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Daldinia eschscholtzii (Ehrenb.: Fr) Rehm, a new record in West Bengal and its mycochemistry, antioxidant contents and activity, lipoxygenase inhibitory activity and molecular docking of its prevalent compound, phloretin, with reactive oxygen species (ROS) producing enzymes

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Abstract

Daldinia eschscholtzii, collected from West Bengal, India, was evaluated for its antioxidant content and activity, as well as its lipoxygenase inhibitory potential from the ethanolic extract. Bioactive compounds were identified using LC-MS, followed by molecular docking between selected compounds and enzymes (lipoxygenase, xanthine oxidase, and CYP1A1). Results indicated significant levels of total phenolic content, total flavonoid content, and total antioxidant activity. LC-MS analysis of DEE (*Daldinia* ethanolic extract) detected 28 compounds in the extract. Molecular docking studies revealed a high binding affinity of phloretin with ROS-producing enzymes, suggesting potential enzyme inhibition. In conclusion, *Daldinia eschscholtzii* is revealed as a good source of antioxidant, anti-cancerous and LOX-inhibiting compounds.

Keywords – Antioxidant Content – Activity – LC-MS – Lipoxygenase inhibition – Molecular Docking – Phloretin

Introduction

Free radicals are commonly known as reactive oxygen species (ROS) when they originate from oxygen, but are called reactive nitrogen species (RNS) when they are generated from nitrogen (Mwangi et al. 2022). ROS, RNS and oxidative stress generated from the glycation, oxidation and peroxidation of proteins, glucose and lipids, respectively, cause damage to DNA, membranes, several enzymes and cellular machines (Arunachalam et al. 2022). Hectic lifestyle and stressful environment are also important factors for ROS and RNS generation (Sachdev et al. 2021). They are linked to premature ageing and the number of various diseases that impact our health (Mwangi et al. 2022). Although, several enzyme systems like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) are present in the human body for scavenging these free radicals, this scavenging work can be performed by exogenous compounds that are known as antioxidants (Mishra et al. 2019). Moreover, there are enzymes in plant and human systems that catalyze some reactions, producing huge ROS. The very prominent enzymes for this function are lipoxygenase

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(LOX), xanthin oxidase (XO) and cytochrome 450 family (CYT 450) (Bredemeier et al. 2018, Veith & Moorthy 2018). Lipoxygenases catalyze the oxidation of polyunsaturated fatty acids (PUFAs) in food to produce hydroperoxides, resulting in off-flavour or off-odour and food spoilage. In our body, ROS stimulates different diseases like cancer, cardiovascular diseases, neurodegeneration, etc. Similarly, xanthin oxidase stimulates ROS production, which causes endothelial dysfunction, leading to cardiovascular diseases like thrombosis and atherosclerosis (Bredemeier et al. 2018). Similarly, cytochrome P450 family enzymes in our liver perform oxidation and reduction, including the metabolism of several drugs we ingest. Therefore, inhibition of these three enzymes by proper inhibitors is an emerging area of research. In modern trends, in the food and cosmetic industries, some synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate are utilized as nutritional supplements and for preventing oxidation (Xu et al. 2021). Due to some reports suggesting their potential as carcinogens, and concerns regarding public safety and economic factors, the continuous use of these synthetic antioxidants has been widely questioned (Park et al. 2019, Xu et al. 2021, Ji et al. 2023). Hopefully, these synthetic antioxidants would be replaced by natural antioxidants in the future (Darwish et al. 2020). Therefore, researchers are exploring natural antioxidants (Shahidi 2000) from several sources, like plants, microbes, and fungi, to replace synthetic antioxidants. Recently, medicinal mushrooms have received more attention globally among many plant-derived preparations (Chaturvedi et al. 2018). Several biologically active molecules extracted from medicinal fungi provide a high source of antioxidants, innovative drugs and functional foods (Zhang et al. 2021). As per the literature, 92,725 described species are present in Ascomycota (Catalog of Life 2021, Senanayake et al. 2022). Some important compounds were extracted from medicinal Ascomycota fungal cultures, and they were reported as anti-inflammatory, anticancer and antimicrobial agents (Rajamanikyam et al. 2018, Al-Fakih & Almaqtri 2019, Wu et al. 2019b). Seven fungi like *Monascus purpureus*, *Cordyceps* spp, *Shiraia bambusicola*, *Xylaria nigripes* and *Claviceps purpurea*, belonging to Ascomycota, have been marked as antidiabetic (Jiang et al. 2025). Some members of Ascomycota, like *Aspergillus* spp., were reported as a good source of several organic acids (e.g. citric acid, gluconic acid) and enzymes (e.g. amylases and proteases). *Tuber* spp., *Morchella esculenta* and *Hypomyces lactifuorum* are edible, having antioxidant properties (Murcia et al. 2002, Li et al 2023, Marathe et al. 2020). Mycochemistry of *Cordyceps* spp (Ascomycota) has been rigorously done, and several bioactive compounds have been isolated, including cordycepin (3-deoxyadenosine), and the latter demonstrated anticancer activity (Yoon et al. 2018, University of Oxford news and events 2021). Some endophytic Ascomycota like *Muyocopron atromaculans* (Ascomycete sp. F53) and *A. niger* secreted novel compounds lijiquinone 1 and camptothecin, respectively, and exhibited cytotoxicity against human cancer cell lines (myeloma cells and colon cancer cells) (Kousar et al. 2022, Cain et al. 2020, Aswini et al. 2018). Katoch et al. (2017) isolated endophytic fungi belonging to 11 distinct genera in the Ascomycota, and solvent extracts of 28 fungal species exhibited cytotoxic potential against one or more human cancer cell lines. Several researchers reported on the medicinal values of *Daldinia*. It was found that *Daldinia concentrica* extract showed some estrogen-like activities *in vivo* (Kouakou & Benie 2003). Chutulo & Chalannavar (2020) reported that the solvent extract of *Daldinia eschscholtzii* showed antimicrobial, antioxidant, and cytotoxic activities.

In honor of the Swiss monk Agostino Daldini, Cesati & De Notaris (1863) first introduced the genus *Daldinia*. *Daldinia* spp. are known as saprophytic or endophytes, or latent pathogens, belonging to the phylum Ascomycota, class Sordariomycetes, order Xylariales and family Xylariaceae. It is one of the biggest genera in the Xylariaceae. These fungi are found to inhabit woody host plants and stay in a dormant phase until perithecial stromata form or the wood decays (Šrůtka et al. 2007). The global monograph of the genus contained morphological, ultra-structural, and chemotaxonomic data for over a thousand specimens and cultures together with introductory phylogeny based on the ITS sequence data (Stadler et al. 2014, Ahmmed et al. 2023, Yin et al. 2024). In 1820, Ehrenberg reported one fungus known as *Sphaeria eschscholtzii*, which was later recognized as *Daldinia eschscholtzii* (Ehrenb.: Fr.) Rehm. Although the habit of this fungus is

saprophytic on dead logs or endophytic in plants, it has also been found in marine algae, mangroves, human nails, skin, and human blood (Karnchanatat et al. 2007, Zhang et al. 2008, Kongyen et al. 2015, Ng et al. 2016, Helaly et al. 2018). Although the edibility of this *D. eschscholtzii* is not reported, *Daldinia fissa* Lloyd is used by people in Guatemala as food by roasting the fruit bodies with salt and lemon (Morales et al. 2006). Few researchers have tried to establish the pharmacological activity of *Daldinia eschscholtzii* globally (Xu et al. 2024). However, in India, relatively few works have been done on this fungus (Narmani et al. 2019, Chutulo & Chalannavar 2020, Mishra et al. 2020), most probably due to the fact that it is a purely wild mushroom with no culinary importance and is not readily available everywhere at all times. Therefore, due to the lack of available samples, proper investigation into its medical importance and application remains very limited. Keeping this in mind, our objectives were to properly characterize *Daldinia eschscholtzii* morpho-anatomically and molecularly, determine the antioxidant content, antioxidant activity, and lipoxygenase inhibitory activity of *Daldinia* ethanol extract (DEE), and detect bioactive compounds within DEE, utilizing LC-MS, followed by their evaluation of drug-likeness. Moreover, it aimed to investigate the inhibitory function of prominent compounds within DEE against three ROS-stimulating enzymes (lipoxygenase, xanthine oxidase, and cytochrome P450 family 1 (CYP1A1)) by the molecular docking approach.

Materials and methods

Chemicals

Folin-Ciocalteau's (FC) reagent, aluminium chloride ($AlCl_3$), ammonium molybdate, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), ferric chloride ($FeCl_3$), ferrous sulphate ($FeSO_4.7H_2O$), potassium acetate, and potassium ferricyanide were obtained from Merck, India. The 2,2′- diphenyl-1-picrylhydrazyl (DPPH), gallic acid, quercetin, tannic acid and 2, 4, 6-tripyridyl-s-triazine (TPTZ), butylated hydroxy anisole (BHA), thiobarbutyric acid (TBA), 15-LOX (15 lipoxygenase), and linolenic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). The DAPI (4',6-diamidino-2-phenylindole), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), and DMEM (Dulbecco's Modified Eagle Medium) were purchased from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. All other reagents were of analytical grade.

Collection of fungal specimens

Fungal specimens were collected from fallen logs of trees at Bandipur, Doperia Village, Khardaha, North 24 Parganas, West Bengal, India, in September (late monsoon season) 2020 and carried to our Laboratory in a biodegradable polythene bag. Macro-morphological characteristics (color, shape, and size) were recorded; microscopical observations were noted. A voucher specimen was deposited at the herbarium of Ramakrishna Mission VC College (Autonomous), Rahara, Kolkata, India.

Fungal identification

Phenotypical (Morphological and anatomical) and biochemical identification

The collection of the fruit body, preservation, macroscopic, and microscopic investigations were conducted using standard procedures according to the method described by Yuyama et al. (2013). Fruit body and spore color were determined by the color code chart presented by Anonymous (1969). From the fresh specimens (n = 10), the morphological features in terms of shape, stroma size, ostioles and perithecia, stromata color, and concentric zones of the mushrooms were recorded. Microscopic characters were analyzed based on the shape, size, and apical ring of the ascus in Melzer's iodine reagent, as well as the size, shape, and color of ascospores and perispore ornaments in 10% KOH. For ascospore measurements, 100 ascospores were taken. For biochemical identification, KOH (10%), HNO_3, H_2SO_4 and $FeSO_4$ were applied, and color changes were recorded. For morphological identification, key or literature of previous studies were used (Ju et al. 1997, Yuyama et al. 2013).

Molecular identification

Isolation of genomic DNA

The genomic DNA isolation was carried out according to the method described by Chutima et al. (2011), purified by a DNA purification kit (Hi-Media Laboratories Pvt. Ltd., Mumbai, India), and visualized by gel electrophoresis (1% agarose) under UV-transilluminator.

Amplification of the conserved internal transcribed spacers (ITS) region of rDNA by PCR

The PCR was done for the amplification of the internal transcribed spacers region (ITS1-5.8S-ITS2) using DNA amplification reagent kit (GeNei), with the help of fungus-specific forward primer ITS-1(5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes & Bruns 1993), and the reverse primer ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990) purchased from Sigma-Aldrich, St. Louis, Missouri, United States. The DNA purification kit was used to purify the amplified PCR product, run on a 1.2% agarose gel, and visualized under a UV-transilluminator.

Nucleotide sequencing of the ITS1-5.8s-ITS2 region of rDNA

The purified PCR amplicon (ITS1-5.8s-ITS2 region of rDNA) was sent to the GCC Biotech, Kolkata, India, for sequencing. The Sanger dideoxy method was used for this purpose. The resulted nucleotide sequence was analyzed by homology searching (BLASTn) against the GenBank nucleotide database (NCBI 1994). To compare with the reference sequence, the most similar sequences were selected (> 98.5 %) from the online bioinformatics tools of GenBank from NCBI. The obtained sequences were submitted to NCBI GenBank to get the accession number (NCBI 1994).

Solvent extraction of mushroom

Fungal fruiting bodies were washed with distilled water to remove the dirt and air dried at room temperature (27 ± 2 °C) for 3 days. The dried fruit bodies were then cut into small pieces and finely milled by using a mixer grinder to make powder. For extraction procedure, 65 g powder of fruit body of the fungus was washed by hexane (purity - 99%), followed by ethyl acetate (purity - 99.5%) and then wet washed powder material was collected by Whatman No. 1 filter paper, and later it was extracted by ethanol solvent (purity - 99%) by the dipping method in a conical flask with mouth sealed and stirring condition for 72h. After 72 h, the extracted solution was filtered through Whatman No. 4 filter paper, followed by Whatman No. 1 filter paper. Using a rotary vacuum evaporator at 40 °C (SUPERFIT, India), the filtrate was concentrated under reduced pressure, and after that, the concentrated filtrate was lyophilized by using a lyophilizer (BIOBASE, China) and the lyophilized powder was weighed. The final yield was calculated using the obtained data. The lyophilized extract powder (DEE) was kept in an airtight condition in a refrigerator at 4 °C for further use. The DEE (*Daldania* ethanolic extract) was used in this study.

LC-MS (Liquid Chromatography-Mass Spectrometry) study for the characterization of the secondary metabolite

The LC-MS analysis of the DEE was carried out by using the protocol described by Hajji et al. (2010). The analysis of DEE was done using a Liquid Chromatography-Mass Spectrometry 3000, LTQXL model (Thermo Scientific, USA) system, consisting of a degasser, binary pump, auto sampler, and column heater. The detector used was a Photodiode array (PDA) with a stationary phase C:18 column. The reverse phase C18 column was used for the chromatographic separation. For 1 min, the column was maintained at 95% solvent A (0.1% formic acid in water) and 5% solvent B (0.1% formic acid in acetonitrile). After that, an 11 min step gradient was applied from 5% B to 100% B, and then 100% B was set for 4 min. In the end, a linear gradient from 100% B to 5% B for 2 min was used to achieve the elution. In the column, a 5 μL volume of sample was injected, and the flow rate was 0.5 mL/min. The run time was 0–18 min. All MS experiments were conducted using the following parameters throughout all the experiments: the capillary voltage was

maintained to 3.5 kV for positive ion polarity electrospray ionization, the drying temperature was set to 350°C, the nebulizer pressure to 40 psi, and the drying gas flow to 10 l/min. The compounds in the sample were characterized according to their UV-Vis spectra and identified by their mass spectra from the Mass Bank database.

Determination of antioxidant content

Total phenolic content (TPC) determination

The total phenolic content of the DEE was determined by the Folin-Ciocalteau method (Ainsworth & Gillespie 2007). In a nutshell, 0.5 mL of sample (from 1 mg/mL stock) of the DEE was added with 0.5 mL of distilled water and 1 mL of FC reagent (10 times diluted with distilled water). After keeping it at room temperature (27 ± 2 °C) for 5 min, 2 mL of sodium carbonate (700 mM) was added to each test tube and mixed properly. Test tubes were then placed in the dark at room temperature for 45 min. After this incubation period, the absorbance was measured using a UV-Vis spectrophotometer at 765 nm (UV-1800, SHIMADZU, UV SPECTROPHOTOMETER). A calibration curve ($r^2 = 0.980$) was made using a solution of gallic acid standard (concentration ranging from 10–80 μg/mL). From the calibration curve, the TPC of the DEE was determined and expressed as mg gallic acid equivalent (GAE) per g of the DEE. The experiment was done in triplicate, and the mean value was taken.

Total flavonoid content (TFC) determination

The total flavonoid content of the DEE was determined according to the Dowd method (Arvouet-Grand et al. 1994). In this method, 1 mL sample (from 1 mg/mL stock) of the DEE was mixed with 0.2 mL $AlCl_3$ solution (10%, w/v) in methanol, 0.2 mL potassium acetate (1 M) and 5.6 mL distilled water. Then, the mixture was subjected to dark incubation at room temperature (27 ± 2 °C) for 30 min. After that, absorbance was measured at 415 nm wavelength using a UV-Vis spectrophotometer against a reagent blank. The TFC of the DEE was measured using a calibration curve of quercetin (concentration ranging from 10–400 μg/mL). The data (total flavonoid content) was expressed as mg quercetin equivalent (QE) per g of the DEE.

Total tannin content (TTC) determination

Total tannin content was determined by the method of Price & Butler (1977). In brief, 0.5 mL DEE (1 mg/mL stock) was added to 0.5 mL $FeCl_3$ (0.1 M), 0.5 mL potassium ferricyanide (8 mM), and 8 mL distilled water. Then, the reaction mixture was incubated for 10 min at room temperature (27 ± 2 °C), and the absorbance was taken against a reagent blank using a spectrophotometer at 720 nm. The TTC was estimated from the calibration curve at different concentrations of tannic acid standard (concentration ranging from 5–25 μg/mL). The total tannin content was expressed as mg tannic acid equivalent (TAE) per g of dry weight of the DEE. The experiment was done in triplicate, and the mean value was taken.

Total antioxidant content (TAC) determination

The total antioxidant content of the DEE was estimated by the Phospho-Molybdenum method (Prieto et al. 1999). In a 10 mL test tube, 0.5 mL sample (1 mg/mL stock) from the extract was mixed with 3 mL reagent solution (28 mM sodium phosphate, 4mM ammonium molybdate and 0.6 M sulfuric acid) and 0.5 mL distilled water. Then, the tubes were kept in a water bath at 90 ± 1 °C with the caps on for 90 min incubation. After that, the experimental setup was allowed to cool to the room temperature (27 ± 2 °C). The absorbance of the reaction mixture was measured using a spectrophotometer at 695 nm against a reagent blank. The amount of total antioxidant content of the sample was calculated from a calibration curve of ascorbic acid standard at various concentrations (10–140 μg/mL), and total antioxidant content (TAC) was expressed as mg ascorbic acid equivalent (AAE) per g of the DEE.

Determination of antioxidant activity

DPPH free radical scavenging activity assay

The ability of the DEE to scavenge free radicals was estimated by the DPPH (2,2′- diphenyl-1-picrylhydrazyl) assay to evaluate antioxidant activity (Brand-Williams et al. 1995). In brief, 50 µL samples of the DEE at different concentrations (0.1 to 5 mg/mL) were added separately with 1.2 mL of DPPH solution (6×10^{-5} M) and mixed properly by vortexing. Then, the resultant mixtures were incubated for 15 min in the dark at room temperature (27 ± 2°C). After that, the absorbance was measured using a spectrophotometer at 517 nm against a blank (Methanol). The radical scavenging activity of the extract was determined by the percentage of DPPH• radical scavenged, and it was estimated according to the following equation:

$$\text{DPPH• scavenging activity (\%)} = \frac{(Abs_{control} - Abs_{sample})}{Abs_{control}} \times 100$$

where $Abs_{control}$ = Absorbance of the control, Abs_{sample} = Absorbance of the test sample.

The EC_{50} or 50% scavenging of the DPPH• radical was calculated from a graphical plot where the scavenging percentage was plotted against different concentrations of an extract. Butylated hydroxy anisole (BHA) was used as a standard.

Hydroxyl radical scavenging assay

According to the method of Smirnoff & Cumbes (1989), the hydroxyl radical scavenging activity of the DEE was assayed. In brief, 3 mL of the reaction mixture contained the extract at different concentrations (0.1 to 5 mg/mL), 0.3 mL sodium salicylate (20 mM), 0.7 mL hydrogen peroxide (6 mM) and 1 mL $FeSO_4$ (1.5 mM). The tubes were vortexed and incubated for 1 h at 37°C. After that, the absorbance of the hydroxylated salicylate complex was taken at 562 nm. The hydroxyl radical scavenging activity was measured according to the following formula:

$$\text{Hydroxyl radical scavenging activity (\%)} = [1-\frac{(Abs_1 - Abs_2)}{Abs_0}] \times 100$$

where Abs_1 = Absorbance presence of the extract, Abs_2 = Absorbance without sodium salicylate and Abs_0 = Absorbance of the control.

The EC_{50} or 50% scavenging of the hydroxyl radical was calculated from a graphical plot where the scavenging percentage was plotted against different concentrations of DEE. The butylated hydroxy anisole (BHA) was used as a standard.

Lipid peroxidation inhibition assay

Lipid peroxidation inhibition was assayed according to the method described by Damien Dorman et al. (1995) with little modification. Egg yolk homogenate was used as a lipid-rich medium to measure the lipid peroxide formed. In this method, egg yolk homogenate (10% v/v) was prepared in 1.15% (w/v) KCl. In a test tube, the homogenate (0.5 mL) was added to 0.1 mL of different concentrations (0.1 to 5 mg/mL) of the test sample, and the volume was made up to 1 mL with double-distilled water. After that, 0.05 mL of 0.07 M $FeSO_4$ was added to each test tube to induce lipid peroxidation and the mixture was incubated at room temperature for 30 min. After incubation, 1.5 mL of TCA (tri-chloro acetic acid), followed by 1.5 mL of thiobarbutyric acid (TBA) (0.06 M) in 0.04 M sodium dodecyl sulphate, was added to the tubes. The mixture was mixed properly by vortexing and heated for 1 h at 95 °C. After that, tubes were placed for cooling and after cooling, 5 mL butanol was added to each tube and centrifuged for 10 min at 3000 rpm. The color intensity of the malondialdehyde-thiobarbutyric acid (MDA-TBA) complex of the upper organic layer was measured by using a spectrophotometer at 532 nm. In the case of control, the same procedure was followed, but the exception was that 0.1 mL of SDS was used instead of the test sample. The lipid peroxidation inhibition activity of the extract was estimated according to the following equation:

$$\text{Lipid peroxidation inhibition (\%)} = \frac{(Abs_{control} - Abs_{sample})}{Abs_{control}} \times 100$$

where $Abs_{control}$ = Absorbance of the control, Abs_{sample} = Absorbance of the test sample. The butylated hydroxy anisole (BHA) was used as a standard.

Reducing power capacity determination

The reducing power of the DEE was assayed according to the method of Oyaizu (1986). In a 10 mL test tube, different concentrations of DEE (2.5 mL) were mixed with 200 mM/L (2.5 mL) sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide (2.5 mL). After that, the reaction mixture was mixed properly by vortexing and incubated for 20 min at 50°C. After the addition of 10% trichloro acetic acid (TCA) (2.5 mL) in each tube, the reaction mixture was centrifuged for 10 min at 650 rpm. From each tube, 5 mL upper layer was taken carefully in a separate tube and mixed with the same volume of deionised water (5 mL) and 0.1% ferric chloride (0.1%). Then, the absorbance was measured at 700 nm in a spectrophotometer. The higher absorbance suggests a higher reducing power. The assay was carried out in triplicate. The DEE concentration at which absorbance reached 0.5 (EC_{50}) was calculated from the graph of absorbance of different DEE concentrations at 700 nm. The butylated hydroxyanisole (BHA) was used as a standard.

Ferric reducing antioxidant potential (FRAP) assay

According to the method of Benzie & Strain (1996), the ferric reducing power of the DEE was determined, and this assay determines the antioxidant effect of a substance as its reducing ability. In this method, at low pH, the electron-donating antioxidants reduced the colourless ferric complex (Fe^{3+}) to a blue-coloured ferrous complex (Fe^{4+}). The freshly prepared FRAP reagent contained 10 volumes of 300 mM sodium acetate buffer (pH 3.6), 1 volume of 20 mM $FeCl_3$ and 1 volume of 10 mM TPTZ in 40 mM hydrochloric acid. It was warmed at 37 ^oC before use. In brief, 300 µL of sample (from 1 mg/mL stock) of DEE was mixed with 2.7 mL of FRAP reagent and then incubated for 5 min at 37 ^oC. After that, absorbance was measured at the 593 nm wavelength. The FRAP values were calculated from the calibration curve at different concentrations of ferrous sulphate, and the values were expressed as mM Fe^{2+}/mg of sample.

Lipoxygenase inhibition assay

Lipoxygenase (15-Lox) inhibitory effect of DEE was performed following the method proposed by Malterud & Rydland (2000) in a borate buffer (0.2 M, pH 9.00). Absorbance was taken at 234 nm wavelength by UV-vis spectrophotometer for 5 min at an interval of 30 sec after the addition of 15-LOX, using linoleic acid (134 µM) as substrate. The final enzyme concentration was 165 U/mL DEE was dissolved in DMSO solutions. DMSO was used as the negative control. The enzyme solution was kept on ice, and controls (1.6% DMSO) were measured at intervals throughout the experiment to ensure that the activity was constant.

All measurements were done at least thrice. The experiment was repeated thrice. In each case, three parallels of controls and three parallels for each concentration of DEE were checked. Quercetin was used as the positive control against 15-LOX.

Calculation of enzyme activity was carried out by the formula:

$$\%\ of\ Inhibition = \frac{\text{Ac} - \text{Atc}}{Ac} \times 100$$

where Ac = absorbance of the control, Atc = absorbance of the test compound

IC_{50} values were estimated by linear interpolation between the measuring points closest to 50% activity. Values are expressed as means ± SD.

Toxicity test on a normal human cell line

The normal kidney cell line of human, HEK 293T (passage no 21) was cultured in DMEM supplemented with L-glutamine, 10% (v/v) fetal bovine serum, 100 µg/mL streptomycin, and 250 IU/mL penicillin (Hi-Media Laboratories Pvt. Ltd., Mumbai, India) in 75-mm tissue culture flasks at 37 °C and 5% CO_2 in CO_2 incubator. Cultures were passaged once a week for maintenance, and the culture medium was changed twice a week (Freshney 2015). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (Hi-Media Laboratories Pvt. Ltd., Mumbai, India) assay was used to assess the effect of DEE on cytotoxicity or cell proliferation in the HEK 293T cell line according to the method described by Ghosh et al. (2020). The changes of cell morphology study under a phase contrast microscope and nuclear morphology study by DAPI (4',6-diamidino-2-phenylindole) staining under a fluorescence microscope after treatment of DEE were done by the method described by Ghosh et al. (2020). For nuclear morphology examination, treated cells were stained with a fluorochrome stain and observed under a fluorescence microscope.

Prediction of physio-chemical properties, lipophilicity, water solubility, pharmacokinetics, drug-likeness and medicinal chemistry of the compounds

In our study, eight bioactive compounds (phloretin, myricetin, phloridzin, quercetin-3-glucuronide, luteolin-7-O-glucuronide, myricetin-3-o-galactoside, quercetin-3-(6"-malonyl)-glucoside, and amentoflavone) from the LC-MS result of DEE were selected because they have antioxidant properties as reported in previous studies (Kamdi et al. 2021, Kenouche et al. 2022, Ridgway et al. 1997, Shirai et al. 2001, Petrović et al. 2017, Hayder et al. 2008, Katsube et al. 2006, Li et al. 2020).

For the screening of chemical properties of the bioactive compounds, the Swiss ADME predictor (Daina et al. 2017), AdmetSAR (Yang et al. 2019), and pkCSM predictor (Pires et al. 2015) were used. Both give details about the physio-chemical properties, lipophilicity, water solubility, pharmacokinetics, drug-likeness and medicinal chemistry of the bioactive compounds. Lipinski's Rule of Five and other filters of different researchers (Lipinski et al. 1997, Ghose et al. 1999, Veber et al. 2002, Egan et al. 2000, Muegge et al. 2001) were used to screen the analyzed compounds, and then a molecular docking study was done.

Molecular docking study of DEE bioactive compound with ROS-generating enzymes

For the molecular docking study of DEE bioactive compound, protein selection, protein preparation, ligand selection, ligand preparation, grid generation and docking calculations were done.

Receptor preparation

The crystal form of human lipoxygenase (PDB ID: 1HU9) (Ghansenyuy et al. 2023), xanthine oxidase (PDB ID: 3NVY) (Cao et al. 2014) and cytochrome P450 family 1 (CYP1A1 (PDB ID: 4I8V)) (Walsh et al. 2013) were downloaded from PDB (RCSB Protein Data Bank 1971) and saved. AutoDoc Vina (AutoDock Vina v1.2.x 2021) software was used to remove all imported foreign substances like water and heteroatoms, but polar hydrogens and Kollman charges were added, and amino acid sequences, if missing, were recovered by this software. Images of prepared receptors (lipoxygenase, xanthine oxidase, and CYP1A1) were visualized in Chimera 1.16 and saved. The target protein was then imported into SwissDoc (Swissdock.ch 2024) software for molecular docking purposes.

Ligand preparation

Phloretin was selected on the basis of chemical properties screened by SwissADME predictor (Daina et al. 2017) and pkCSM predictor (Pires et al. 2015), and the literature search for its functions. It was selected as a ligand for molecular docking against lipoxygenase, xanthine oxidase and CYP1A1 receptors. It was obtained in its stable conformation from PubChem, and then it was

converted from SDF to mol2 file using Open Babel software before being imported into SwissDock for molecular docking.

Docking of receptor and ligand

The automated docking tool SwissDock was used for the molecular docking study. The three-dimensional protein structure was enhanced with polar hydrogen atoms and Gasteiger partial charges. Before molecular docking, the protein structures were saved in pdb files. Pre-calculation of atomic affinities was done using AutoGrid. For the docking simulation, grid box was integrated as 20(x) × 20(y) × 20(z) and centered at -2(x) × 43(y) × 5(z) for a pocket of the active site of lipoxygenase, -33(x) × 90(y) × -23(z) for oxanthine oxidase and -41(x) × 82(y) × -23(z) for CYP1A1, respectively**.** The Protein-ligand dock complexes were analyzed by SwissDock software for post-docking studies, visualized by the Discovery Studio Visualizer 4.1 client, and saved as images (Sravani et al. 2021).

Statistical analysis

Data was presented as mean ± standard deviation (SD). Statistical analysis was done by following Bhattacharjee et al. (2023), with required modifications. The Pearson correlation coefficient test was used to understand the relationship between the concentrations of DEE and radical scavenging percentage, lipid peroxidation inhibition percentage and reducing power ($p<0.05$). Depending on the data, the Spearman rank correlation test was performed to understand the possible relationship between the following parameters: TPC, TFC, TTC, TAA, EC_{50} of DPPH radical scavenging, EC_{50} of OH radical scavenging, EC_{50} of lipid peroxidation inhibition, EC_{50} of reducing power and FRAP. The statistical analysis and all graphs were developed using GraphPad Prism 9 software.

Results and discussion

Identification of mushroom: Phenotypical (Morphological and anatomical) and biochemical identification of the fungus

Description:

Ecology (Habitat) - Rotten log of trees; Growth pattern - solitary or sometimes aggregated; Spore print - Brown; Biochemical test - KOH negative; Morphology (Fruit body) - Hemispherical and sessile; Size - 3.2–6.5 cm diam × 1.3–3.6 cm high ($\bar{x}$ = 5.05 × 2.42 cm; n=10); Surface – Smooth; Colour – Brown/Pinkish; Anatomy - Stromata – Ring; Ostiole – Present, apapillated. Ascus - 8 ascospores. Ascospore - Unicellular, ellipsoid-inequilateral, dark brown; Size - 11.28–13.84 × 5.04–6.38 µm ($\bar{x}$= 12.15 × 5.57 µm; n=100); Germ slit – Straight on convex side; Perispore – Ornaments/ smooth; Edibility – Not reported (Fig. 1)

Daldinia species were identified by the presence of the internal concentric zones below the perithecial layer in their stroma and by the existence of KOH extractable pigments on and under their stromatal surface (Ju et al. 1997). In our specimen, the perispore of ascospore ornamentation was found to be a coil-like structure, supported by Van der Gucht (1993). In contrast, *D. concentrica* bears a smooth perispore (Van der Gucht 1993). The ascospore size of our specimen was smaller than *D. concentrica* (13–17 × 6–7.5 µm), and our specimen had an apapillate ostiole, while the latter contained a papillate ostiole (Ju et al. 1997). Similarly, our specimen is KOH negative, as supported by Ju et al. (1997) and Stadler et al. (2014). Moreover, our morphological and biochemical data were matched with published key or manual (Ju et al. 1997, Yuyama et al. 2013). Therefore, our fungal specimen was identified as *Daldinia eschscholtzii* morphologically and biochemically (Phylum - Ascomycota; Order - Xylariales; Family - Xylariaceae). For the confirmation of identification, we have proceeded with molecular identification (ITS1-5.8S-ITS2).

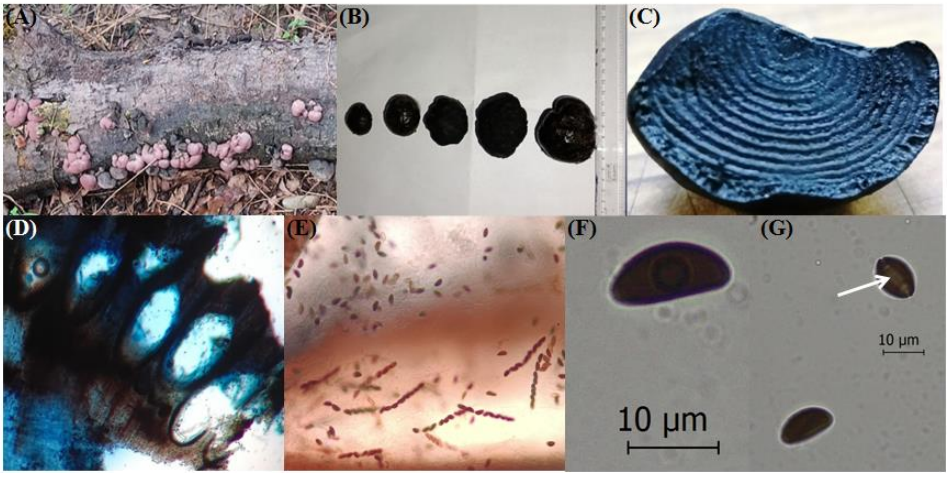

Fig. 1 – Morpho-anatomy of *Daldinia eschscholzii* (Ehrenb.: Fr.) Rehm A Habit on log. B Different sized fruiting bodies. C Section of the fruit body showing concentric rings. D A section showing stroma in ring. E Asci with eight ascospores in each. F A spore with the surface character. G Spores showing spore-length straight germ slit on convex side pointed by the arrow (at 100×magnification; Compound microscope, Olympus Corporation, Tokyo, Japan).

Molecular identification of the fungus

The result showed that the genomic DNA band of this fungus was near 3 kb, compared to the DNA ladder (Figure not shown). When compared to the DNA ladder, the PCR product of the ITS amplification displayed a band at 600 bp (Figure not shown). After getting the nucleotide sequence (n = 562 bp) in the FASTA file (not shown), it was loaded for BLASTn in the NCBI database to search the sequence similarity. After running the homology search, the genus and the species of the fungus were identified. The fungal species was identified as *Daldinia eschscholtzii* on the basis of the query coverage (99%) and the percent of identity (100%) (Fig. S1). The nucleotide sequence was submitted to NCBI GenBank to get the accession number, and the accession number of the fungus was PP024880.1 (*Daldinia eschscholtzii* strain SKGTBON19). The phylogenetic tree was constructed by the Neighbour-Joining method (0.05 Max Seq Difference), and the results showed its taxonomic position with other *Daldinia* species and the ascomycetes group (Fig. S2). The ITS region of rDNA is widely used for species identification of fungi (Bera & Ghosh 2024).

Yield and LC-MS of DEE

The extraction yield of DEE was 2.84 ± 0.03%. The identification of secondary metabolites in DEE was based on the retention time of liquid chromatography and analysis of mass spectra (Fig. 2). The identification of metabolites was done with the help of the mass spectra database (MassBank Europe Mass Spectral Data Base) and also with the help of literature (Table 1). The identified peaks are tentatively mentioned here because the natural products are present also as isomeric forms, like isomerization of aglycones (e.g., isoflavones and flavones) or as isobaric compounds with distinct element composition but identical molecular weight. Acylating groups may also coexist in different configurations with the sugar moieties. Total of 28 peaks were detected from the LCMS chromatogram but 25 peaks/ compounds were identified in DEE; among them, 11 flavonoids (Myricetin, Myricetin-3-O-xyloside, Myricetin-3-O-galactoside, Quercetin-3-(6"-malonyl)-glucoside, Quercetin-3-glucuronide, Chalcone base + 3O, 1prenyl, Amentoflavone, Isoflavone base + 1O, 2MeO, O-Hex+C7H12NO, Naringenin-7-O-glucoside, Tricin, and Luteolin-7-O-glucuronide), two polyphenols (Phloretin and Phloridzin), three alkaloids (Lycoctonine, Neoxaline, and Mesaconitine), two terpenoids ((-)-Curcumol, 13-Episclareol), one triterpene glycoside (Glycyrrhetic acid, Me ester), one lignin (Eudesmin), one lignan (Dihydrohesperetin-7-O-Neohesperidoside), one spermidine (Dicaffeoyl coumaroyl spermidine) and one benzoxazinoid (DIBOA + O-Hex-Hex) compounds were identified. The other two compounds were identified as

Phloretin + C-Hex, C-Hex and 3,5-Dichloro-2-hydroxybenzophenone. Three peaks of the chromatograms of compounds were not identified as they were not available in the database. Liu et al. (2019) reported two new compounds from the EtOH extract of *D. eschscholzii* like eschscholin A, and 3-ene-2-methyl-2H-1-benzopyran-5-ol by semipreparative HPLC. Liao et al. (2019) reported five tetralones like daldiniones A–E, three new chromones such as 7-hydroxy-5-methoxy-2,3-dimethylchromone, 5-methoxy-2-propylchromone, and 7-ethyl-8-hydroxy-6-methoxy-2,3-dimethylchromone, and also two new lactones like helicascolides D and E were isolated from the fungus *Daldinia eschscholtzii* HJ004 (mangrove-derived) by GC-MS. However, the literature survey exhibited that none of the studies ventured to detect compounds in the DEE of *D. eschscholzii* through LC-MS. Therefore, as per our knowledge, it may be the first report of the compounds from *D. eschscholzii*. Similarly, a few scientists reported bioactive compounds from other species of *Daldinia,* like *D. concentrica,* and their solvent extracts were different. Qin & Liu (2004) from China found some bioactive compounds like 3-henicosyl-1-4benzoquinone, 3-docosyl-5-methoxy-2-methyl-1,4-benzoquinone and 5-methoxy-2-methyl-3-tricosyl-1,4-benzoquinone in $CHCl_3$ extract. Furthermore, they isolated a few other compounds like Friedin, Ergosta-4,5,8,22-tetraen-3-one, Ergosta-7,22-dien-3-one, (22E,24R)-ergosta-7,22-dien-3β-ol and Ergosta-5,5,7,22-trien-3β-ol. Zhang et al. (2008) isolated two polyketides (Dalesconlo A and B), which were immunosuppressive, from *D. eschscholtzii*. Later on, other bioactive compounds were isolated from this species by Zhang et al. (2011) and Tarman et al. (2012). We have searched the literature for the bioactivity of these compounds, which were isolated from different sources by previous studies, and it is interesting to note that most of the compounds have antioxidant as well as anti-cancerous properties. The functions of eight compounds out of 25 were unavailable in the database. Important anti-cancerous compounds like Phloretin (Hytti et al. 2023, Kim et al. 2022), (-)-Curcumol (Wang et al. 2018), Mesaconitine (Guan Xin et al. 2017), 13-Episclareol (Sashidhara et al. 2007), Neoxaline (Koizumi et al. 2004), Glycyrrhetic acid Me ester (Hibasami et al. 2006), Naringenin-7-O-glucoside (Han et al. 2008), DIBOA + O-Hex-Hex (Adhikari et al. 2015), Quercetin-3-glucuronide (Wu et al. 2018), Tricin (Oyama et al. 2009, Han et al. 2016), Eudesmin (Patel & Patel 2022, Jiang et al. 2017) and Myricetin (Kumar et al. 2023, Li et al. 2022) were found to be present in DEE. Particularly, myricetin has attracted attention and exhibited different bioactivities, such as anticancer, immunomodulatory, anti-inflammatory, antiplatelet aggregation, and anti-photoaging activities, and it protects us from several health complications, including cardiovascular diseases (Semwal et al. 2016, Agraharam et al. 2022). Previous studies recorded that Amentoflavone (Li et al. 2020), Quercetin-3-(6"-malonyl)-glucoside (Katsube et al. 2006), Phloridzin (Ridgway et al. 1997), Luteolin-7-O-glucuronide (Petrović et al. 2017), Myricetin-3-O-galactoside (Hayder et al. 2008), Phloretin (Kamdi et al. 2021), Myricetin (Kenouche et al. 2022) and Quercetin-3-glucuronide (Shirai et al. 2001) were antioxidant. However, Phloretin, Myricetin and Quercetin-3-glucuronide had both antioxidant activity and anticancer potential. Therefore, this fungus or DEE (*Daldinia* ethanolic extract) is a good source for both anti-cancer and antioxidant compounds.

Table 1 Identification of bioactive compounds with their bioactivity present in the DEE (*Daldinia* ethanolic extract) by the LC-MS

No of peak	Experimental m/z	Tentative Bioactive compound	Retention time	Type of compound	Bioactivity	Reference
1	274.09	Phloretin	1.11	Polyphenol	Anti-inflammatory, Anticancer and Antioxidant	Hytti et al. (2023), Kim et al. (2022), Kamdi et al. (2021)
2	236.16	(-)-Curcumol	1.17	Sesquiterpenoid	Anticancer	Wang et al. (2018)
3	467.37	Lycoctonine	2.02	Alkaloid	NA	NA

Table 1 Continued.

No of peak	Experimental m/z	Tentative Bioactive compound	Retention time	Type of compound	Bioactivity	Reference
4	451.36	Myricetin-3-O-xyloside	2.75	Flavonol	NA	NA
5	629.46	Not found in the library	2.88	NA	NA	NA
6	523.45	Not found in the library	3.65	NA	NA	NA
7	324.40	Chalcone base + 3O, 1Prenyl	4.43	Flavonoid	NA	NA
8	537.46	Amentoflavone	5.16	Biflavonoid	Antioxidant	Li et al. (2020)
9	631.36	Mesaconitine	8.34	Diester-diterpenoid alkaloid	Anticancer	Guan Xin et al. (2017)
10	602.45	Not found in the library	8.34	NA	NA	NA
11	585.45	Isoflavone base + 1O, 2MeO, O-Hex+C7H12NO	8.83	Flavonoid	NA	NA
12	549.23	Quercetin-3-(6"-malonyl)-glucoside	8.99	Flavonoid	Antioxidant	Katsube et al. (2006)
13	615.50	Dicaffeoyl Coumaroyl Spermidine	9.44	Spermidine	NA	NA
14	308.37	13-Episclareol	9.44	Diterpene	Anticancer	Sashidhara et al. (2007)
15	599.54	Phloretin + C-Hex, C-Hex	10.17	Polyphenol	NA	NA
16	435.37	Neoxaline	10.57	Alkaloid	Anticancer	Koizumi et al. (2004)
17	484.27	Glycyrrhetic acid, Me ester	11.00	Triterpene glycoside	Anticancer	Hibasami et al. (2006)
18	434.41	Naringenin-7-O-glucoside	11.56	Flavonoid	Anticancer	Han et al. (2008)
19	505.48	DIBOA + O-Hex-Hex	11.80	Benzoxazinoids	Anticancer	Adhikari et al. (2015)
20	318.31	Myricetin	12.58	Flavonoid	Anticancer and Antioxidant	Kumar et al. (2023), Li et al. (2022), Kenouche et al. (2022)
21	478.39	Quercetin-3-glucuronide	13.10	Flavonoid	Antioxidant and Anticancer	Shirai et al. (2001), Wu et al. (2018)
22	330.36	Tricin	13.65	Flavonoid	Anticancer and Antiangiogenic	Oyama et al. (2009), Han et al. (2016)
23	436.40	Phloridzin	14.98	Polyphenol	Antioxidant	Ridgway et al. (1997)
24	386.38	Eudesmin	15.48	Lignin	Anticancer	Patel & Patel (2022), Jiang et al. (2017)
25	462.39	Luteolin-7-O-glucuronide	16.74	Flavonoid	Antioxidant	Petrović et al. (2017)
26	480.27	Myricetin-3-O-galactoside	16.74	Flavonoid	Antioxidant	Hayder et al. (2008)
27	265.22	3,5-Dichloro-2-hydroxybenzophenone	17.22	Unknown	NA	NA
28	612.42	Dihydrohesperetin-7-O-neohesperidoside	17.68	Lignans	NA	NA

Note: NA means not available.

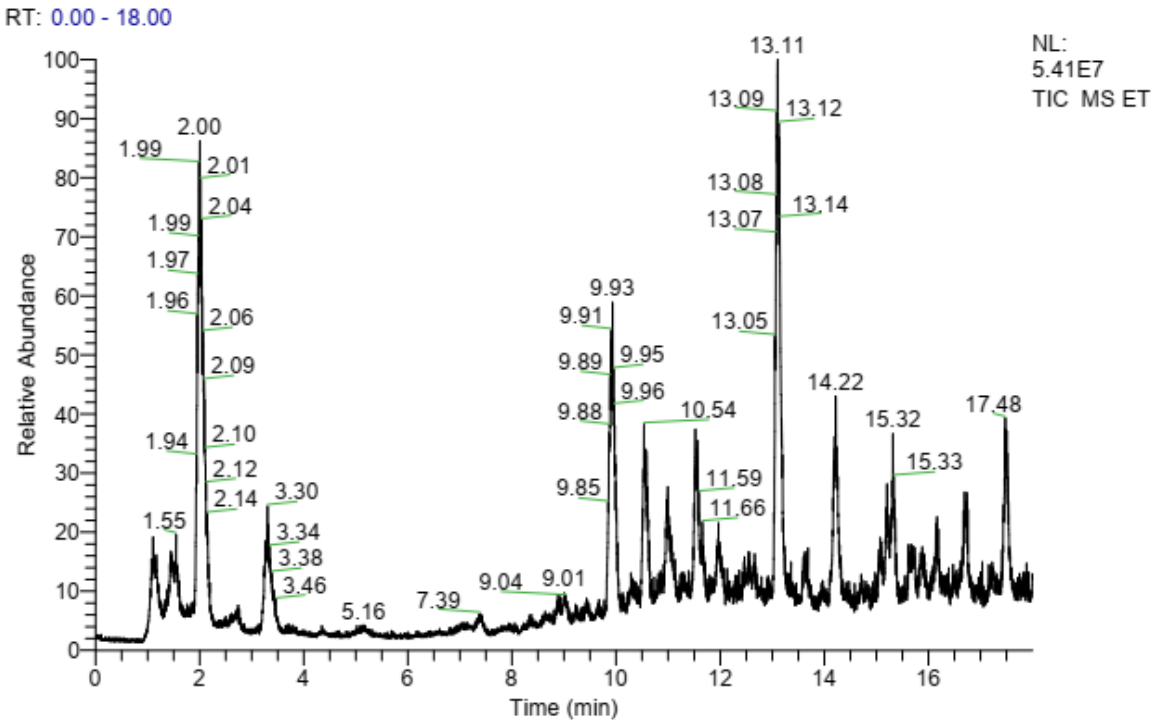

Fig. 2 – Total ion chromatogram of DEE (*Daldinia* ethanolic extract) separated by LCMS

Estimation of total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC) and total antioxidant activity (TAA) of DEE

In our experiment, the TPC of *Daldinia eschscholtzii* ethanolic extract (DEE) was 74.55 ± 1.44 mg GAE/g dry weight of the mushroom extract (DEE) (Data not shown in the table). Phenolic compounds (a large group of secondary metabolites) are involved in the oxidation process. These compounds also show antioxidant properties by scavenging free radicals, donating hydrogen atoms, and quenching singlet oxygen (Croft 1999, Sifat et al. 2020). Eating foods rich in polyphenols and phenolics can also help to protect against cancer and heart disease (Jayakumar et al. 2009). According to Mishra et al. (2019), the TPC of the ethyl acetate extract of the endophytic fungus *D. eschscholtzii* was 87.32 ± 8.96 µg GAE/mL. Chutulo & Chalannavar (2020) also reported that the TPC of the mixture of ethyl acetate and acetone solvent extract of *D. eschscholtzii* was 43.853056 ± 0.05894 GAE/g of the extract. Therefore, our DEE contained more TPC. It might be due to the fact that Chutulo & Chalannavar (2020) conducted their work on the culture extract of *Daldinia eschscholtzii*, whereas our experiment used the fruiting bodies of the fungus. This could be a probable cause for the difference in the same species. The antioxidant ability of phenolic compounds is positively associated with the hydroxyl content present in them.

The TFC of DEE was found to be 24.5 ± 0.55 mg QE/g dry weight of the DEE (Data not shown in the table). Similarly, Chutulo & Chalannavar (2020) reported that the TFC of *Daldinia eschscholtzii* extract was 27.4763 ± 0.68423 QE/g, which was nearly the same as our result. Flavonoids are the most widespread and broadly dispersed group of phenolic compounds (Yanishlieva-Maslarova 2001). The TPC and TFC of the extract of *Xylaria* sp were 65.91 ± 0.7 µg GAE/mg and 81.25 ± 1.9 µg QE/mgT (Chen et al. 2024, Pham et al. 2024). The TPC of the ethanolic extract of *X. papulis* was evaluated to be 43.17 mg GAE/g (De Leon et al. 2020). Cui et al. (2015) suggested that the antioxidant action of the extract might be associated with the presence of flavonoids in the extract. Depending on their molecular structure, flavonoids show antioxidant activity. The position of hydroxyl groups and other aspects in their chemical configuration, flavonoids show free radical scavenging activities and antioxidant activity (Saxena et al. 2012), and they are responsible for lipid stabilization, along with phenolic compounds (Liu et al. 2007).

Tannins are known as polyphenolic compounds and have been shown to have several biological activities, like antitumor, antimicrobial, and antioxidant activities (Hatano et al. 1989). In

our experiment, the total tannin content (TTC) found in DEE was 15.83±0.47 mg TAE/g dry weight of extract (Data not shown in table). Yilmaz et al. (2017) reported that the tannin content of the *Pleurotus ostreatus* mushroom cultivated on walnut sawdust was 1.011 ± 0.088 CE mg/g. This value is lower than our result. Our value is also higher than the previously reported work on some wild mushrooms like *Hygrocybe conica* (0.251 ± 0.011 CE mg/g), *Schizophyllum commune* (0.280 ± 0.024 CE mg/g), *Lentinus ciliatus* (0.343 ± 0.030 CE mg/g), and the cultivated mushroom *Pleurotus ostreatus* (0.326 ± 0.025 CE mg/g) (Hip et al. 2009). Tannin contents in QUENCHER extracts and water extract of *Terfezia boudieri* (Ascomycota) were 8.12 mg TAE/g DW and 4.34 mg TAE/g DW, respectively (Hammami et al. 2023). In our experiment, the total antioxidant content of the DEE was measured by phosphomolybdenum assay spectrophotometrically, and it was found to be 135.9 ± 1.09 mg AAE/g dry weight of the extract (Data not shown in the table). It is commonly known that the development of chronic diseases linked to oxidative stress can be reduced by eating foods rich in phytochemicals with strong antioxidant activity (Khan et al. 2016). The total antioxidant content of mushroom extract is based on its ability to reduce Mo (VI) to Mo(V) and is estimated by the subsequent development of a green phosphate/Mo(V) complex at an acidic pH (Sudha et al. 2012).

Antioxidant activity

DPPH free radical scavenging activity

The result showed that the DPPH radical-scavenging activity of DEE was concentration-dependent (Fig. 3). EC_{50} value of DEE was 3.13 ± 0.14 mg/mL, and Butylated hydroxy anisole (BHA), the commercial synthetic antioxidant, was used as a standard, and the EC_{50} of BHA was 0.0364 ± 0.0002 mg/mL for scavenging DPPH free radical (Table 2). The Pearson correlation coefficient test result showed that the relationship between the concentration of DEE and DPPH free radical scavenging percentage was statistically significant ($p<0.05$). DEE was seen to scavenge the stable DPPH radical directly to varying degrees across a concentration range of 0.1–5 mg/mL with a scavenging percentage of 2.9–68.3%. DPPH is a stable free radical. To what extent free radicals are scavenged by an antioxidant in a sample is determined by DPPH radical scavenging. Because it takes a short time to complete the analysis, the DPPH free radical scavenging assay is widely used (Mwangi et al. 2022). It was reported by Chutulo & Chalannavar (2020) that the DPPH radical scavenging percentages of the crude extract of *Daldinia eschscholtzii* were concentration-dependent, and at the highest concentration, the scavenging percentage was 80.298 ± 0.0608%. According to Yildiz et al. (2015), the EC_{50} values of methanolic extract of four different wild mushrooms, like *Morchella esculenta* (Ascomycota), *Hericium erinaceus*, *Lentinula edodes*, and *Ganoderma lucidum* (Basidiomycota), were 1.923 ± 0.46 mg/mL, 8.386 ± 0.43 mg/mL, 4.994 ± 0.51 mg/mL, and 1.763 ± 0.38 mg/mL, respectively. The ethanolic extracts of *Xylaria nigripes* exhibited EC_{50} values of DPPH radical scavenging activity ranging from 2.0 ± 0.1 to 2.7 ± 0.1 mg/mL (Divate et al. 2017). According to Ko et al. (2009), water and ethanol extracts of *X. nigripes* showed that the EC_{50} values of DPPH were 62.07 µg/mL and 73.49 µg/mL, respectively. The antioxidant potential of all ethyl acetate extracts from ten isolates of *Xylaria* species was estimated using the DPPH and ABTS radical scavenging assays, and data exhibited that all extracts had antioxidant activities, but out of them, *X. vinacea* SWUF18-2.3 had the lowest EC_{50} values (0.020 ± 0.004 to 0.194 ± 0.031 mg/mL) (Wangsawat et al. 2021). The effect of antioxidants on DPPH radical scavenging is due to their hydrogen-donating capacity, which may be due to phenolic contents (Tepe et al. 2011) and flavonoid contents (Saxena et al. 2012).

Reducing power activity

The reducing power assay is used to evaluate the ability of fungal extract to donate electrons. Through the donation of an electron, antioxidants reduce the Fe^{3+}-ferricyanide complex to the ferrous form (Fe^{2+}), and the resultant Perl's Prussian blue color for the formation of ferrous ions (Fe^{2+}) can be monitored spectrophotometrically at 700 nm. Greater reducing power is indicated by

the higher absorbance (Öztürk et al. 2007).

In our study, it was found that DEE showed dose-dependent reducing power activity (Fig. 3). The reducing power of DEE ranged from 0.011 to 0.983 in the concentration range of 0.1–5 mg/mL, and the reducing capacity of DEE increased with increasing concentrations. The EC_{50} value was 2.47 ± 0.05 mg/mL, whereas the EC_{50} of BHA (standard synthetic antioxidant) for reducing power activity was 0.039 ± 0.0003 mg/mL (Table 2), which was higher than the DEE. The Pearson correlation coefficient test result showed that the relationship between the concentration of DEE and reducing power was statistically significant ($p<0.05$). The reducing power of ethanolic extract of the four endophytic ascomycetes (*Phomopsis* sp., *Xylaria* sp., *Diaporthe* sp. and *Epacris* sp.) was determined, and the results showed significant activity (Nath et al. 2012). Sudha et al. (2012) reported that the reducing power activity of the methanolic extract of the edible mushroom *Pleurotus eous* was 0.587 at 2 mg/mL. This result was relatively similar to ours.

Ferric-reducing antioxidant potential (FRAP) activity

The FRAP assay is a very simple and quick technique to measure the antioxidant activity of a sample. Antioxidants have the ability to reduce TPTZ-Fe^{3+} to a blue color TPTZ-Fe^{2+} complex at low pH with an absorption maximum at 593 nm (Benzie & Strain 1996). The ferric-reducing antioxidant potential of DEE was 0.921±0.005 mM Fe^{2+}/mg of the extract (Table 2). It was reported by Sudha et al. (2012) that the ferric reducing power of the ethyl acetate extract of *Pleurotus eous* was 0.635, and for the methanol extract of *Pleurotus eous*, the ferric reducing power was 0.250 at 2 mg/mL. The FRAP assay of the extract of *Diaporthe fraxini* ED2 (Ascomycota) exhibited TAC of 188.41 ± 18.67 µg AAE/mg extract (Tan et al. 2022). As antioxidants are the reducing agents and FRAP values are based on reducing ferric ions, the higher FRAP values show the higher antioxidant capacity. For reduction, antioxidants are able to donate a single electron or hydrogen atom. Therefore, the higher the total phenolic content, the higher the antioxidant activity (Yildiz et al. 2015).

Hydroxyl radical scavenging activity

The hydroxyl radical is the most reactive radical among the ROS because it has the potential to generate severe injury to adjacent biomolecules (Yang et al. 2014). In an attempt to balance the configuration of the unpaired electrons, hydroxyl free radicals (OH) produce a crucial oxidizing species that can attack and oxidize molecules within their vicinity. Thus, hydroxyl radicals react violently with oxidative systems because of their strong oxidizing power (Herraiz & Galisteo 2015). In our study, DEE was shown to scavenge hydroxyl radicals by 8.13-66.35% within a concentration range of 0.1-2 mg/mL (Fig. 3). The EC_{50} was 1.57 ± 0.06 mg/mL. To compare the hydroxyl radical scavenging activity of DEE with the standard synthetic antioxidant, BHA was used, and the EC_{50} of HBA was 0.296 ± 0.002 mg/mL (Table 2). The Pearson correlation coefficient test result showed that the concentrations of DEE were statistically significant with the hydroxyl radical scavenging percentages ($p<0.05$). The hydroxyl radical scavenging activity of ethyl acetate extract of *Xylaria* sp. was recorded by Pham et al. (2024), and it was 39.7 ± 2.6%. Hasnat et al. (2014) reported that the ethanolic extract of *Russula virescens* showed a scavenging potential against hydroxyl radical ranging between 39.69-63.71% at 0.125-2 mg/mL concentrations. It is well recognized that hydroxyl radicals have the ability to remove hydrogen atoms from the membrane and induce lipid peroxidation (Kitada et al. 1979). Therefore, it has been expected that DEE could be able to show activity against lipid peroxidation on membranes as it has scavenging activity against hydroxyl radicals.

Lipid peroxidation inhibition activity

The assembly of the membrane can be interrupted by lipid peroxidation, and that may cause changes in fluidity, permeability, alter the transport of ions and inhibit metabolic processes. More ROS are generated when lipid peroxidation damages the mitochondria (Mwangi et al. 2022). The removal of a hydrogen atom from the unsaturated fatty acyl chain initiates the process of lipid

peroxidation, and then it spreads as a chain reaction (Kohen & Nyska 2002). Therefore, the inhibition of lipid peroxidation is a great area of research for the development of a new drug. In our study, it was found that DEE was able to inhibit lipid peroxidation when egg yolk homogenate was used as a lipid-rich medium to measure the lipid peroxide, and the lipid peroxidation was induced by $FeSO_4$. The DEE was shown to inhibit lipid peroxidation from 9.93% to 63.02% in the concentration range of 0.1–5 mg/mL (Fig. 3). The Pearson correlation coefficient test result showed that the concentrations of DEE were statistically significant with the percentages of lipid peroxidation inhibition ($p<0.05$). The EC_{50} was found 2.71 ± 0.09 mg/mL (Table 2), whereas in the case of BHA, the EC_{50} was found to be 0.093 ± 0.0004 mg/mL. The inhibition of lipid peroxidation of two truffles (*Terfezia* sp. and *Picoa* sp.) (Ascomycota) was noted by and they were 87.8% and 94.3%, respectively (Murcia et al. 2002). It was reported by Sudha et al. (2012) that the methanolic extract of *Pleurotus eous* mushroom showed lipid peroxidation inhibition in liver homogenate and an inhibition percentage ranging from 16.92–79.23% at 2–10 mg/mL. EC_{50} values less than 10 mg/mL generally indicate the strong antioxidant properties of the extract (Sudha et al. 2012).

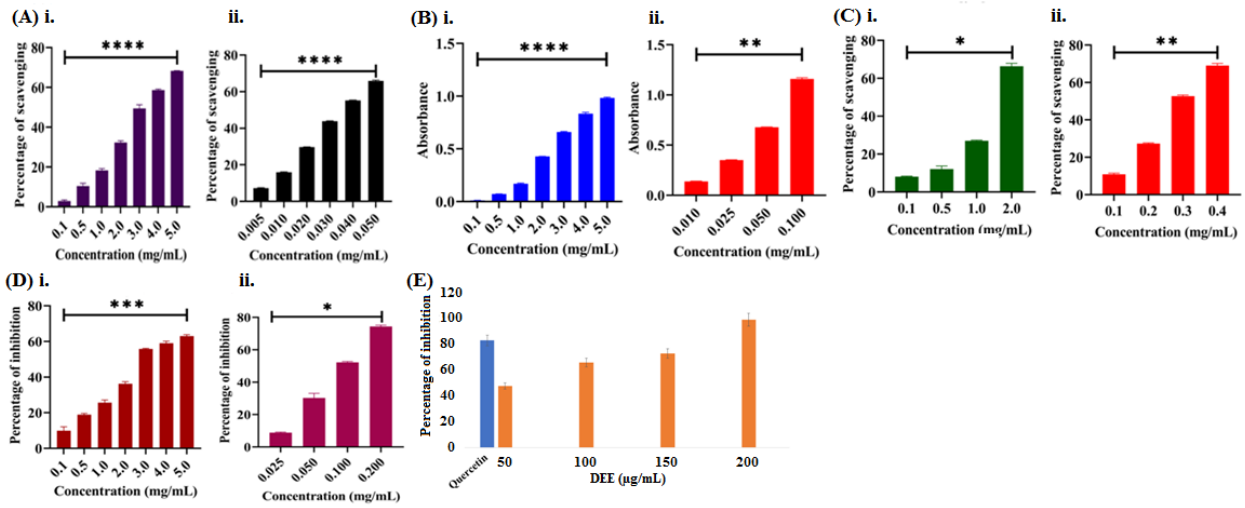

Fig. 3 – A DDPH radical scavenging activity by (i) DEE and (ii) BHA. B Reducing power activity of (i) DEE and (ii) BHA. C Hydroxyl radical scavenging activity of (i) DEE and (ii) BHA. D Lipid peroxidation inhibition activity of (i) DEE and (ii) BHA. and E Percentage of inhibition of LOX by DEE (50-200 µg/mL) and positive control (Quercetin (50 µg/mL)) (Mean ± SD inserted at the top of each bar) ($p<0.05$ in all experiments).

Table 2 EC_{50} values and FRAP activity of DEE for antioxidant property.

Sample name	DPPH (mg/mL)	OH radical (mg/mL)	Reducing power (mg/mL)	Lipid peroxidation inhibition (mg/mL)	FRAP (mM Fe^{2+}/mg extract)
DEE	3.13 ± 0.14	1.57 ± 0.06	2.47 ± 0.05	2.71 ± 0.09	0.921 ± 0.005
BHA	0.0364 ± 0.0002	0.296 ± 0.002	0.039 ± 0.0003	0.093 ± 0.0004	--

Spearman rank correlation test

The Spearman rank correlation was done to correlate the antioxidant content (TPC, TFC, TTC, and TAA) with the EC_{50} value of DPPH free radical scavenging, OH radical scavenging, lipid peroxidation inhibition reducing power, and the FRAP activity of DEE (Fig. 4). Positive correlation was also found between FRAP and the EC_{50} of reducing power and between TPC with TTC and

TAA, and TFC with TAA. Negative correlations were also noticed in this correlation study. The relationship was found to be negative between EC_{50} of OH radical scavenging, with EC_{50} of DPPH radical scavenging, EC_{50} of reducing power and FRAP and EC_{50} of lipid peroxidation inhibition, with EC_{50} of DPPH radical scavenging.

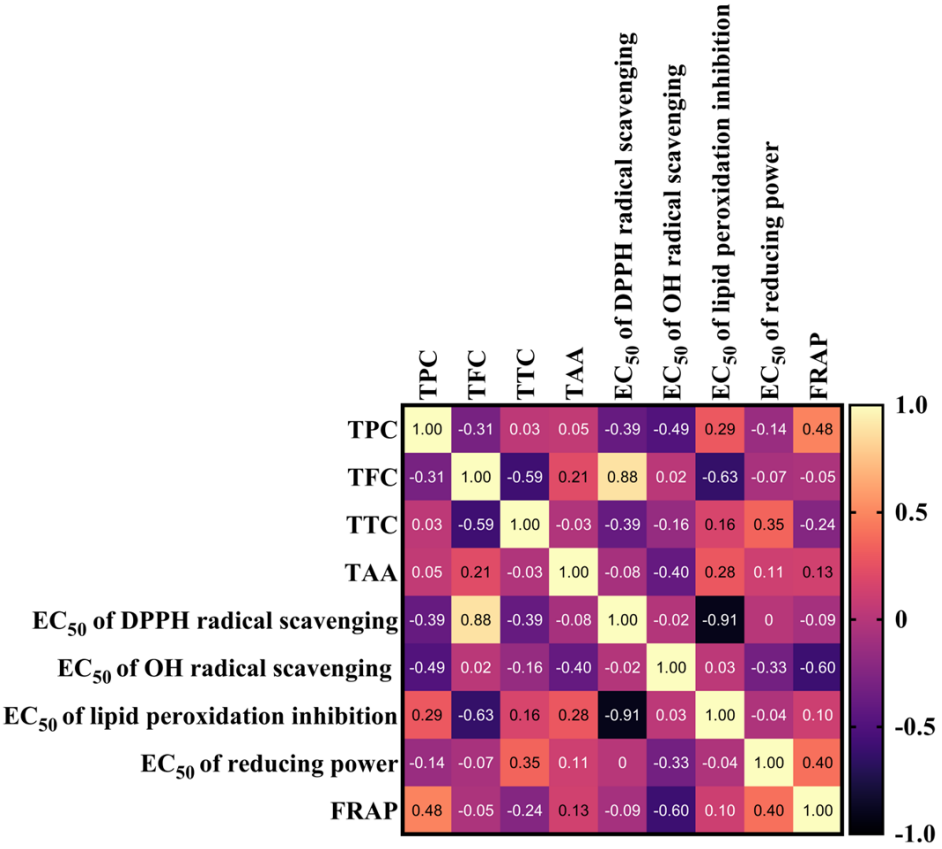

Fig. 4 – Spearman rank correlation test between antioxidant content (TPC, TFC, TTC and TAA) and EC_{50} of DPPH, OH radical scavenging activity, lipid peroxidation inhibition, reducing power and FRAP.

Lipoxygenase inhibitory test

The results showed that (Fig. 3) DEE at the highest concentration (200 µg/mL) gave 98.65± 10 % inhibition of the LOX while the lowest concentration (50 µg/mL) gave 47.45 ± 7.6% inhibition. Other concentrations (100 and 150 µg/mL) exhibited 65.75 ± 8.78 and 72.67 ± 8.90% of inhibition, respectively. While positive, quercetin (50 µg/mL) control gave 82.78 ± 9.78% inhibition, but the negative control showed no inhibition. The EC_{50} value of DEE was 87.54 ± 12 µg/mL. The EC_{50} value of quercetin was 73 ± 5 µg/mL (Malterud & Rydland 2000). From the literature search, we found that our work on 15-LOX inhibition by DEE is a report for the first time. Lee et al. (2014) conducted research on the LOX inhibitory effects of 335 samples of methanolic extracts of different species of mushrooms having a concentration of 100 µg/mL. From their results, we found that 37 spp. belonging to 32 genera of mushrooms exhibited inhibitory effects against the LOX enzyme, and the percentage (%) of inhibition ranged from 33.1-100 %. The highest inhibition (100%) was found by methanolic extract of *Phellinus baumii* followed by *Inonotus makadoi* (85.2%), *Collybia maculata* (73.3%), *Phellinus gilbus* (66.7%), *Strobilomyces confuses* (52.4%), while the lowest was found in *Clitocybe gibba* and *Phomitopsis pinicola* (each 31.1%). The IC_{50} values for the LOX inhibition assay were 368.5, 147.3 and 40.2 µg/mL for the petroleum ether extract, ethanol extract of *Tuber aestivum* (Ascomycota), and the positive control ascorbic acid, respectively (Marathe et al. 2020).

From our LC-MS data of DEE, we found that DEE was a mixture of many flavonoids,

phenolic, terpenoids and some new compounds. Among the flavonoids, Myricetin, Myricetin-3-O-xyloside, Myricetin-3-O-galactoside, Quercetin-3-(6"-malonyl)-glucoside, and Quercetin-3-glucuronide. were inhibitors of LOX as reported by earlier scientists (Lyckander & Malterud 1992, Malterud & Rydland 2000).

Toxicity test on a normal human cell line

The results of the toxicity test on human normal cell line HEK293T exhibited that the highest concentration of 1000 µg/mL had no effect on cellular morphology and nuclear morphology (Fig. not shown), and similarly, no inhibition of cell growth in contrast to the negative control (Figure and data not shown).

Properties of some compounds identified by the LC-MS

Based on Swiss ADMEs, AdmetSAR and pkCSM prediction web servers, the physio-chemical, lipophilicity, water solubility, pharmacokinetics, drug-likeness and medicinal chemistry properties of some compounds or ligands were studied (Table not shown). These properties are extremely linked with intestinal permeability and solubility in the initial step of oral bioavailability. If the number of violations of law for a molecule is more than two, the molecule can't be a drug molecule (Lipinski, 2004). Christopher Lipinski from Pfizer in 1997 created and introduced an easy scientific rule where he described the "Rule of 5" as the parameter cut-off values all contained 5's. There are only four parameters (https://www.pharmainformatic.com/html/rule_of_5.html). Lipinski's Rule of Five is used to determine the drug-likeness, which is the consistency of orally active drugs in humans (Lipinski 2004, Lipinski et al. 1997). To be potentially used as a drug, the bioactive compounds must not violate more than one parameter for oral administration. The absorption or permeation of a drug will be poor if molecular weight (MW) > 500 g/mol, the number of H-bond acceptors >10, the number of H-bond donors > 5, MlogP>4.15 (Lipinski et al. 1997). The drug score is used to select the bioactive compounds. The greater drug score value of the bioactive compounds denotes better drug candidates (Oduselu et al. 2019). Furthermore, Ghose filter (Ghose et al. 1999), Veber filter (Veber et al. 2002), Egan filter (Egan et al. 2000 and Muegge filter (Muegge et al. 2001) were applied for validation of drug-likeness of any compound. Web predictors like Swiss ADMEs (Daina et al. 2017), AdmetSAR (Yang et al. 2019) and pkCSM (Pires et al. 2015) prediction were used to screen eight ligands (phloretin, myricetin, phloridzin, quercetin-3-glucuronide, luteolin-7-O-glucuronide, myricetin-3-o-galactoside, quercetin-3-(6"-malonyl)-glucoside and amentoflavone) identified by LCMS study of DEE.

The results (Table not shown) showed that, among the eight ligands, only the phloretin compound fulfilled all the criteria of different filters (Lipinski filter, Ghose filter, Veber filter, Egan filter and Muegge filter). Based on SwissADME result, it was a low molecular weight water-soluble compound (MW 274.27 g/mol) with high gastrointestinal (GI) absorption and was able to inhibit CYP1A2 protein (over-expressed in tumor cells than the normal cells). Based on pkCSM predictor, phloretin was found as a nontoxic compound (Table not shown). Its hepatotoxicity, skin sensitization activity, hERG I and hERG II inhibition activity were not found through this pkCSM predictor. As the other seven compounds did not qualify the above filters, therefore, phloretin was only selected for further studies like molecular docking against human lipoxygenase, xanthine oxidase and cytochrome P450 family 1 (CYP1A1) protein. Based on the SwissADME predictor and pkCSM predictor, it was found that only phloretin was best fitted for oral drug preparation.

Molecular docking of phloretin with lipoxygenase, xanthine oxidase and cytochrome P450 family 1 (CYP1A1)

Reactive oxygen species (ROS) are produced in mammalian cells through a variety of cellular oxidative processes by xanthine oxidases and lipoxygenases (LOX) (Cho et al. 2011). Cytochrome P450 (CYP) is also responsible for the generation of ROS through their reaction cycle (Veith & Moorthy 2018) and also metabolizes the drugs ingested in the liver. Therefore, for the docking study of the selected ligand with these proteins, the crystal structure of human lipoxygenase (PDB

ID: 1HU9) (Ghansenyuy et al. 2023), xanthine oxidase (PDB ID: 3NVY) (Cao et al. 2014) and cytochrome P450family 1 (CYP1A1 (PDB ID: 4I8V)) (Walsh et al. 2013) were obtained from the protein data bank (http://www.rcsb.org) in PDB format. Though cytochrome P450 is responsible for the generation of ROS, CYP1A1 (family 1 of cytochromes P450) was chosen because it can also generate ROS (Stading et al. 2020) and is over-expressed in tumor cells compared to normal cells (Nebert & Russell 2002).

To study the binding interaction, affinity and stability of phloretin with different amino acids of the active site of lipoxygenase, xanthine oxidase and cytochrome P450 family 1 (CYP1A1), phloretin was docked with the above proteins. It was found that phloretin had a significant potential as a ligand in the binding domain of the lipoxygenase, xanthine oxidase and cytochrome P450 family 1. The calculated affinities (kcal/mol) were -8.7, -8.6 and -8.1kcal/mol for the binding of phloretin with the active site of lipoxygenase, xanthine oxidase and cytochrome P450 family 1 (CYP1A1), respectively (Table 3). The docking result showed that there were two hydrogen bonds observed between phloretin and lipoxygenase (Fig. 5A). In the case of phloretin and xanthine oxidase, four hydrogen bonds were found (Fig. 5B) and five hydrogen bonds were shown between phloretin and cytochrome P450 family 1 (CYP1A1) (Fig. 5C). The amino acids in the active site of lipoxygenase were Asn142, Phe157, Phe158, Glu257, Asp256, Asn529, Val534, Tyr539, Phe543 and the amino acids in the active site in xanthine oxidase were Leu257, Val259, Asn261, Glu263, Ile264, Ala301, Gly350, and Thr354. In the case of CYP1A1, the amino acids in the active site were Val116, Ala117, Arg128, Leu298, Ala301, Gly302, Cys439, Phe440, Leu444, and Ala445. The above docking results indicated that this phloretin compound was able to bind with the active site of these proteins (lipoxygenase, xanthine oxidase and CYP1A1) and inactivated or inhibited these proteins for the generation of ROS. Similarly, some scientists predicted the affinity of some compounds with the target receptor in three enzymes (lipoxygenase, xanthine oxidase and CYP1A1) and they also found their compounds inactivated these enzymes (Ghansenyuy et al. 2023, Cao et al. 2014, Walsh et al. 2013). Our wet lab experiments, like antioxidant activities, also showed that DEE was able to scavenge the free radicals, which justified this result. One interesting fact was that the SwissADME result also supported the inhibition activity of phloretin to cytochrome P450 family 1 (Table 3).

Table 3 – Docking results of phloretin with lipoxygenase, xanthine oxidase and cytochrome P450 family 1 (CYP1A1)

Target protein	**Bioactive compound**	**Calculated affinity (kcal/mol)**	**No. of hydrogen bond**	**Amino acids**
Lipoxygenase (PDB ID: 1HU9)	Phloretin	-8.7	2	Asn 142, Phe 157, Phe 158, Glu 257, Asp 256, Asn 529, Val 534, Tyr 539 and Phe 543
Xanthine Oxidase (PDB ID: 3NVY)	Phloretin	-8.6	4	Leu 257, Val 259, Asn 261, Glu 263, Ile 264, Ala 301, Gly 350 and Thr 354
Cytochrome P450 (CYP1A1 (PDB ID: 4I8V)	Phloretin	-8.1	5	Val 116, Ala 117, Arg 128, Leu 298, Ala 301, Gly 302, Cys 439, Phe 440, Leu 444 and Ala 445

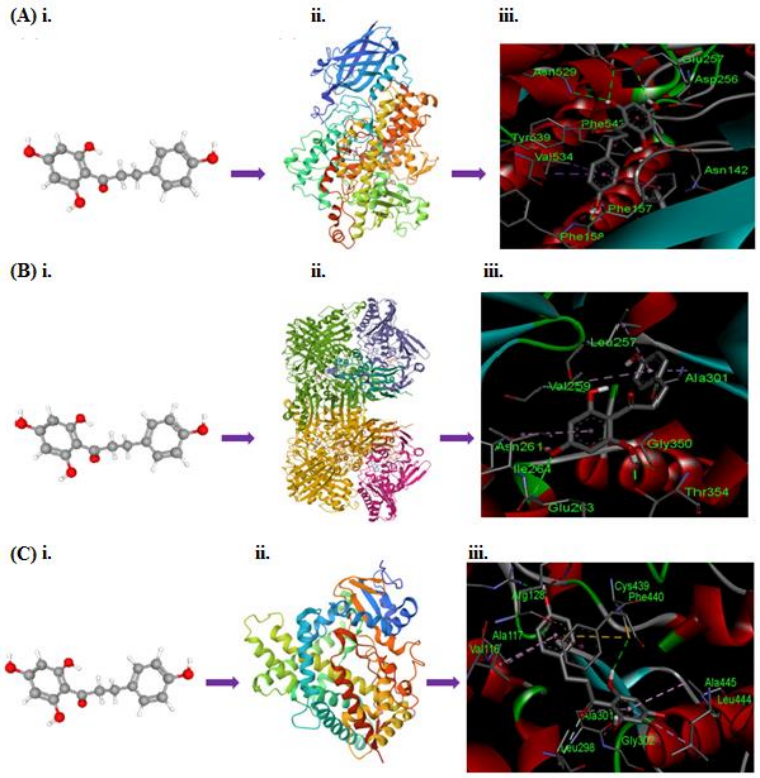

Fig. 5 – Molecular docking of phloretin with A lipoxygenase (i) 3D conformer of phloretin (ii) 2D structure of lipoxygenase, and (iii) interaction of phloretin with the active site of lipoxygenase. B xanthine oxidase (i) 3D conformer of phloretin (ii) 2D structure of xanthine oxidase and (iii) interaction of phloretin with the active site of xanthine oxidase. and C CYP1A1 (i) 3D conformer of phloretin (ii) 2D structure of CYP1A1and (iii) interaction of phloretin with the active site of CYP1A1

Conclusions

Daldinia eschscholtzii is an Ascomycota fungus, which has no culinary importance in West Bengal, as well as in India and other countries. Although some researchers have isolated this fungus as an endophyte from several sources (medicinal plants, human nails, blood, skin, etc.), we have collected its fruit body from a fallen log. Our experiments on antioxidant content and activities showed that the solvent extract (DEE) of fruit bodies of this fungus has a good amount of antioxidant contents (total phenol, total flavonoid, total tannin) and good scavenging properties of ROS, and these are comparable with other medicinal fungi or mushrooms. The DEE showed good inhibitory property against 15-lipoxygenase. Approximately 25 bioactive compounds have been detected by LC-MS from DEE, and literature shows that some are important anti-cancerous, some are antioxidants, and a few belong to both categories of compounds. Out of them, we have searched the chemical properties of eight compounds by SwissADME predictor and pkCSM predictor, and found that only phloretin was best fitted for oral drug preparation. In addition, as per our molecular docking study, we have justified that phloretin, the main compound present in the DEE, has high molecular affinity or binding capacity with our ROS-producing enzymes, and most probably, it inactivated or inhibited them from producing ROS. The novelty of this work is that it reports, for the first time, the antioxidant content, antioxidant activity, lipoxygenase inhibitor activity, 25 bioactive compounds of this mushroom, including Phloretin, and molecular interaction with ROS-producing enzymes in human. Furthermore, DEE showed no toxicity on the normal human cell line HEK 293T. Therefore, this research discovers that this fungus or DEE is a good source of antioxidants, inhibitors of ROS-stimulating enzymes and anti-cancerous bioactive compounds, including Phloretin, (-)-Curcumol, and Tricin, that can be utilized for oral drug preparation or as a food additive.

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Credit authorship contribution statement

T.B has done the experimental works, data collection, statistical analysis, figure preparation and manuscript preparation, and S.K.G has advised on study design and edited the manuscript. R.C has done the LCMS experiment and literature search. All authors have viewed the manuscript.

Data availability statement

All data generated or analyzed during this study are included in this article or are available from the corresponding author on reasonable request.

Competing financial and non-competing financial interest

Authors declare they have no competing financial and non-financial interests.

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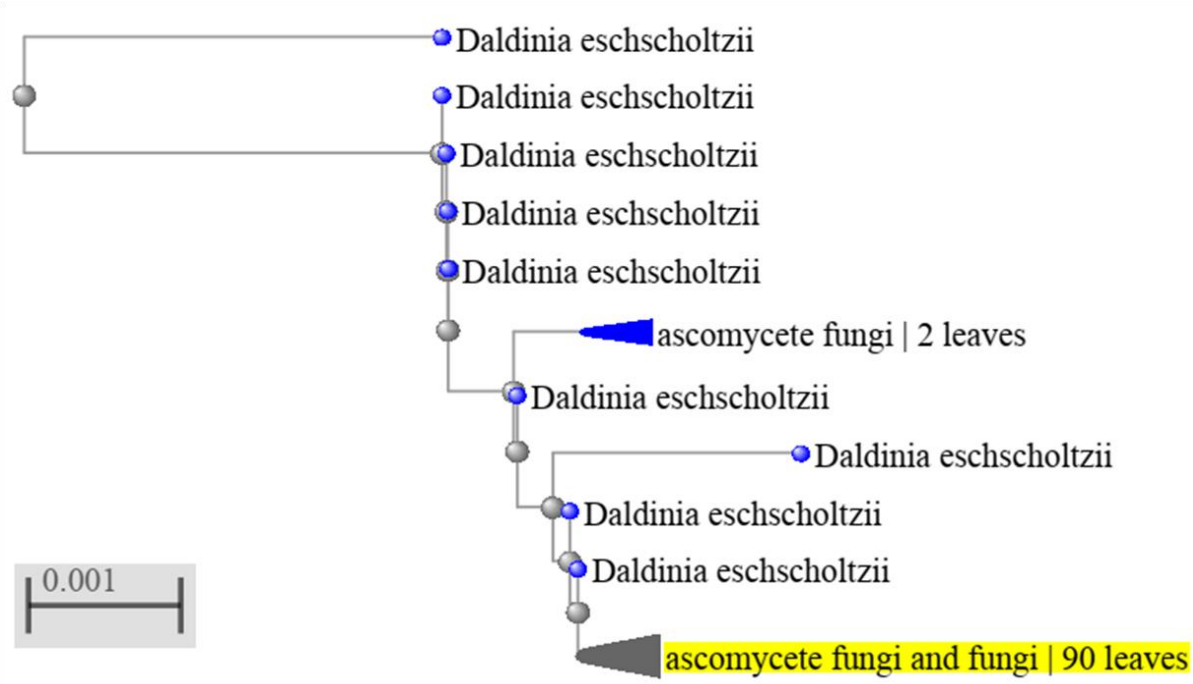
Supplementary Figures

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Daldinia eschscholtzii isolate 2a small subunit ribosomal RNA gene, partial sequence; internal transcribed sp...	Daldinia eschsc...	965	965	99%	0.0	100.00%	603	MK849924.1
Daldinia eschscholtzii isolate VOLN2 small subunit ribosomal RNA gene, partial sequence; internal transcribe...	Daldinia eschsc...	965	965	99%	0.0	100.00%	571	KX621971.1
Daldinia eschscholtzii isolate Endolichenic small subunit ribosomal RNA gene, partial sequence; internal tran...	Daldinia eschsc...	965	965	99%	0.0	100.00%	555	OR506241.1
Fungal endophyte isolate 856 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and ...	fungal endophyte	965	965	99%	0.0	100.00%	543	KR016924.1
Daldinia eschscholtzii strain RF 110 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1...	Daldinia eschsc...	965	965	99%	0.0	100.00%	626	KT151596.1
Daldinia eschscholtzii strain JF 20 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 ...	Daldinia eschsc...	965	965	99%	0.0	100.00%	649	KT151578.1
Daldinia eschscholtzii isolate B001 small subunit ribosomal RNA gene, partial sequence; internal transcribed ...	Daldinia eschsc...	965	965	99%	0.0	100.00%	592	OP413056.1
Daldinia eschscholtzii isolate HNNUZCJ-065 small subunit ribosomal RNA gene, partial sequence; internal tr...	Daldinia eschsc...	965	965	99%	0.0	100.00%	584	OM278371.
Daldinia eschscholtzii isolate HNNUZCJ-052 small subunit ribosomal RNA gene, partial sequence; internal tr...	Daldinia eschsc...	965	965	99%	0.0	100.00%	585	OM278358.
Daldinia eschscholtzii isolate HL-3231 18S ribosomal RNA gene, partial sequence; internal transcribed space...	Daldinia eschsc...	965	965	99%	0.0	100.00%	586	KJ466979.1
Daldinia eschscholtzii isolate PH29 small subunit ribosomal RNA gene, partial sequence; internal transcribed ...	Daldinia eschsc...	965	965	99%	0.0	100.00%	587	MN613135.
Daldinia eschscholtzii strain CFE-70 small subunit ribosomal RNA gene, partial sequence; internal transcribe...	Daldinia eschsc...	965	965	99%	0.0	100.00%	545	MW045594.

Supplementary Fig. 1 – Homology searching of nucleotide sequence by BLASTn (NCBI data base)



Supplementary Fig. S2 – Phylogenetic tree constructed by Neighbor Joining method (Max Seq Difference 0.05)