuries. In addition, many medicinal macrofungi, including species of *Sanghuangporus*, continue to be of significant interest for their medicinal properties and their traditional uses in ethnomedicine across East and Southeast Asia[19].

S. lonicerinus, also known as the "Reishi of the East", is a medicinal mushroom that has been used in traditional Chinese medicine for over two centuries[8]. Its widespread use in the Hubei province of China and various other regions, particularly among the Tujia ethnic minority, has been well documented[29]. Recent pharmacological studies have shown that *S. lonicerinus* exhibits diverse biological effects[18]. These include antiproliferative properties, as demonstrated by Wang et al.[30–32], cytotoxic activity, as reported by Zhang et al.[29], and both estrogenic and anti-estrogenic effects, as seen by Hu et al.[33]. Additionally, hepatoprotective effects have been observed[34]. However, there is limited scientific literature on *S. lonicerinus*, and its mycochemical composition remains poorly characterised. To date, few compounds have been identified, including polyphenols (hispolon, vanillin, and 3,4-dihydroxybenzalacetone), a fatty acid (docosanoic acid), and sterols (ergosta-6,22-dien-3β,5β,8β-triol, ergosterol, and β-sitosterol)[30–32]. Among these, hispolon, a bioactive styrylpyrone polyphenolic compound, has garnered significant attention for its therapeutic potential, particularly in the treatment of cancer and neurodegenerative disorders[16,18,35].

Despite its historical use and potential, research on *S. lonicerinus* has been slow, hindered by classification problems and a lack of systematic studies[20]. Oxidative stress, characterised by an imbalance between reactive oxygen species (ROS) production and antioxidant defences, is a major contributor to the pathogenesis of various diseases, such as diabetes and cancer. In diabetes, high glucose levels increase ROS production, causing oxidative damage to pancreatic β-cells and impairing insulin signalling[36]. In cancer, ROS-mediated DNA damage, lipid peroxidation, and protein oxidation drive tumour initiation, progression, and metastasis[37,38]. The potential of natural compounds to combat oxidative stress has garnered significant interest[38]. Extracts of *S. lonicerinus*, rich in phenolic content, exhibit potent antioxidant properties, offering promise as preventive agents against diabetes and cancer. By scavenging ROS and modulating oxidative stress pathways, these extracts may help mitigate disease progression and provide a natural, effective approach to disease management. Hormone-dependent cancers, such as breast and prostate cancer, contribute to a large number of cancer-related deaths globally[39,40]. Since their growth is stimulated by specific steroid hormones, blocking the synthesis and action of these hormones represents a promising treatment strategy. Despite significant advancements in endocrine therapies, there remains a need for the development of more selective and effective modulators targeting steroid receptors and steroid-transforming enzymes. To reduce side effects associated with synthetic anticancer drugs, natural products, such as those derived from mushrooms, are being explored for their potential in cancer treatment[41]. Additionally, mushrooms are increasingly recognised for their importance in this field[30,42]. Based on available literature, hispolon, a polyphenol from *S. lonicerinus*, has demonstrated promising anticancer properties by targeting various signalling pathways; and has exhibited antiproliferative activity against various breast and prostate cancer cell lines[35].

Through this multifaceted approach, we aim to highlight the significance of conducting mycochemical studies on the neglected macrofungal species of Uzbekistan, showcasing their potential applications in medicinal and therapeutic contexts. Green chemistry (GC) and green analytical chemistry (GAC) have become critical considerations in modern scientific research, ensuring the environmental impact of chemical products and processes is minimised[43]. The concept of GC emerged prominently in the 1990s, with GAC gaining increasing importance in analytical chemistry as standardised tools for evaluating "green profiles" became essential. Notable methods include the Analytical GREEnness Metric approach (AGREE), AGREEprep, and the Green Analytical Procedure Index (GAPI), which provide frameworks for assessing the greenness of methodologies. Recent advancements, such as the modified GAPI (MoGAPI), enhance this evaluation, allowing for detailed comparisons between methods using software tools that combine metrics like the analytical Eco-Scale with GAPI[44]. Such tools are vital for ensuring that analytical procedures, such as those used in this study, meet both scientific and environmental standards[43,44].

In this study, *S. lonicerinus* fruiting bodies were sectioned, air-dried, and stored until extraction. Following established protocols, hot water extracts (H_2O) were prepared by boiling dried material, filtering, and lyophilising. Hydroethanolic (EtOH) and chloroform ($CHCl_3$) extracts were obtained by macerating samples in 70% ethanol (EtOH) or $CHCl_3$, followed by filtration and evaporation under reduced pressure. Dried residues were dissolved in dimethyl sulphoxide (DMSO) for storage and further analysis. These methods align with GC principles, balancing environmental impact with analytical efficiency as suggested by Locatelli et al.[43] and Mansour et al.[44].

In this study, we conducted LC-MS/MS analysis to determine the mycochemical profile of *S. lonicerinus*. The antioxidant capacity

of the extracts was assessed using *in vitro* assays, including anti-2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-3-e thylbenzothiazoline-6-sulfonic acid (ABTS), NO radicals, and FRAP. Additionally, enzyme inhibitory effects were evaluated for their hypoglycaemic potential.

We also investigated the relative binding affinities of EtOH and H_2O extracts of *S. lonicerinus* for the ligand-binding domains (LBDs) of estrogen receptor α (ERα), estrogen receptor β (ERβ), androgen receptor (AR), and glucocorticoid receptor (GR) using a fluorescent yeast screen *in vitro*. The potential of *S. lonicerinus* extracts to inhibit specific isoforms of human aldo-keto reductase 1C, which are therapeutic targets for hormone-dependent diseases, was investigated using fluorescence spectroscopy. Lastly, to establish a structural basis for the experimental results and predict the potential binding mechanisms of the quantified polyphenolics with AKR1C3, we performed molecular docking simulations *in silico*.

Material and methods

Sample collection and specimen examination

In June 2021, *S. lonicerinus* specimens were collected from mixed broadleaf wild fruit forests in the Western Tien-Shan and northern slopes of the Alay range, Pamir-Alay Mountains, Uzbekistan. The samples were transported to the Mycology Laboratory at the Institute of Botany and stored for further analysis. Macroscopic features were examined using a Leica M165 FC stereomicroscope, while microscopic observations were performed on dried samples with a Leica DM 1000 microscope (up to 1000× magnification). The identification of *S. lonicerinus* was confirmed by Yusufjon Gafforov from the Institute of Botany, Uzbekistan Academy of Sciences, and a voucher specimen (TASM6174) was deposited at the Tashkent Mycological Herbarium (TASM), Uzbekistan Academy of Sciences.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from fruiting body tissue using the BioSprint 96 DNA Plant Kit (QIAGEN, Germany) with the KingFisher Flex system (Thermo Fisher Scientific, Waltham, MA). PCR amplification targeted the ITS, LSU, and *TEF1* regions, using primers ITS1F/ITS4B, ITS1/ITS4[45,46], LR/LROR[47], and EF-983F/EF-1567R[48]. Amplicons were sequenced at the Senckenberg Biodiversity and Climate Research Centre, Goethe University, and the Swedish University of Agricultural Sciences, using the same primers. The assembled sequences were deposited in NCBI GenBank (Supplementary Table 1).

To confirm the identification of the medicinal *S. lonicerinus* from Uzbekistan, sequences for the ITS, LSU, and *TEF1* regions were obtained and combined with additional sequences sourced from GenBank to build a dataset (Supplementary Table 1). *Tropicoporus dependens* (Murrill) L.W. Zhou, Y.C. Dai & Vlasák and *T. guanacastensis* L.W. Zhou, Y.C. Dai & Vlasák, both from the Hymenochaetaceae, were used as outgroup taxa. The ITS, LSU, and *TEF1* regions were aligned using MAFFT version 7, applying the 'G-INS-i' algorithm[49].

Phylogenetic study

Phylogenetic analysis was conducted in PAUP* version 4.0b10[50], applying the heuristic search method with TBR branch swapping, 1000 random sequence additions, and setting max-trees to 5000. Zero-length branches were collapsed, and all parsimonious trees were retained. Clade stability was evaluated using 1000 bootstrap replicates[51]. Phylogenetic statistics such as tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were calculated for each maximum parsimonious tree (MPT) produced, and phylogenetic trees were visualised with Treeview[52]. MrModeltest 2.3[53] was employed to select the most suitable evolutionary model for the Bayesian inference (BI) analysis. BI was executed with MrBayes3.1.2[54] using a general time reversible (GTR) model with a gamma distribution and invariable sites. Four Markov chains were run for 2 million generations, with sampling every 100th generation until the split frequency value was <0.01. The first 25% of the trees were discarded as burn-in, and a majority-rule consensus tree was constructed. Treeview[52] was used to visualise the phylogenetic trees, and branch support for maximum likelihood (BS), maximum parsimony (MP), and Bayesian posterior probabilities (BPP) was indicated for values of ≥75% (MP) and 0.95 (BPP).

Preparation of the extracts for mycochemical study and tested biological activities

Fruiting bodies of *S. lonicerinus* were first divided into sections, air-dried, and stored in opaque containers at room temperature until further processing. All three extracts were prepared following the procedure outlined by Karaman et al.[55] and Rašeta et al.[56].

Hot water extracts (H_2O) were prepared by boiling dried mushroom parts in water (1:10) and incubating at 80°C for 1h. After filtration and cooling, the extracts were stored at −20°C, lyophilised for 24h, and further dried in an ice condenser at −70°C.

Hydroethanolic (EtOH) and chloroform ($CHCl_3$) extracts were obtained by maceration of mushroom material in 70% ethanol or $CHCl_3$ (1:10) for 72h at room temperature. The mixtures were then filtered and evaporated under reduced pressure at 40°C until dry.

Dried residues were dissolved in DMSO and stored at +4°C for further analysis.

Mycochemical characterisation

LC-MS/MS analysis of phenolic compounds

Quinic acid and 44 phenolic compounds (14 phenolic acids, 25 flavonoids, three coumarins, and two lignans) were analysed using LC-MS/MS, based on a previously established method[57]. The analysis was performed using an Agilent 1200 Series HPLC coupled with a 6410A Triple Quad MS, controlled by MassHunter software. Standards were sourced from Sigma-Aldrich, Fluka Chemie, and ChromaDex, and both samples and standards were prepared in 50% methanol (25 mg/mL). A 5 μL injection was used, with separation on a Zorbax Eclipse XDB-C18 column. Data were collected in dynamic MRM mode, and peak areas quantified using MassHunter. Calibration curves were created using OriginPro software. Detailed retention times and MS parameters are available in Supplementary Table 2.

Quantification of total phenolic, flavonoid, protein, and carbohydrate contents

The total phenolic content (TP) was measured using the Folin–Ciocalteu (FC) method as per Singleton et al.[58], using an absorbance wavelength of 760 nm. Results were expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE/g d.w.), based on a standard curve.

The total flavonoid content (TF) was determined using an $AlCl_3$ complexation method, with absorbance at 415 nm and quercetin

as the standard. TF values were reported as mg quercetin equivalents per gram of dry weight (mg QE/g d.w.)[59].

The Lowry method[60] was used to assess total protein content (TPR), based on the reaction of Cu^{2+} with Folin reagent. Absorbance was recorded at 740 nm, and TPR was calculated using a BSA standard curve, expressed as mg BSA equivalents per gram of dry weight (mg BSAE/g d.w.).

Total carbohydrate content (TC) was evaluated using the phenol–sulphuric acid method, following Rašeta et al.[56], with absorbance measured at 490 nm. A glucose calibration curve was used to express TC as mg glucose equivalents per gram of dry weight (mg GluE/g d.w.).

Examination of biological activities

Antioxidant activity

Antioxidant activity was evaluated using ABTS[61], DPPH[62], NO radical scavenging[63], and FRAP assays[64]. In the ABTS assay, 290 μL of ABTS reagent (7 mM ABTS and 2.45 mM $K_2S_2O_8$) was added to 10 μL of extract or standard, incubated for 5 min, and absorbance was recorded at 734 nm[61]. For DPPH, 60 μL of 90 μM DPPH solution, 180 μL MeOH, and 10 μL extract/standard were mixed, incubated for 30 min in the dark, and absorbance was measured at 515 nm[62]. NO scavenging was monitored by mixing 15 μL of extract/standard with 250 μL of sodium nitroprusside and phosphate buffer. Following a 90-min incubation period, 500 μL of Griess reagent was added and absorbance was measured at 546 nm[63]. The FRAP assay involved mixing 10 μL of extract/standard with 225 μL FRAP reagent and 22.5 μL distilled water (dH_2O), followed by measurement of absorbance at 593 nm after 6 min incubation[64]. Results were expressed as mg Trolox equivalents per gram of dry weight (mg TE/g d.w.) for ABTS, DPPH, and NO assays, and as mg ascorbic acid equivalents per gram of dry weight (mg AAE/g d.w.) for FRAP.

Hypoglycaemic effect

The in vitro hypoglycaemic effect was evaluated by assessing the extracts' ability to inhibit α-amylase and α-glucosidase, obtained from Sigma-Aldrich (Steinheim, Germany).

Inhibition of α-amylase was determined using the procedure of Yang et al.[65], where 90 μL of α-amylase (porcine pancreas Type VI-B, glucose) was mixed with 80 μL of 0.05% starch in 20 mM phosphate buffer (pH 6.9) and 10 μL of extract or acarbose as standard. After incubating for 10 min at 37 °C, the reaction was stopped with 100 μL of cold 1 M HCl and 20 μL of Lugol solution, and absorbance was measured at 620 nm. Results were expressed as % of enzyme inhibition.

For evaluation of α-glucosidase inhibition, the method from Palanisamy et al.[66] was used, where 100 μL of 0.1 M phosphate buffer (pH 6.8) was combined with 10 μL of α-glucosidase (Saccharomyces cerevisiae Type I), 20 μL of extract or acarbose as standard, and 20 μL of p-nitrophenyl α-D-glucoside. The mixture was incubated for 15 min at 37 °C, then 80 μL of 0.2 M Na_2CO_3 was added, and absorbance was measured at 400 nm. Results were reported as % of enzyme inhibition.

Anticancer activity

Chemicals. Tryptone, yeast extract, and agar were purchased from Torlak Institute. Synthetic drop-out medium supplement without tryptophan, yeast nitrogen base without amino acids and ammonium sulphate, galactose, kanamycin sulphate, lysozyme Tris,

components for phosphate buffer and 9,10-phenanthrenequinone (PQ) were from Sigma. Bio-Gel P-10 was from Bio-Rad and HisTrap HP columns were from Cytiva. Imidazole, NADPH and sodium chloride were supplied by Carl Roth. DMSO was purchased from Lach-Ner and IPTG from Fisher Scientific. Stock solutions of both extracts were prepared in DMSO and stored at 4 °C.

Fluorescent screen in yeast

Relative binding affinities of EtOH and H_2O extracts of S. lonicerinus for LBDs of ERα, ERβ, AR, and GR were evaluated using a yeast-based fluorescent assay according to our previously described procedure with some modifications[67–70]. S. cerevisiae FY250 strain (MATα, ura3-52, his3Δ200, leu2Δ1, and trp1Δ63) was transformed with either pRF4–6-ERα LBD-EYFP, pRF4–6-ERβ LBD-EYFP, pRF4–6-AR LBD-EYFP, or pRF4–6-GR LBD-EYFP[71], using a lithium acetate method[72]. Recombinant yeast cells were preincubated with shaking in a Biosan orbital shaker-incubator ES-20/60 for 40 h at 28 °C in a tryptophan-selective liquid medium containing 2% raffinose. Cells were then diluted in the same medium to an OD_{600nm} 0.15 and cultured until they reached logarithmic phase. LBD-YFP protein expression was induced by shifting from raffinose-containing medium to medium supplemented with 2% galactose, to enable activation of the GAL1 promoter. Stock solutions of steroid compounds and fungal extracts were prepared in DMSO. DMSO was added to each tube in the same volume of 10 μL ensuring that its concentration did not exceed 1%. Uninduced, solvent, positive and negative controls were included for each binding assay. In ER- and AR-binding assays cells were exposed to steroid compounds at a final concentration of 10 μM, while in the GR-binding assay control ligands were tested at 10 times higher concentration because the biosensor for this isoform is less sensitive. H_2O and EtOH extracts of S. lonicerinus were tested at final concentration of 3 μg/mL in ERα/β- and AR-LBD expressing cells, while GR expressing cells were treated with extracts at final concentration of 30 μg/mL. Treated cells were incubated at 22 °C for 14–16 h in the dark. Fluorescence was measured in 96-well format in the Fluoroskan Ascent FL fluorimeter using excitation at 485 nm and measuring emission at 538 nm. All measurements were performed in triplicate. Relative binding affinity was expressed as the fold fluorescence change between ligand-treated and ligand-free recombinant yeast cells exposed to DMSO solvent only (or oestradiol-treated cells in the case of GR-assay), normalised to 1. The binding data were analysed in Origin (OriginLab, Northampton, MA), version 9.0. Error bars correspond to the propagated standard errors of the mean.

Heterologous expression, purification and enzyme assay of AKR1C3 and AKR1C4

For protein expression in E. coli, the strain BL21 (DE3) was purchased from Novagen (Merck, Germany). Plasmid constructs pET28b(+)-AKR1C3 and pET28b(+)-AKR1C4 were obtained from Dr. Chris Bunce (University of Birmingham)[73]. Expression, purification, and in vitro enzyme activity assays of recombinant AKR1C3 and AKR1C4 were performed as described in our previous work[69,74,75]. The pET28b(+) vectors carrying a gene encoding 6 × His-tagged human AKR1C3 or AKR1C4 under the control of a T7 promoter were used to transform chemically competent BL21(DE3) E. coli cells via a heat shock procedure. Transformed cells were then grown overnight in LB medium supplemented with 50 μg/mL kanamycin in Biosan orbital shaker-incubator ES-20/60 at 37 °C. Protein expression was induced by adding 0.5 mM isopropyl 1-thio-D-galactopyranoside (IPTG) and

cultures were incubated for an additional 20h at 23 °C. Cells were harvested, resuspended in 20 mM Tris–HCl pH 8.0, 5 mM imidazole and lysed by a combination of lysozyme treatment and sonication (Soniprep 150, MSE, Crawley, West Sussex, England). Lysate was centrifuged (12000×g, 4 °C, 45 min) and the supernatant was loaded onto a HisTrap HP column previously equilibrated with the buffer solution (20 mM Tris–HCl pH 8.0, 0.5 M NaCl). After removal of the flow through fraction, the affinity resin was washed with five column volumes of binding buffer with 20 mM imidazole and 6 × His His-tagged protein was eluted with 5 ml of elution buffer containing 400 mM imidazole. As a final step, residual imidazole was removed from the eluted protein by size-exclusion chromatography using a Bio-Gel P-10 column (Bio-Rad, Hercules, CA, exclusion limit 20 000 daltons) equilibrated in 20 mM Tris–HCl pH 8.0, 0.1 M NaCl. The elution profile was monitored by Bradford assay[76]. Protein-containing fractions were pooled and frozen in small aliquots in 20 mM Tris–HCl pH 8.0, 0.1 M NaCl, 10% glycerol and 1 mM DTT at −80 °C for future use in enzyme assays. Following our previously published protocols for testing the AKR1C inhibitory effects of steroid derivatives[69,75,77], the potential of H_2O and EtOH extracts of *S. lonicerinus* to inhibit AKR1C3 and AKR1C4 was examined. AKR1C activity was assayed by fluorimetric measurement of NADPH consumption using a Fluoroskan Ascent FL fluorimeter. Enzyme (80 µg/mL AKR1C3 or 160 µg/mL AKR1C4) was incubated with NADPH (250 µM) and 9,10 PQ (4 µM in AKR1C3 assay and 17 µM in AKR1C4 assay) in potassium phosphate buffer (100 mM, pH 6.0) and reaction progress was monitored by fluorescence spectroscopy with excitation/emission wavelengths set to 340/460 nm at 37 °C every 30 s for 10 min. The catalytic activity of AKR1C was also tested in the presence of 33 µM ibuprofen (IBU) as a known inhibitor. Fungal extracts were screened for inhibition potential at a final concentration of 3 µg/mL. Blank was conducted in the absence of enzymes, while in the reaction control (Reaction) no inhibitor was added and the slope of the curve from fluorescence vs. time plots was defined as 100% enzymatic activity. All measurements were performed in triplicate. The inhibition potential of tested fungal extracts was analysed in Origin (OriginLab), version 9.0.

In silico molecular docking study of secondary metabolites from S. lonicerinus

Seventeen phenolic compounds that were detected in EtOH extracts of *S. lonicerinus* were chosen for further analysis by molecular docking against human AKR1C3. The three-dimensional coordinates of each compound were obtained from PubChem[78] and verified in the program AVOGADRO[79]. The geometries of three-dimensional models of these compounds were energy minimised in AVOGADRO using the conjugate gradients algorithm in an MMF94 force field with a convergence setting of $10e^{-7}$. Gasteiger partial charges were calculated for ligand atoms and ligand coordinate files were converted to PDBQT format for molecular docking using the script, "ligand.c", in the program VEGA ZZ version 3.2.1.33. For molecular docking, the "receptor" used was an X-ray structure of AKR1C3 in complex with ibuprofen, a non-steroidal inhibitor (PDB 3R8G)[80]. Using the script 'receptor.c' in VEGA ZZ, hydrogen atoms were added to the receptor, Gasteiger partial charges were calculated and ligand coordinate files were converted to PDBQT format[81]. Molecular docking simulations were conducted for each of the 17 compounds in Autodock Vina using the program PyRx[82,83]. Docking simulations were centred on the AKR1C3 active site (x = 1.80, y = 30.34, z = 30.25) with a search space of 25x25x25 Å and an exhaustiveness setting of 16. As a positive control, ibuprofen, the ligand present in the X-ray structure of

AKR1C3 (PDB 3R8G) was redocked into the active site with an R.M.SD of ≤ 0.6 Å using the same parameters. The resulting predicted binding energy for ibuprofen (−8.4 kcal/mol) was considered to be a threshold energy to identify compounds that could be potential AKR1C3 inhibitors. Compounds with binding energies ≥ −8.4 kcal/mol were then further analysed in the program PyMol. Docking results were analysed by superposition onto the experimental X-ray structure of AKR1C3 in complex with ibuprofen. 3D images of docking results were created in PyMol[84].

Statistical analysis

Data with a normal distribution were analysed using univariate (ANOVA) and multivariate (MANOVA) methods, with Tukey's HSD test to determine significant differences ($p < 0.05$). Pearson's correlation was applied for correlation analysis. Statistical analyses were performed using IBM SPSS version 22.0 (IBM SPSS Statistics, Armonk, NY) and Statistica version 12.01 (Tulsa, OK), while a heat map was created in Microsoft Excel (2016). Principal component analysis (PCA) was conducted using Past 4 Project software version 1.0.0.0.

Results and discussion

Phylogenetic analyses

The combined ITS + LSU + *TEF1* dataset of *S. lonicerinus* and related species included 14 specimens representing 12 taxa. The dataset had an aligned length of 1823 characters, of which 1478 characters are constant, 156 are variable and parsimony-uninformative, and 189 are parsimony-informative. Maximum parsimony analysis yielded two equally parsimonious trees (TL = 492, CI = 0.811, RI = 0.739, RC = 0.599, HI = 0.189). The best model for the combined ITS + LSU + *TEF1* sequences dataset estimated and applied in the Bayesian analysis was: GTR + I+ G, lset nst = 6, rates = invgamma; prset statefreqpr = dirichlet (1,1,1,1). Bayesian analysis resulted in the same topology with an average standard deviation of split frequencies = 0.003781. Two sampled isolates of the species, *S. lonicerinus* formed a well-supported lineage (100% MP and 1 BPPs) (Figure 1). The morphology of the specimens collected in Uzbekistan, Central Asia was generally uniform with descriptions of the type specimen[24,85]. Also, the phylogenetic relationship of *Sanghuangporus* was the same as that previously reported[8,12] and our specimen matched the type sequence of *S. lonicerinus* (Figure 1). Therefore, both morphology and phylogeny support identification of the specimen as *S. lonicerinus*.

Mycochemical characterisation

The mycochemical analysis of EtOH, H_2O, and $CHCl_3$ extracts from *S. lonicerinus* entailed quantifying quinic acid and 44 selected phenolic compounds using LC-MS/MS. Furthermore, spectrophotometric techniques were utilised to assess the phenolic profile by quantifying total phenolics content (TP) and total flavonoid content (TF). Additionally, TPR and total carbohydrate content (TC) were quantified using the same approach.

LC-MS/MS quantification of selected phenolic compounds

Based on LC-MS/MS quantitative analysis, only 26 compounds were detected among the 45 targeted compounds, with the most abundance in the EtOH extract (Table 1).
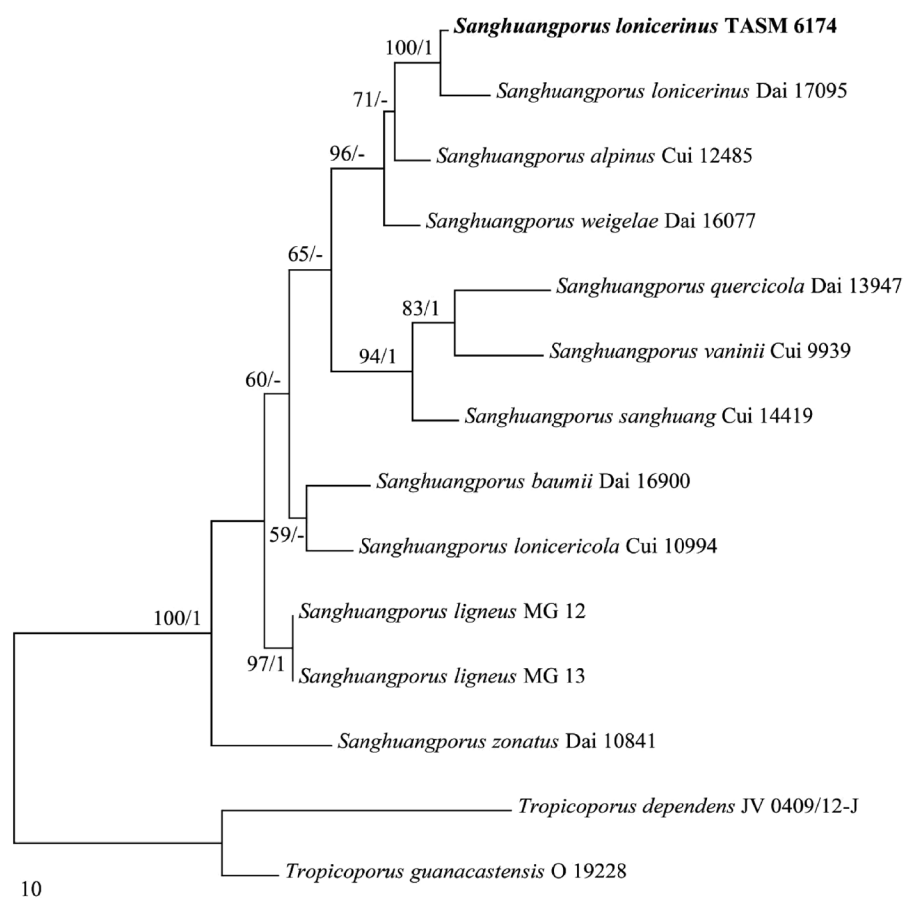
Figure 1. Phylogeny of *Sanghuangporus* and related species generated by maximum parsimony analysis based on combined ITS + nLSU + *TEF1* sequences. Branches are labelled with parsimony bootstrap values ≥50% and Bayesian posterior probabilities ≥0.95.

These compounds were classified into different groups, including 13 flavonoids, 11 phenolic acids, quinic acid and esculetin, a coumarin derivative. Among the identified compounds, only two flavonoids, baicalein and amentoflavone, were detected in all three extracts, but both higher than the limit of detection (LoD) and beneath the limit of quantification (LoQ), while the most abundant flavonoid was quercetin-3-O-Glc + Gal (761.73 ± 45.70 ng/g d.w.), followed by luteolin (414.92 ± 20.75 ng/g d.w.) and kaempferol-3-O-glucoside (391.18 ± 15.65 ng/g d.w.) detected in EtOH extract. Conversely, apiin and rutin were only quantified in CHCl$_3$ extract (77.06 ± 3.85 and 112.99 ± 3.39 ng/g d.w., respectively). Various phenolic acids were also identified in the extracts of *S. lonicerinus,* including caffeic acid, protocatechuic acid, cinnamic acid and quinic acid among others. These compounds were found to be present in relatively higher concentrations in the EtOH extract compared to the other two analysed extracts. Notably, the EtOH extract exhibited higher levels of all detected phenolic compounds compared to the H$_2$O and CHCl$_3$ extracts. Overall, the LC-MS/MS analysis demonstrated distinct phenolic profiles for each extract of *S. lonicerinus*, suggesting that the choice of solvent for extraction significantly influences the phenolic composition of these extracts.

Importantly, the results obtained in this study represent a groundbreaking account of LC-MS/MS quantification for this fungal species. This is particularly significant, as there is no existing literature data on the phenolic profile of *S. lonicerinus* or its synonyms (*Fomes lonicerinus*, *Cryptoderma lonicerinum*, *Phellinus lonicerinus*, *Porodaedalea lonicerina*, *Inonotus lonicerus*).

Determination of total contents: phenolics (TP), flavonoids (TF), proteins (TPR), and carbohydrates (TC)

The results revealed that the analysed *S. lonicerinus* extracts where rich in phenolic compounds among others (Table 2).

The EtOH extract exhibited the highest TP and TF (143.15 ± 6.70 mg GAE/g d.w. and 149.24 ± 2.74 mg QE/g d.w., respectively), followed by the H$_2$O extract (63.18 ± 0.71 mg GAE/g d.w. and 5.66 ± 0.45 mg QE/g d.w., respectively). The same trend was observed in TPR, quantified using the Lowry assay as well as for TC, quantified by the phenol-sulphuric assay (Table 2). These differences suggest that the choice of solvent for extraction plays a crucial role in extracting specific biochemical constituents from the fungal extracts, as mentioned above. Obtained results are in accordance with previous studies of *Sanghuangporus* spp.[32,86–88], but no comparison with *S. lonicerinus* in the form of crude extracts is possible, since there is lack of literature data. Furthermore, this is the first report of TC and TPR for this fungal species.

Biological activities of S. lonicerinus

Antioxidant activity
The antioxidant activity of the EtOH, H$_2$O, and CHCl$_3$ extracts of *S. lonicerinus* was evaluated using four different assays: DPPH radical scavenging assay, FRAP assay, NO scavenging assay, and ABTS radical scavenging assay. Among the extracts, the EtOH extract exhibited the highest DPPH and ABTS radical scavenging activity (34.18 ± 1.04 mg TE/g d.w. and 128.89 ± 1.01 mg TE/g d.w.,

Table 1. Concentrations of selected phenolic compounds and quinic acid (ng/g d.w.).

Class	Standard compounds	Extract type (content ng/g d.w.)		
		EtOH	H_2O	$CHCl_3$
Hydroxybenzoic acids	Gallic acid	418.77 ± 37.69	294.74 ± 26.53	221.14 ± 19.90
	p-Hydroxybenzoic acid	3266.04 ± 195.96	1239.34 ± 74.36	120.50 ± 7.23
	Protocatechuic acid	58480.07 ± 4678.41	18634.78 ± 1490.78	3291.68 ± 263.33
	Syringic acid	6407.39 ± 1281.48	3535.40 ± 707.08	<960.00[a]
	Vanillic acid	8040.34 ± 2412.10	5945.77 ± 1783.73	<1960.00[a]
Hydroxycinnamic acids	Caffeic acid	132947.04 ± 9306.29	5382.44 ± 376.77	5713.50 ± 399.95
	Chlorogenic acid	315.45 ± 15.77	75.58 ± 3.78	<60.00[a]
	Cinnamic acid	8072.02 ± 1614.40	<960.00[a]	<960.00[a]
	o-Coumaric acid	nd	nd	nd
	p-Coumaric acid	5934.04 ± 534.06	568.76 ± 51.19	499.83 ± 44.99
	3,4-Dimethoxycinnamic acid	nd	nd	nd
	Ferulic acid	1095.57 ± 109.56	324.20 ± 32.42	2079.34 ± 207.93
	Gentisic acid	nd	nd	nd
	Sinapic acid	267.09 ± 26.71	nd	nd
Cyclohexanecarboxylic acid	Quinic acid	7298.28 ± 729.83	5406.27 ± 540.63	2300.50 ± 230.05
Catechin	Epigallocatechin gallate	nd	nd	nd
Coumarins	Esculetin	1565.11 ± 93.91	<240.00[a]	333.93 ± 20.04
	Scopoletin	nd	nd	nd
	Umbelliferone	nd	nd	nd
Biflavonoid	Amentoflavone	<3920.00[a]	<3920.00[a]	<3920.00[a]
Flavanols	Catechin	nd	nd	nd
	Epicatechin	nd	nd	nd
	Kaempferol	nd	nd	nd
	Luteolin	414.92 ± 20.75	nd	141.17 ± 7.06
Flavonols	Isorhamnetin	nd	nd	nd
	Kaempferol-3-O-glucoside	391.18 ± 15.65	nd	<60.00[a]
	Luteolin-7-O-glucoside	nd	nd	<60.00[a]
	Myricetin	nd	nd	nd
	Quercetin	nd	nd	nd
	Quercetin-3-O-Glc + Gal	761.73 ± 45.70	nd	<240.00[a]
	Quercitrin	<60.00[a]	nd	<60.00[a]
	Rutin	nd	nd	112.99 ± 3.39
Flavanone	Naringenin	nd	nd	nd
Flavones	Apigenin	<960.00[a]	nd	nd
	Apigenin 7-O-glucoside	nd	nd	<60.00[a]
	Apiin	<60.00[a]	nd	77.06 ± 3.85
	Baicalein	<7800.00[a]	<7800.00[a]	<7800.00[a]
	Baicalin	nd	nd	nd
	Chrysoeriol	nd	nd	<240.00[a]
	Vitexin	nd	nd	<60.00[a]
Isoflavonoids	Daidzein	nd	nd	nd
	Genistein	nd	nd	nd
Lignans	Secoisolariciresinol	nd	nd	nd

[a]values marked with the symbol "<"—<LOQ: detected compound – peak observed, with concentration lower than the limit of quantification (LoQ) but higher than the limit of detection (LoD). Here are the abbreviations for the parameters analysed: EtOH: hydroethanolic; H_2O: hot water; $CHCl_3$: chloroform; nd: not detected, peak not observed.

Table 2. Antioxidant activity, contents of total phenolics (TP), total flavonoids (TF), total proteins (TPR) and total carbohydrates (TC) and hypoglycaemic activity in analysed *S. lonicerinus* extracts.

Parameter	Analysed extract		
	EtOH	H_2O	$CHCl_3$
DPPH (mg TE/g d.w.)	34.18 ± 1.04	4.47 ± 0.18	na
ABTS (mg TE/g d.w.)	128.89 ± 1.01	116.86 ± 6.87	40.94 ± 1.17
NO (mg TE/g d.w.)	320.55 ± 23.47	345.77 ± 18.16	n.a.
FRAP (mg AAE/g d.w.)	408.10 ± 9.42	92.74 ± 2.64	57.43 ± 2.22
TP (mg GAE/g d.w.)	143.15 ± 6.70	63.18 ± 0.71	12.70 ± 0.14
TF (mg QE/g d.w.)	149.24 ± 2.74	5.66 ± 0.45	1.21 ± 0.15
TPR (mg BSAE/g d.w.)	501.75 ± 2.58	322.12 ± 1.00	21.41 ± 0.55
TC (mg GE/g d.w.)	53.61 ± 5.53	31.89 ± 1.41	n.a.
α-amylase (% inhibition)	41.21 ± 0.79	11.02 ± 0.03	41.51 ± 1.96
α-glucosidase (% inhibition)	85.29 ± 5.58	na	39.45 ± 0.61

Here are the abbreviations for the parameters analysed: EtOH: hydroethanolic; H_2O: hot water; $CHCl_3$: chloroform; DPPH: radical scavenger capacity against 2,2-diphenyl-1-picrylhydrazyl radical, DPPH˙; ABTS: radical scavenger capacity against 2,2′-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid, $ABTS^{·+}$; NO: radical scavenger capacity against NO radical; FRAP: ferric-reducing antioxidant power; TP: total phenolic content; TF: total flavonoid content; TPR: total protein content; TC: total carbohydrate content; n.a.: not analysed; na: non active.

respectively) and reduction potential (408.10 ± 9.42 mg AAE/g d.w.), with the exception of NO scavenging ability where H_2O extract was notable (345.77 ± 18.16 mg TE/g d.w.) (Table 2). In general, $CHCl_3$ extracts showed the lowest antioxidant potential, which is in accordance with the observed mycochemical profile, since this extract had the lowest content of polyphenolic compounds.

Correlation analysis showed high positive correlation among observed mychochemical profile and antioxidant activity. In particular, primary metabolites (TPR and TC) showed the highest positive correlation with ABTS and NO scavenging ability of the analysed extracts ($r^2 = 0.97$ and $r^2 = 0.96$, respectively) which is in accordance with a previous report by Kozarski et al.[89], while secondary metabolites were probably more responsible for the observed high DPPH activity and reduction potential, since r^2 was in the range of 0.95 – 1.00 (Supplementary Figure 1(A,B).

However, a high positive correlation was observed for both primary and secondary metabolites and antioxidant activity, which could suggest possible synergistic effects, as noted in previous studies of other fungal species[10,55,90–93]. On the other hand, a

negative correlation was noted solely for ferulic acid, apiin, and rutin, particularly evident in ABTS and NO scavenging activities, with coefficients of determination (r^2) ranging from −0.84 to −1.00.

The observed correlation was supported by PCA analysis where PC1 accounted for the highest proportion of variance (98.99%) and was primarily associated with phenolic composition, while PC2 captured additional variability related to antioxidant activities (1.01%). The score plot revealed distinct clustering patterns among the extracts, indicating differences in their biochemical compositions, since EtOH extract formed a separate cluster with the most of quantified compounds, with the exception of protocatechuic acid and caffeic acid (Supplementary Figure 2). Furthermore, there was a degree of intersection noted between ferulic acid and the CHCl$_3$ extract, in agreement with LC-MS/MS findings, since this particular phenolic acid exhibited the highest abundance within this extract (2079.34 ± 207.93 ng/g d.w.). Notably, correlation analysis indicated a negative association between this acid and the antioxidant profile. The same trend can be clearly seen in the loading plot where the contribution of each variable is displayed, since ferulic acid and rutin were positioned opposite, suggesting a negative correlation with antioxidant potential.

To our knowledge, the results obtained constitute the first documentation of the antioxidant profile of crude extracts derived from *S. lonicerinus*. Previous studies have primarily explored antioxidant activity in other *Sanghuangporus* species, with a particular emphasis on mycelia[88] and submerged fermentation[87].

Hypoglycaemic effect

Diabetes also referred to as diabetes mellitus, remains one of the most widespread epidemic illnesses globally. Because it is believed to be preventable, type 2 diabetes garners more attention compared to type 1 diabetes[94]. Management of type 2 diabetes involves dietary and lifestyle changes to regulate plasma glucose levels, achieved by stimulating insulin secretion or slowing starch digestion to reduce blood sugar absorption[94]. Inhibition of α-amylase and α-glucosidase activity is crucial for regulating blood glucose levels by reducing the rate of sugar absorption[94]. While conventional inhibitors like acarbose, miglitol, and voglibose are effective, alternative solutions are sought to mitigate their potential side effects, including particular gastrointestinal issues[94]. As potential alternative sources, nature has long provided a plethora of valuable compounds associated with human health benefits[95]. Given that mushrooms are recognised for their abundance of secondary metabolites[5], which include flavonoids, phenolic acids, carbohydrates, proteins, and fatty acids, among others, and are known to possess inhibitory effects on α-amylase and α-glucosidase, we sought to investigate *S. lonicerinus* extracts as potential antidiabetic agents.

The *in vitro* α-glucosidase and α-amylase inhibition activities of three *S. lonicerinus* extracts were assessed to gauge their potential antidiabetic properties. Importantly, these results represent the first documentation of antidiabetic activity in this fungal species. The findings revealed significant inhibition of both α-glucosidase and α-amylase enzymes by all three fungal extracts, except for the H$_2$O extract, which showed no activity against α-glucosidase. EtOH extract exhibited the highest inhibition potential against both enzymes, inhibiting 85.29 ± 5.58% of α-glucosidase activity, which is comparable to the medication acarbose that was used as a standard (86.10 ± 2.64%) (Table 2).

Moreover, CHCl$_3$ and EtOH extracts demonstrated similar inhibition effects against α-amylase (41.51 ± 1.96 and 41.21 ± 0.79%, respectively). These findings suggest that the EtOH extract possesses the most potent antidiabetic activity among the three extracts tested, potentially due to its unique chemical composition since the highest amounts of tested polyphenols were found in this extract (Table 2).

Through correlation analysis, a strong positive correlation was identified between TP and TF and inhibition of α-glucosidase, whereas only TF was found to be associated with inhibition against α-amylase, as indicated by a heat map (Supplementary Figure 3(A)), which is in accordance with literature data[96–98].

This was confirmed with detected polyphenols, since a strong positive correlation (r^2 was in range from 0.86 to 0.99) was observed among phenolic acids and flavonoids, including coumarin esculetin and inhibition of α-glucosidase (Supplementary Figure 3(A,B)). For example, the highest correlation was between luteolin (flavonoid) and quercetin-3-*O*-Glc + Gal and inhibition of α-glucosidase enzyme where r^2 was 0.99. Lim et al.[99] outlined the structural prerequisites for three flavonoids, including quercetin and luteolin as natural antidiabetic inhibitors. They highlighted the significance of the double bond between C$_2$ and C$_3$ of the C-ring in quercetin and luteolin for inhibiting porcine pancreatic α-amylase, while the hydroxyl group (OH) at C$_3$ of the C-ring in quercetin was associated with inhibition of α-glucosidases from rat intestines. Also, molecular docking simulations indicated that luteolin has the potential to bind within the active site of α-glucosidase and engage with critical amino acid residues, while subsequent *in vivo* experiments provided additional evidence that luteolin effectively reduced the absorption of glucose and triacylglyceride in rats through the inhibition of both α-glucosidase and pancreatic lipase[100]. Furthermore, Kumar et al.[98] highlighted that quercetin and acarbose emerged as the most potent inhibitors of α-amylase. They proposed that the increased inhibitory efficacy of these compounds might stem from the existence of a double bond between C$_2$ and C$_3$ in the C rings, as revealed by various analyses including docking, as outlined in their study. Similarly, inhibition of α-amylase showed the strongest correlation with two flavonoids, flavone glycoside apiin and flavone luteolin (r^2 = 0.98 and r^2 = 0.76, respectively), in agreement with the findings of Lim et al.[96], who noted that the chemical composition of flavonoids and the presence of a double bond between C$_2$ and C$_3$, along with OH groups at A$_5$ and B$_3$, play crucial roles in α-amylase inhibition. Conversely, the OH groups at B$_3$ and C$_3$ are significant for α-glucosidase inhibition, enabling specific entry of the B-ring into the catalytic active site of α-glucosidase[96]. Also, a notable correlation with antidiabetic activity (inhibition of α-amylase) was observed for certain hydroxybenzoic acids, with the most prominent correlation observed for ferulic acid (a hydroxycinnamic acid), which exhibited an r^2 of 0.83 (Supplementary Figure 3(B)). On the other hand, greater concentrations of phenolic acids were required for α-amylase inhibition following prior interaction with starch[97]. This was attributed partially to the absorption of phenolic acids by starch during gelatinisation, suggesting unique inhibition mechanisms for these compounds in the α-amylase assay Aleixandre et al.[97]. Results of correlation analysis were confirmed by PCA analysis, where the total variance was 100% (PC1 98.99% and PC2 1.01%), since most of the detected polyphenols clustered in the same quadrant, together with the EtOH extract (Supplementary Figure 4). This implies that polyphenols contribute to the observed antidiabetic activity in the EtOH extract, whereas the significant antidiabetic activity of the CHCl$_3$ extract likely stems from the bioactivity of other compounds, given its distinct grouping in the third quadrant. Conversely, caffeic and protocatechuic acid were grouped distinctly from compounds exhibiting antidiabetic activity, consistent with prior research indicating that phenolic acids containing multiple hydroxyl (OH) groups demonstrated reduced inhibition of

antidiabetic effects Aleixandre et al.[97]. However, further studies are warranted to elucidate the mechanisms underlying these inhibitory effects and to explore the therapeutic potential of these fungal extracts in managing diabetes.

In vitro anticancer activity of S. lonicerinus
Relative binding affinities of extracts of *S. lonicerinus* for the ligand binding domains of ERα, ERβ, AR, and GR.

In order to test the relative binding affinities of EtOH and H₂O extracts of *S. lonicerinus* for the LBDs of ERα, ERβ, AR, and GR, we used a yeast-based fluorescent assay that was optimised for preliminary screening of library of steroid derivatives, natural phenolic compounds, and phytoestrogen-rich plant extracts, as previously described[67–70]. Results obtained here are used to estimate the hormonal effects of the tested fungal extracts based on their steroid receptor-binding affinity. In this assay, yeast cells expressing the LBD of a hormone receptor fused to a yellow fluorescent protein (YFP) were treated with steroid hormones or fungal extract. Ligand-binding induced hormone receptor dimerization and facilitated fluorescence resonance energy transfer (FRET) between YFP monomers, resulting in a measurable increase in fluorescence intensity. Relative binding affinity was expressed as fold fluorescence change between ligand-treated and recombinant yeast cells exposed to DMSO in AR- and ER-binding assays or estradiol in GR-binding assay, with higher values corresponding to stronger affinity. As can be seen in Figure 2, both tested extracts of *S. lonicerinus* have negligible binding affinity for AR-LBD and ERα-LBD. The absence of androgenic and estrogenic properties makes these extracts promising candidates for further research in the development of therapeutics targeting androgen- and estrogen-dependent cancers, as well as other diseases where cell proliferation is stimulated by hormones. Following treatment with EtOH extract of *S. lonicerinus*, recombinant yeast cells expressing GR-LBD showed fold fluorescence values lower than the threshold value of 1.2, suggesting weak interaction with this receptor isoform at the tested

Figure 2. *In vitro* AKR1C3 inhibitory potential of hydroethanolic (EtOH) and hot water (H₂O) extracts of *S. lonicerinus* evaluated by NADPH consumption assay. AKR1C3 activities in the presence of known inhibitor ibuprofen (IBU) or tested fungal extracts (3 μg/mL). Blank was conducted in the absence of enzyme. AKR1C3 enzyme activity in the absence of inhibitor (Reaction) was defined as 100%. Reaction mixture components included 80 μg/mL recombinant AKR1C3, 4 μM PQ and 250 μM NADPH in 100 mM potassium phosphate buffer pH 6.0. Kinetic measurements were taken at 30 s intervals during 10 min at 37 °C.

concentration. Among tested extracts of *S. lonicerinus*, the EtOH extract displayed stronger binding to ERβ-LBD compared to the H₂O extract. The EtOH extract of *S. lonicerinus* displayed moderate binding affinity for ERβ-LBD, with a fold fluorescence enhancement of ~1.3. This extract appears to be selective for ERβ over the ERα isoform. While ERα is a trigger for proliferation of breast cancer cells, ERβ plays a protective, inhibitory role. Although researchers predominately focus on antagonists of ERα, due to its role in promoting tumour growth, recent studies suggest that highly selective ERβ agonists lacking affinity for ERα can effectively block the proliferation of ER⁺ breast cancer cells[101], indicating the potential of this extract in the treatment of estrogen-mediated breast cancer. However, further studies are necessary to determine whether the ligands identified here exhibit agonist or antagonist properties towards ERβ. Due to side effects associated with estrogen replacement therapy, there is also a growing interest in fungal extracts as new natural sources of compounds with estrogen-like activity that could offer beneficial effects in menopause and osteoporosis and provide safer alternative treatment options[30]. Our results are in agreement with the findings of Wang et al.[30] who reported that hispolon isolated from *S. lonicerinus*, exhibited stronger binding affinity for ERβ than ERα in a yeast two hybrid assay. This biologically active polyphenol is also known for its significant antiproliferative activity against ER⁺, as well as estrogen-independent breast cancer cell lines[30,35]. Hispolon is a structural analogue of cinnamic acid that was identified in high concentrations in our EtOH extract (Table 2). Performing *in vitro* studies Hu et al.[33] confirmed the estrogenic and antiestrogenic properties of EtOH extracts of *S. lonicerinus*[33], consistent with the ERβ-binding ability we detected using our fluorescent biosensor in yeast. Furthermore, investigating the chemical composition of *S. lonicerinus*, Wang et al.[102] identified sterols that are reported to modulate the activity of nuclear receptors, such as ER and GR[103], supporting our binding assay results.

In vitro inhibition of human recombinant AKR1C3 and AKR1C4 activity by S. lonicerinus extracts
The AKR superfamily of oxidoreductases plays a crucial role in detoxification, redox transformations of carbonyl-containing substrates, and biosynthesis and metabolism of steroid hormones. However, some members of the AKR1C subfamily are also involved in the development of certain pathological conditions, such as breast, prostate and endometrial cancer and acute myeloid leukemia[104,105]. In addition to their overexpression in many types of cancers, AKR1C3 also contribute to drug resistance by metabolising chemotherapeutic agents, and inhibiting this isoform has been shown to restore chemosensitivity in drug-resistant cancer cells[106]. On the other hand, AKR1C4 preferentially acts as a 3-α-hydroxysteroid dehydrogenase and plays a role in xenobiotic metabolism in the liver, and inhibition of AKR1C4-mediated activity may be undesirable[106]. The interest in finding selective AKR1C3 inhibitors particularly in the context of cancer therapy is growing; however, none have yet been approved for clinical use. In our previous studies[69,75,77] several synthesised steroid derivatives have shown significant inhibition of the AKR1C3 isoform, however, published data on the inhibition of AKRs by fungal extracts is limited. There are only available reports on the aldose reductase inhibitory effects of fungal extracts[107,108]. For example, hispolon has been identified as a potent inhibitor of aldose reductase, a metabolic target for the treatment of diabetes[35]. To our knowledge, this study is the first to investigate the inhibitory effects of extracts of *S. lonicerinus* on AKR1C3 and AKR1C4 activity. Since AKRs utilise NADPH as a cofactor, to monitor the AKR1C activity we used a method based on
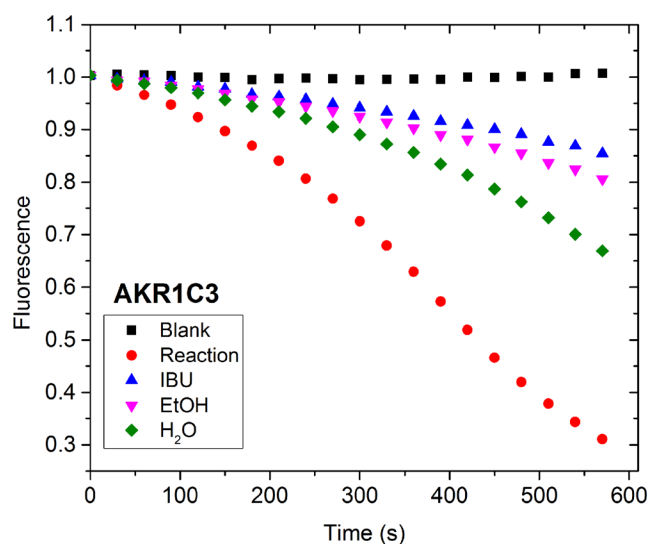
fluorimetric measurement of NADPH consumption. To evaluate the inhibition of fungal extracts, we measured their effects on the time course of reduction of 9,10-phenantrenequinone by recombinant AKR1C3 and AKR1C4 and results are shown in Figures 3 and 4. A lower slope indicates stronger inhibition. In the absence of enzyme (Blank) there was no change in NADPH fluorescence, as expected. Based on our enzymatic assay, EtOH extract of *S. lonicerinus* has been identified as a strong AKR1C3 inhibitor, inhibiting the target enzyme by 73.9%, in the range of that identified for the known AKR1C inhibitor, ibuprofen (IBU). H_2O extract of *S. lonicerinus* exhibited moderate inhibition (56.3%) against AKR1C3. Flavonoids such as 2–hydroxyflavanone, naringenin, 7-hydroxyflavone and caffeic acid phenethyl ester have been previously identified as highly potent inhibitors of the AKR1C3 isoform[109,110]. A possible explanation for the observed high AKR1C3 inhibition potential of the EtOH extract of *S. lonicerinus* in this study may be attributed to the presence of caffeic acid in high concentrations (Table 2), as it has been reported to inhibit this isoform[110]. Additionally, luteolin has also been quantified in our extract and there is data suggesting its potential to inhibit AKR1C3 as well[109]. On the other hand, IC_{50} values for *p*-hydroxybenzonic acid against AKR1C3 were found to be >1000 μM[110]. In the presence of EtOH and H_2O extracts of *S. lonicerinus*, AKR1C4 activity was weakly inhibited by 21.6% and 13.1% respectively, suggesting selectivity for the AKR1C3 isoform. Our findings indicate that extracts of *S. lonicerinus* may represent a promising source for isolating bioactive compounds to develop potential drug candidates.

In silico analysis of quantified polyphenolics against AKR1C3

As shown above, the *in vitro* AKR1C3 inhibitory potential of EtOH extracts of *S. lonicerinus* appears to be stronger than that of H_2O extracts. To identify potential AKR1C3 inhibitors present among phenolic compounds detected in *S. lonicerinus* EtOH extracts, we conducted molecular docking simulations in the program Autodock Vina[82]. As a positive control, the known AKR1C3 inhibitor ibuprofen was redocked into the X-ray crystal structure of AKR1C3 in complex with ibuprofen (PDB 3R8G). As shown in Figure 5, Autodock Vina was able to correctly predict the binding geometry of ibuprofen from the X-ray structure with an R.M.S.D of less than 0.6 Å and a favourable binding energy of −8.4 kcal/mol. Using the same parameters, 17 phenolic compounds present in EtOH extracts of *S. lonicerinus* were docked against AKR1C3. Binding energies predicted by Autodock Vina for the top-ranking poses are listed in Supplementary Table 3.

Compounds are ranked by concentration (content ng/g d.w.) detected in EtOH extracts from *S. lonicerinus* based on Table 1. Binding energies predicted by Autodock Vina for the top-ranking pose for each compound are shown in kcal/mol, where negative values indicate energetically favourable molecular interactions. Compounds previously shown to have AKR1C3 inhibitory activity are marked with a (+), along with their reported IC_{50} values and relevant reference in the literature if available. Compounds that are uniquely present in EtOH extracts are marked in bold. Compounds with binding energies ≥ −8.4 kcal/mol (the threshold binding energy predicted for ibuprofen), are shown in bold.

Many of the phenolic compounds detected in EtOH extracts from *S. lonicerinus* in this study were previously reported in the literature to have some level of AKR1C3 inhibitory activity
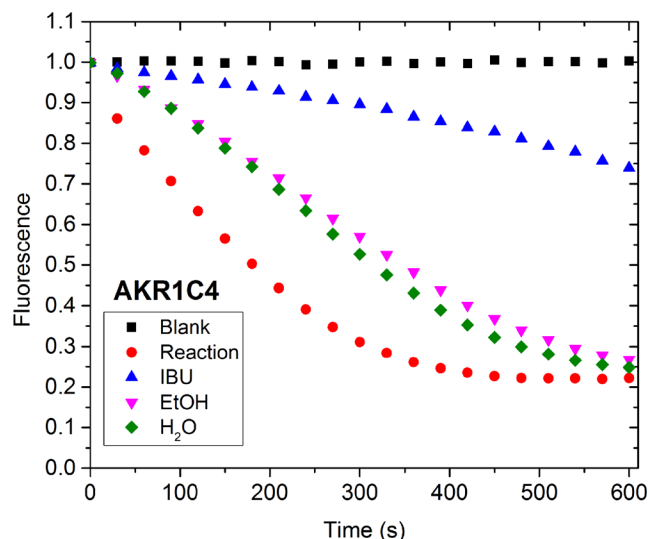

Figure 3. *In vitro* AKR1C4 inhibitory potential of hydroethanolic (EtOH) and hot water (H_2O) extracts of *S. lonicerinus* evaluated by NADPH consumption assay. AKR1C4 activities in the presence of known inhibitor ibuprofen (IBU) or tested fungal extracts (3 μg/mL). Blank was conducted in the absence of enzyme. AKR1C4 enzyme activity in the absence of inhibitor (Reaction) was defined as 100%. Reaction mixture components included 160 μg/mL recombinant AKR1C4, 17 μM PQ and 250 μM NADPH in 100 mM potassium phosphate buffer pH 6.0. Kinetic measurements were taken at 30 s intervals during 10 min at 37 °C.

(Supplementary Table 3). As can be seen, five phenolic compounds are predicted by molecular docking to bind with similar or greater affinity than ibuprofen (≥ −8.4 kcal/mol). All of these five compounds are present in the EtOH extract and were not detected in the H_2O extract. Thus, it is possible that these compounds contribute to the greater AKR1C3 inhibitory potential of EtOH extracts of *S. lonicerinus* observed experimentally. Of these, luteolin was previously reported to inhibit AKR1C3 activity with an IC_{50} of 37 μM, similar to the micromolar IC_{50} value reported for ibuprofen (10 μM)[111,112]. Molecular docking predicts that luteolin could be a competitive inhibitor that interacts with the NADP cofactor and catalytically essential amino acids Y55 and H117, in a manner similar to ibuprofen (Figure 5 panels B and C). Based on X-ray crystal structure analysis of over 60 AKR1C3-ligand complexes, AKR1C3 enzymes contain three conserved subpockets (SP1, SP2 and SP3) that are very important for ligand recognition[113,114]. Additional interactions are visible between luteolin and the sub-pocket known as SP1, *via* residues S118, N167, F306, and Y319. SP1 is involved in inhibitor binding in all known structures of AKR1C3 to date, and these molecular interactions suggest luteolin could contribute to the AKR1C3 potential of EtOH extracts of *S. lonicerinus*.

As can be seen in Supplementary Table 3, both kaempferol-3-*O*-glucoside (-9.1 kcal/mol) and quercetin-3-*O*-glucoside (−9.0 kcal/mol) are predicted by Autodock Vina to have the highest binding affinity for AKR1C3 among the tested compounds. The top-ranking binding geometries for these compounds were visualised in the program PyMol (Figure 6). Both compounds have similar chemical and three-dimensional structures, and may interact with catalytically important H117 and the NADP cofactor. Additional interactions are found with residues N167 and F306 of the AKR1C3 SP1 binding pocket, as well as Y24 and Q222 in a larger subpocket known as SP3 and W86 in the SP2 subpocket. Although further studies are
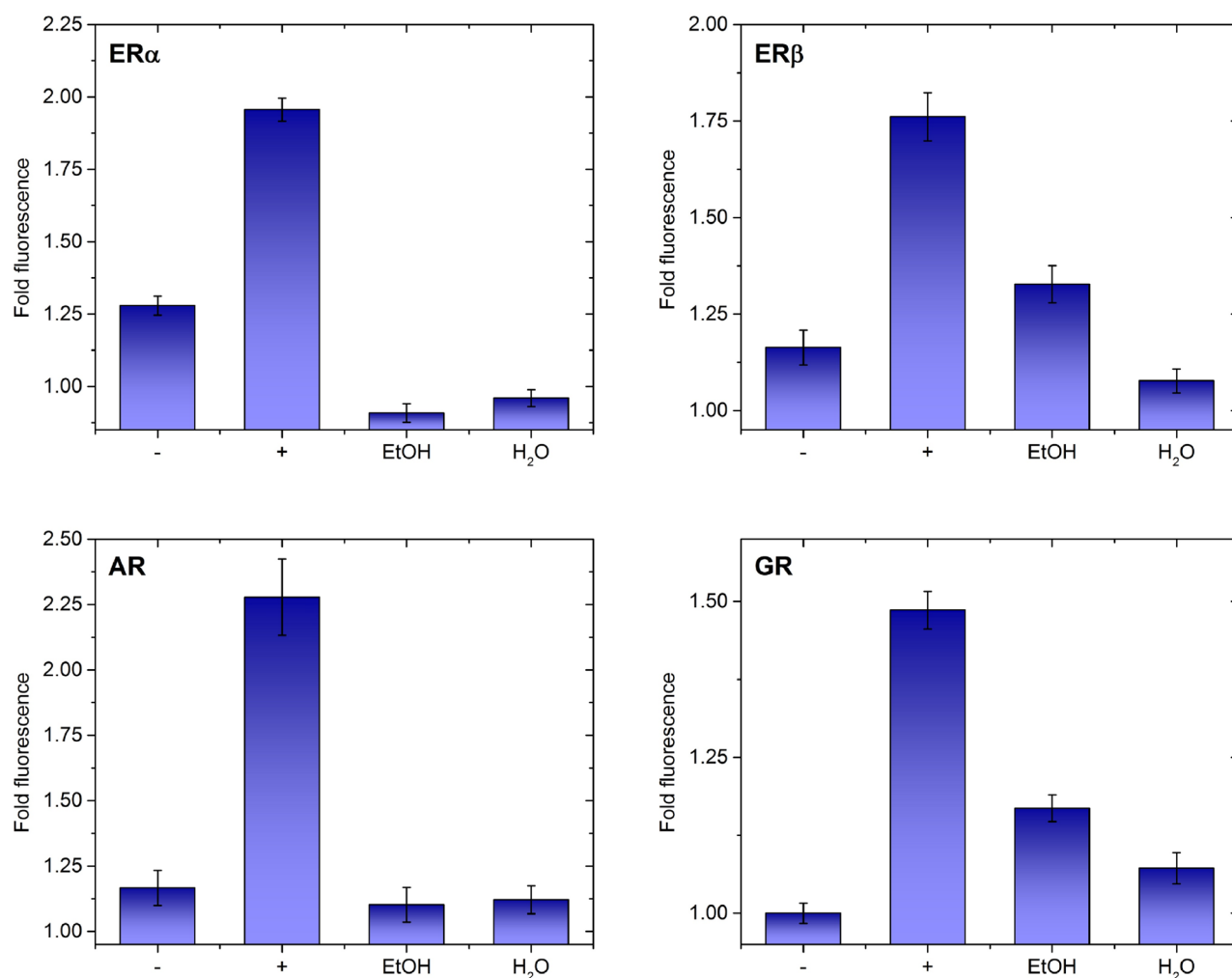
Figure 4. Relative binding affinities of hydroethanolic (EtOH) and hot water (H₂O) extracts of *S. lonicerinus* for ligand binding domains of estrogen receptor α (ERα), estrogen receptor β (ERβ), androgen receptor (AR) and glucocorticoid receptor (GR) measured by fluorescent screen in yeast. Relative binding affinity was expressed as fold fluorescence change between ligand-treated and recombinant yeast cells exposed to DMSO in AR- and ER-binding assays or estradiol in GR-binding assay (normalised to 1, not shown). Negative control (-) for yeast cells expressing ERα/β, AR- and GR-LBD were androstenedione, estrone and estradiol, while positive control (+) for yeast cells expressing ERα/β-, AR- and GR-LBD were estrone, androstenedione and prednisolone, respectively. H₂O and EtOH extracts of *S. lonicerinus* were tested at final concentration of 3 μg/mL in all binding assays, except in the GR assay where they were added at 30 μg/mL.

necessary, the phenolic compounds identified by molecular docking could be responsible for the AKR1C3 inhibitory potential of EtOH extracts of *S. lonicerinus*. Moreover, the fact that some of these compounds could interact with SP1, SP2 and SP3 suggests they might be candidates for design of AKR1C3 inhibitors with high specificity. Future studies could focus on structure-function analysis of purified individual phenolic compounds as AKR1C3 inhibitors.

Conclusions

This study presents a detailed mycochemical profile of *S. lonicerinus*, highlighting its phenolic, carbohydrate, and protein content, along with the biological activities of its extracts. The EtOH extract, which is rich in quinic acid and other polyphenolic compounds, demonstrated superior antioxidant, antidiabetic, and antiproliferative properties compared to CHCl₃ and H₂O extracts. Moreover, the EtOH extract displayed significant inhibition of α-glucosidase and α-amylase activities, underscoring its potential as a natural hypoglycaemic agent. The significant inhibition of α-glucosidase and α-amylase, along with strong AKR1C3 inhibitory activity and moderate binding to ERβ-LBD, suggests its promise in treating estrogen-mediated cancers and other hormone-related conditions. The phenolic compounds identified in the EtOH extract of *S. lonicerinus*, particularly luteolin, kaempferol-3-*O*-glucoside, and quercetin-3-*O*-glucoside, demonstrate significant AKR1C3 inhibitory activity, suggesting their potential for the development of specific AKR1C3 inhibitors. These findings, supported by molecular docking analyses, pave the way for future research into the development of nutraceutical and pharmaceutical developments derived from *S. lonicerinus*.

This study underscores a critical need to explore under-researched fungal species like *S. lonicerinus* to fully understand their health-promoting properties, particularly in Central Asia, where this species has been studied for the first time. Our ongoing efforts aim to expand the knowledge and understanding of
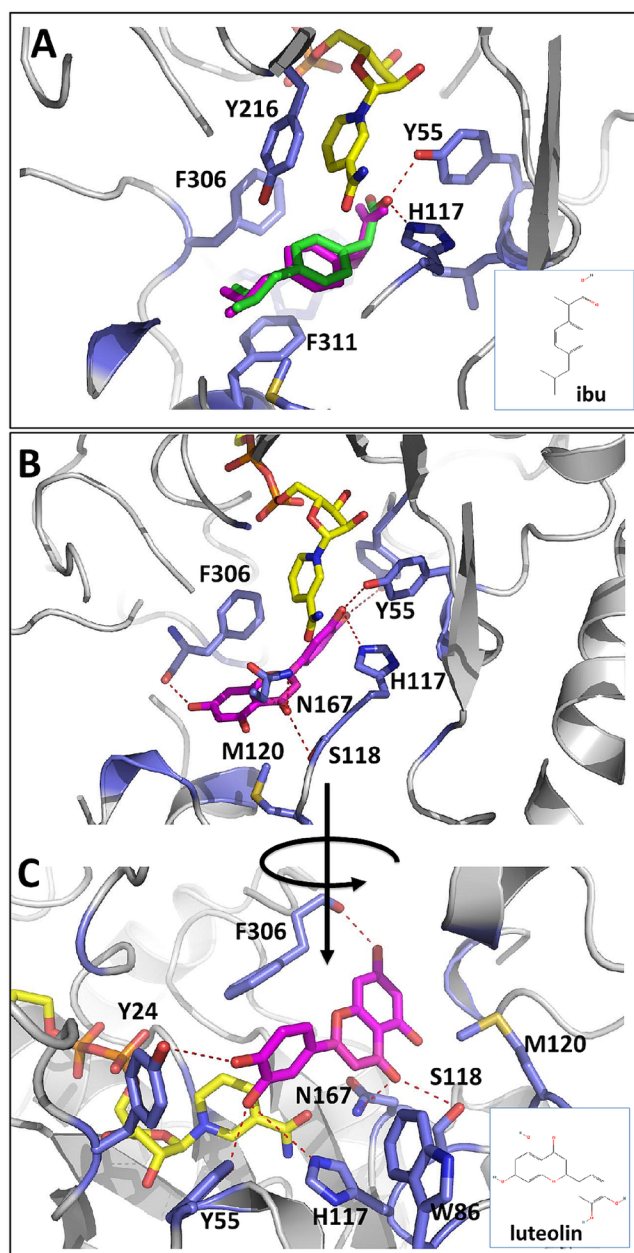
Figure 5. Molecular docking of luteolin against human AKR1C3. Panel A) Top ranking pose predicted by Autodock Vina during control redocking of ibuprofen (magenta sticks) against the X-ray structure of AKR1C3 in complex with ibuprofen (green sticks). Panels B and C) Top ranking pose predicted by Autodock Vina for luteolin (magenta sticks) shown in two orientations to enable visualisation of intermolecular interactions. Amino acids involved in binding are shown as blue sticks and labelled. The NADP cofactor is shown as yellow sticks.

Figure 6. Predicted molecular interactions for the top two ranking phenolic compounds based on molecular docking. Panel A) Top ranking pose predicted by Autodock Vina for kaempferol-3-*O*-glucoside (magenta sticks). Panel B) Top ranking pose predicted by Autodock Vina for quercetin-3-*O*-Glc. Amino acids involved in binding are shown as blue sticks and labelled. NADP cofactor is shown as yellow sticks.

Author contributions statement

YG: Conceptualisation, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualisation, Writing –original draft, Writing – review and editing. SB: Data curation, Formal analysis, Methodology, Resources, Visualisation, Writing – original draft.

these valuable fungi, with the goal of bringing greater awareness to their significant nutraceutical and pharmaceutical potential. By highlighting the antioxidant, hypoglycaemic, and anticancer potential of this species, our research paves the way for further investigation into both polar and non-polar bioactive compounds, with the aim of unlocking new therapeutic applications.
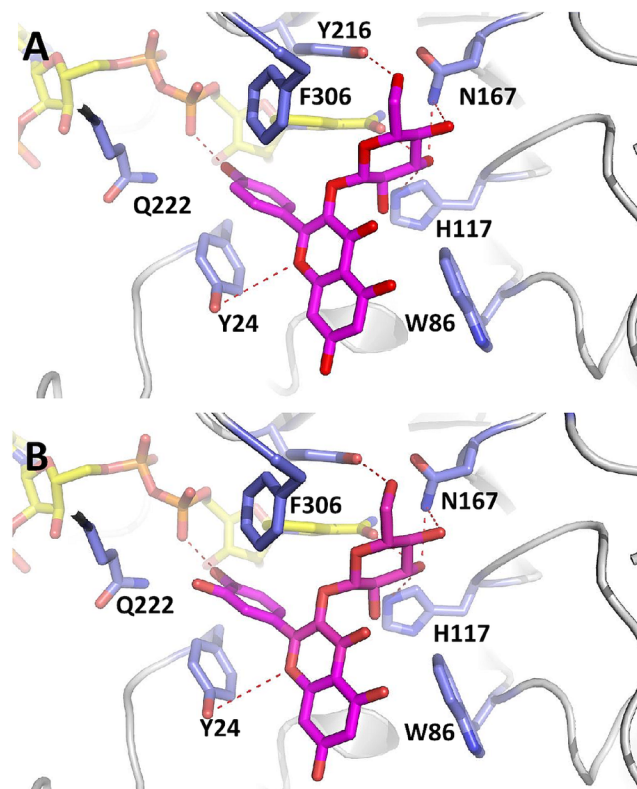
MY: Formal analysis, Methodology, Writing – review and editing. JM: Data curation, Investigation, Visualisation, Writing – original draft. NŽ: Data curation, Formal analysis, Methodology, Writing – review and editing. JJC: Formal analysis, Methodology, Writing – review and editing. EP: Data curation, Formal analysis, Methodology, Validation, Visualisation, Writing – original draft, Writing – review and editing. BA: Methodology, Data analysis. SR: Investigation, Data curation, Writing – review and editing. YWL: Investigation, Data analysis, Writing – review and editing. IA: Investigation, Data analysis, Methodology, Resources, Writing – review and editing. AMA: Data analysis, Methodology, Visualisation, Writing – original draft, Writing – review and editing. SG: Investigation, Data analysis, Writing – review and editing. WAAQIWM: Investigation, Visualisation, Writing – review and editing. MR: Conceptualisation, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualisation, Writing – original draft, Writing – review and editing. All authors have read the final manuscript version and approved it.

Disclosure statement

The authors declare that they have no known competing financial interests or personal relationships that might have influenced the work reported in this paper.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article or its supplementary materials.

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