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RESEARCH ARTICLE

GC-MS profiling, antioxidant evaluation and *in silico* pharmacological assessment of bioactive compounds from *Aspergillus welwitschiae* imrd1 extract

Farjana Akter Koly[1], Fabiha Zaman Neetu[2], Oaindrila Bhattacharjee[3], Rajib Sarkar[4], Jannatul Ferdous[1], Sumaiya Islam Chowdhury[1], Abdullah Muhammad Waazid[3], M Jakir Hossain[5] & Saiful Islam[1*]

[1]Industrial Microbiology Research Division, Bangladesh Council of Scientific and Industrial Research Chattogram Laboratories, Chittagong 4220, Bangladesh
[2]Daegu Gyeongbuk Institute of Science and Technology, Daegu 42988, South Korea
[3]Department of Microbiology, University of Chittagong, Chittagong 4331, Bangladesh
[4]Institute of Food Science and Technology, BCSIR, Dhaka 1205, Bangladesh
[5]Forest Chemistry Division, Bangladesh Forest Research Institute, Chittagong 4211, Bangladesh

*Correspondence email - saifulctg@bcsir.gov.bd

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Abstract

The present study evaluated the significance of *Aspergillus welwitschiae* imrd1 extracts in terms of total phenolic content (TPC), total flavonoid content (TFC), GC-MS profiling, antioxidant activity and *in silico* pharmacological properties. Metabolites were extracted using methanol and ethyl acetate, where the ethyl acetate extract exhibited higher levels of TPC (8.57±0.31 mg GAE/g) and TFC (12.43±1.23 mg QE/g). Further experiment on ethyl acetate extract revealed an IC50 value of 38.79 µg/mL in the DPPH scavenging assay. GC-MS analysis detected twenty-two compounds, among which four components-methyl stearate, hexadecanoic acid methyl ester, 9,12-octadecadienoic acid methyl ester and 11-octadecenoic acid methyl ester-were selected for molecular docking against NADPH oxidase. These ligands showed desired physicochemical properties and acceptable binding affinities of -5.0 to -5.7 Kcal/mol while examined *in silico*. These findings are evidence of the efficacy of *Aspergillus welwitschiae* imrd1 extracts as a promising source of natural antioxidants in developing alternative therapeutics.

Keywords: antioxidant; *Aspergillus welwitschiae* ; extract; GC-MS; molecular docking ; pharmacokinetic properties

Introduction

Oxidative stress (OS) is a pathophysiological condition detrimental to human health, primarily arising from the redundant production and persistence of reactive oxygen species (ROS). Excessive accumulation of ROS also leads to oxidative damage to proteins, lipids and DNA, contributing to the onset and progression of cardiovascular diseases, diabetes, rheumatoid arthritis, cancer and neurodegenerative disorders (1). Production of these reactive species rises under stress, UV exposure, infections and several diseases. Various enzymes such as NADPH oxidase, lipoxygenase and cytochrome P450 further contribute to their generation. Favorably, antioxidants help neutralize unstable free radicals and maintain free radical balance (2). When this equilibrium is disturbed, oxidative stress can occur. Consequently, research on antioxidants as free radical scavengers is essential for developing strategies to combat these harmful effects (3).

Natural antioxidants may be obtained from diverse origins, among which secondary metabolites from microbial sources represent a significant repertoire. Secondary metabolites containing components such as phenol, 3,5-bis(1,1-dimethylethyl) (4); hexadecanoic acid, methyl ester; 9,12-octadecadienoic acid, methyl ester; 13-docosenoic acid, methyl ester, (Z)-; methyl stearate; bis (2-ethylhexyl) phthalate; heneicosane and some phenolic derivatives have been studied extensively for their diverse biological activities, highlighting their potential as antioxidant agents (5, 6). Filamentous fungi belonging to the phylum Ascomycota and genus *Aspergillus* (7) are reported to contain relatively good proportions of mycochemicals that have potential antioxidant properties (8, 9). In particular, members of the section *Nigri* are historically proven for their widespread application in biotechnological processes (10, 11). *Aspergillus welwitschiae,* a species of the section *Nigri*, has gained much attention due to its ubiquitous nature (12). This species has previously been studied for its pathogenic nature and production of mycotoxin (13). However, extracts produced by most fungi are often considered safe, as mycotoxins and certain secondary metabolites are typically not produced under controlled and industrially relevant growth conditions (14, 15). The industrial utilization of *Aspergillus welwitschiae* as an amylase producer has already been reported (16). Despite this, studies on the antioxidant potential of secondary metabolites produced by this fungus remain largely unexplored.

On the other hand, the identification and development of drug lead compounds in the laboratory are often laborious and time-consuming. In this context, computer-aided structure-based drug design is an emerging alternative, offering a rapid and cost-effective approach in drug discovery. The integration of *in silico* molecular docking with experimental investigations will provide a specific and efficient way to evaluate and optimize candidate bioactive molecules in the drug development process (17, 18).

In light of the above information, this study first assessed the total phenolic and flavonoid contents of methanol and ethyl acetate extracts from the *Aspergillus welwitschiae* isolate imrd1. The ethyl acetate extract was then selected for determination of antioxidant activity and further chemical profiling using GC-MS analysis. Finally, *in silico* analyses of physicochemical properties and toxicity prediction were carried out to estimate the druggability of these compounds and thereafter compounds having promise of being drug-like were evaluated as inhibitors of NADPH oxidase using molecular docking studies.

Materials and Methods

Study duration and collection of fungi

The study was carried out from July 2023 to October 2024 at the BCSIR Chattogram Laboratories (Industrial microbiology research division). The fungal strain *Aspergillus welwitschiae* imrd1 (Fig. 1), previously submitted to NCBI under accession no. MW332490, was collected from the culture collection of this laboratory.

Media preparation and revival of *Aspergillus welwitschiae* imrd1

Potato Dextrose Agar (PDA) medium (200 g potato, 20 g dextrose, 15 g agar, 1000 mL distilled water) was prepared and sterilized by autoclaving at 121 °C and 15 psi for 20 min. *Aspergillus welwitschiae* isolate imrd1 was revived from glycerol (20 % *v/v*) stock stored at -80 °C. The stock was thawed slowly and cultured onto PDA medium and incubated at 25 ° C for seven days (19) .

Extraction of secondary metabolites

The procedure followed by previous researchers was utilized to carry out the extraction of secondary metabolites with slight modification. The revived organism was grown in potato dextrose broth (PDB) for 21 days using (30×250 mL) conical flasks. Fermentation broth was separated from the fungal biomass by the filtration method (Whatman® qualitative filter paper, Grade 1) and an equal volume of ethyl acetate (100 %) was used to extract the metabolite layer. The desired portion of metabolites was extracted using a separating funnel. The mycelial biomass was dissolved in methanol (100 %) and kept under dark conditions for two days. Methanolic extract was also collected subsequently. The obtained metabolite layers from both solvents were concentrated by evaporation using a rotary evaporator. For further analysis, the dried extracts were stored in tightly closed glass vials in refrigeration (20).

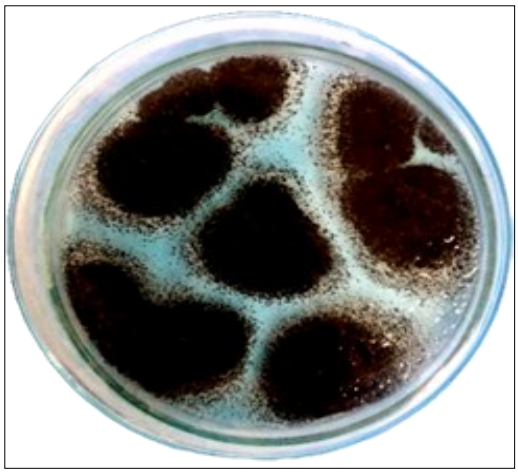

Fig. 1. Cultural characteristics of *Aspergillus welwitschiae* isolate imrd1.

Analysis of total phenolic and flavonoid contents

Determination of Total Phenolic Content (TPC)

TPC was determined using the spectrophotometric method described earlier (21). Briefly, 500 µL of each crude extract (1 mg/mL) was combined with distilled water up to a volume of 3 mL. Then, 0.25 mL of Folin-Ciocalteu reagent was added and mixed properly for 5 min, followed by the incorporation of 1 mL of 7.5 % (w/v) sodium carbonate. The diluted solution was then homogenized and left to stand for 90 min under dark conditions. The absorbance of the reaction mixture was measured at 760 nm. Gallic acid was used to prepare the calibration curve and result of TPC was presented as milligram (mg) gallic acid equivalent (GAE) per gram of extract.

Determination of Total Flavonoid Content (TFC)

TFC of the extracts was measured using the aluminium chloride colorimetric method (21, 22). 0.5 mL of both extracts (1 mg/mL) were taken in separate test tubes and 2 mL of distilled water was combined. Then, 0.15 mL of 5 % $NaNO_2$ was added to each tube. Following 5 min incubation, 0.15 mL of 10 % aluminum chloride was incorporated and the mixture was allowed to stand for 6 min. After that, 1 mL of 1 M NaOH was introduced and the volume was made up to 5 mL with distilled water. Following 10 min of incubation, the absorbance of the resulting solution was recorded at 510 nm. Finally, TFC value was calculated from a calibration curve prepared using quercetin as standard and the result was expressed as mg quercetin equivalent (QE) per gram of extract.

Antioxidant activity of *Aspergillus welwitschiae* imrd1 extracts

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

With slight modification, the DPPH radical scavenging capacity of the ethyl acetate extract was evaluated following the procedure reported by previous researchers (23). A working DPPH (Sigma-Aldrich, Germany) solution (0.2 mM *w/v*) was made using methanol. Then 100 µL of the DPPH solution was combined with 100 µL of serially diluted sample at the concentrations 200, 100, 50, 25, 12.5 and 6.25 µg/mL within the 96 microtiter plate wells. For negative control, DPPH and HPLC-grade methanol (*Honeywell, Germany*) at the same ratio were added to the corresponding wells of the microtiter plate. Ascorbic acid was served as positive control. The mixture was incubated at 37 °C in dark conditions for 30 min. Following incubation, absorbance was measured at a wavelength of 517 nm. The inhibition of the DPPH radical by the active compounds in the sample was examined by estimating the DPPH free radical scavenging efficiency percentage according to the following formula. Finally, IC50 values were calculated.

$$\text{DPPH scavenging rate (\%)} = \frac{\text{Absorbance of DPPH alone} - \text{Absorbance of DPPH in presence of extracts or ascorbic acid}}{\text{Absorbance of DPPH alone}}$$

Profiling of compounds

To examine the chemical composition of the ethyl acetate extract, GC-MS analysis was carried out as described previously (24). The analysis was performed using GCMS-QP2020 (Shimadzu) equipped with a GC-2010 Plus Gas Chromatograph, an AOC-20s autosampler, an AOC-20i autoinjector and an SH-Rxi-5Sil MS capillary column (30 m × 0.25 mm internal diameter × 0.25 µm film thickness; cross bond 5 % diphenyl-95 % dimethylpolysiloxane) interfaced with a Mass Spectrometer. Helium was used as the carrier gas. Samples were injected in splitless mode at an injector temperature of 280 °C. The oven temperature was programmed as follows: 80 °C for 2 min, ramped to 150 °C (7 °C/min) for 5 min and finally increased to 280 °C (7 °C/min) for 5 min. The ion source and interface temperatures were set at 280 °C and 250 °C, respectively. The mass spectra were obtained within the scan range of 50–500 m/z. The chemical constituents and their relative concentrations were identified by comparing the average peak areas with the total area and matching the spectra with the National Institute Standard and Technology (NIST) libraries (NIST08, NIST08s and NIST14).

Computational studies

In silico drug likeness and toxicity predictions

In order to predict the drug-likeness property of the studied compounds, Lipinski's rule of five was applied (25) and pharmacokinetic parameters were predicted using the SwissADME web server (26). The toxicity factors of the compounds were further examined with the OSIRIS property explorer (27) and the ProTox 3.0 web server (28). OSIRIS property explorer was used to assess toxicity risks such as mutagenicity (MUT), irritation (IRR), tumorigenicity (TUM) and reproductive development (REP) toxicity. ProTox 3.0 was used to study the carcinogenicity (CARC), cytotoxicity (CYT) and immunotoxicity (IMM). In addition, ProTox 3.0 server was employed to estimate the rat oral acute toxicity LD50 values and corresponding toxicity classes.

In silico molecular docking

The three-dimensional crystal structure of the NADPH oxidase (PDB ID: 2CDU) was retrieved from Protein Data Bank (www.rcsb.org). All undesirable objects and water molecules were removed using Discovery Studio Visualizer 2020 (29). Polar hydrogens were added and Kollman charges were assigned using MGL Tools 1.5.6 (30). Then, energy minimization of the protein was carried out with the GROMOS96 43B1 force field implementation of Swiss-PdbViewer v4.1.0. (31). The structure of the selected ligands and the standard (ascorbic acid) were obtained from the Pubchem database (https://www.pubchem.ncbi.nlm.nih.gov). Molecular docking was performed using AutoDock Vina integrated in PyRx 0.8 (32). Both the optimized protein (target) and all ligand structures were imported into the PyRx docking wizard. The energy of ligands was minimized and the structure of the target was converted to AutoDock macromolecule. All docking files were prepared in PDBQT format. A grid box enclosing the entire protein was defined with dimensions of X axis: 64.55 Å, Y axis: 63.15 Å and Z axis: 68.98 Å. After docking was completed, conformations were ranked according to their binding energies, with the pose exhibiting the lowest binding energy considered the best docking score. The interactions between the protein and ligands were finally visualized using Discovery Studio Visualizer 2020.

Statistical analysis

All experiments were conducted in three replicates and the results were expressed as mean ± SD (standard deviation). The obtained data were analyzed statistically with Microsoft Excel and R Studio. For IC50 values, linear regression analysis was used.

Results and Discussion

The chemical constituents of secondary metabolites have garnered significant attention in the development of alternative remedies. Secondary metabolites are often referred to as nature's biological response modifiers while possessing compounds with bioactive properties (33). In the present study, the secondary metabolites of *Aspergillus welwitschiae* imrd1 were chemically profiled and antioxidant activity was evaluated by integrating laboratory experiments with in silico analyses.

Morphological characteristics of *Aspergillus welwitschiae* imrd1

Colonies of *Aspergillus welwitschiae* imrd1 grew efficiently on PDA medium at 25 °C, growing to a diameter of 43-45 mm in seven days; white mycelia with black spores were observed. Exudate and soluble pigment were not produced (Fig. 1). The fungus was subsequently subjected to fermentation in PDB. The resulting culture was extracted separately using ethyl acetate and methanol and both extracts were preserved at 4 °C for subsequent experiments.

Estimation of TPC and TFC

Both methanolic and ethyl acetate extracts contained significant quantities of TPC and TFC. The amount was found to be higher in ethyl acetate extract compared to methanolic extract (Fig. 2). Quantitative estimation revealed that the ethyl acetate extract contained 12.43 ± 1.23 mg QE/g of TFC and 8.57 ± 0.31 mg GAE/g of TPC, whereas the methanolic extract showed 7.04 ± 0.82 mg QE/g of TFC and 2.33 ± 0.19 mg GAE/g of TPC.

Previous studies have also demonstrated the presence of mycoconstituents like TPC and TFC in the secondary metabolites of fungi such as *Aspergillus* spp (34, 35). However, the concentrations may vary substantially depending on the cultivation medium and fermentation conditions and the fungal species selected for metabolite production. Moreover, variation can also result from the types of solvent chosen for extraction (36, 37). Previous reports found that lower TPC levels in methanol extracts of *Aspergillus awamori* when using three different solvents (38). Ethyl acetate, in contrast, has been widely reported as an effective solvent for maximizing the bioactivity of fungal extracts, which is consistent with our current findings (39). Former researchers studied fermented cultures of *Aspergillus oryzae* and *Aspergillus niger* to evaluate TPC, flavonoids and antioxidant activities of oats. They used four different solvents (*n*-hexane, ethyl acetate, *n*-butanol and water) and compared the results with unfermented oats. They observed a significant increase in bioactive output in the fermentation products, particularly in the ethyl acetate fraction and demonstrated a clear correlation between TPC and antioxidant activity (34).

The considerable amount of TPC and TFC in our extracts underscores their potential as antioxidants since phenolics and flavonoids are well recognized for their roles in electron transfer reactions and in scavenging reactive oxygen species (ROS) (40, 41). Therefore, ethyl acetate extract, which exhibited the highest levels of TPC and TFC, was selected for subsequent analyses.

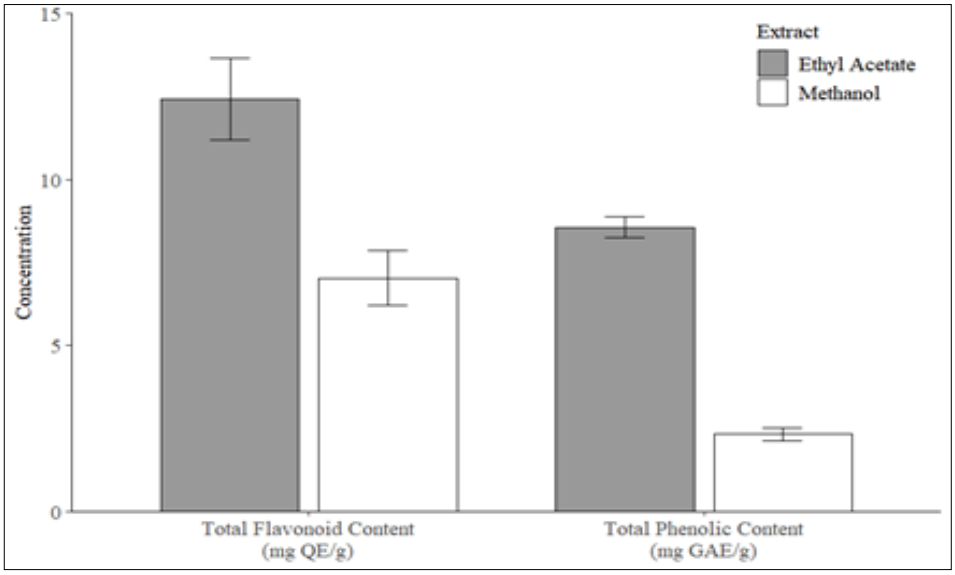

Fig. 2. Estimation of TPC and TFC.

Antioxidant function of *Aspergillus welwitschiae* imrd1 extract

The scavenging activity of ethyl acetate extract was determined using the DPPH radical scavenging assay. This method is a first rate approach to approximate the ability of bioactive components as antioxidants (42). The extract demonstrated a significant radical scavenging capacity compared to the standard ascorbic acid. Table 1 represents the results of DPPH activities, detailing the IC50 values of ethyl acetate extracts of *Aspergillus welwitschiae* imrd1. It was observed that the IC50 value of ethyl acetate extract was 38.79 µg/mL, while ascorbic acid had an IC50 value of 8.29 µg/mL. The highest (74.2327 %) scavenging activity of the sample was recorded at 100 µg/mL.

Table 1. Antioxidant activity of *Aspergillus welwitschiae* imrd1 extract (ethyl acetate)

Sample and Standard	IC50 (µg/mL)
Ethyl acetate extract	38.79
Ascorbic acid	8.29

Scavenging of free radicals by *Aspergillus* spp was found in several earlier studies (38, 42). The IC50 values of DPPH quenching activities of *Aspergillus wentii* EN-48 (5.2 to 99.4 µg/mL) (43) and A*spergillus terreus* (2.8 mg/mL) (44) were reported previously.

Aspergillus spp produce secondary metabolites with a vast repertoire of bioactive compounds, including phenolics, flavonoids, terpenes and alkaloids (45). These metabolites not only facilitate diverse biological activities but also supplement their free radical scavenging properties, thereby enhancing therapeutic value. Several researchers have reported a correlation between the bioactive compounds, particularly phenolic content and their antioxidant activity (8, 46). In this context, the rate of antioxidant activity is proportional to both the type and concentration of bioactive secondary metabolites present in the fungal extracts.

Chemical composition of ethyl acetate extract identified by GC-MS

To further elucidate the specific constituents, the ethyl acetate extract of *Aspergillus welwitschiae* imrd1 was subjected to GC-MS analysis (Fig. 3). The identified twenty-two compounds, together with their PubChem Compound Identifier (CID), molecular formula, retention time and peak area, are presented in Table 2. The key components detected include phenol, 3,5-bis(1,1-dimethylethyl)-; estragole; hexadecanoic acid, methyl ester; 9,12-octadecadienoic acid, methyl ester; 11-octadecenoic acid, methyl ester; tetracosane, methyl stearate; heneicosane; di-n-octyl phthalate; bis(2-ethylhexyl) phthalate etc. Several of these

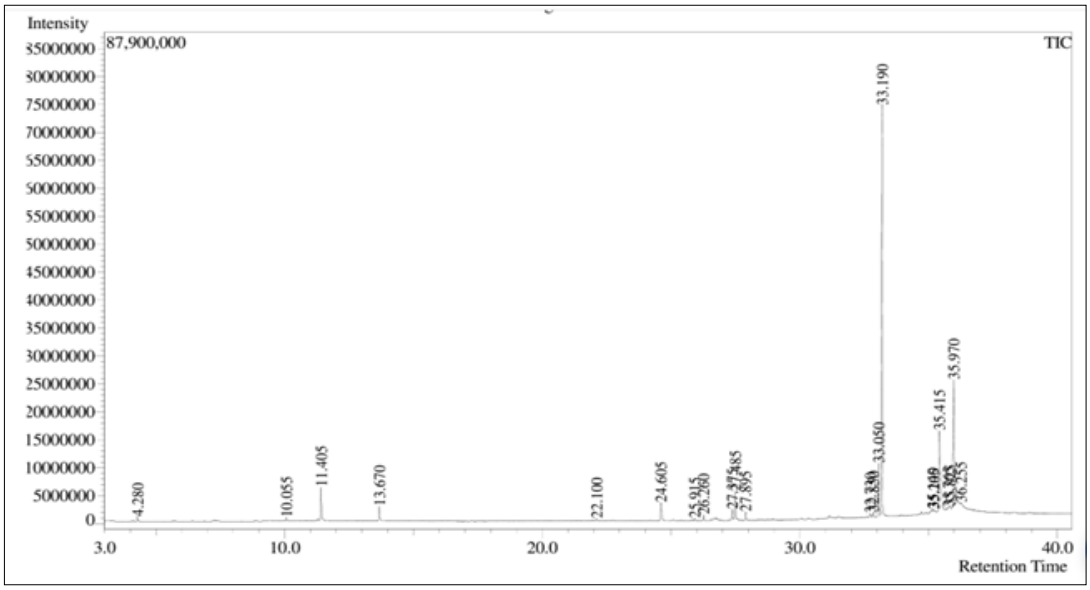

Fig. 3. Chromatogram of the active compounds from ethyl acetate extracts of *Aspergillus welwitschiae* imrd1.

Table 2. GC-MS analysis of ethyl acetate extract of *Aspergillus welwitschiae* isolate imrd1

CID	Name	Retention Time	Area	Molecular Formula
7720	2-Ethyl-1-hexanol	4.280	743228	$C_8H_{18}O$
5370526	9-Methoxybicyclo(6.1.0)nona-2,4,6-triene	10.055	192223	$C_{10}H_{12}O$
8815	Estragole	11.405	2036263	$C_{10}H_{12}O$
70825	Phenol, 3,5-bis(1,1-dimethylethyl)-	13.667	2523912	$C_{14}H_{22}O$
12403	Heneicosane	22.100	333447	$C_{21}H_{44}$
8181	Hexadecanoic acid, methyl ester	24.603	2096885	$C_{17}H_{34}O_2$
12592	Tetracosane	25.917	257320	$C_{24}H_{50}$
596027	Methyl 2-ethylhexyl phthalate	26.261	707565	$C_{17}H_{24}O_4$
8203	9,12-Octadecadienoic acid, methyl ester	27.377	502897	$C_{19}H_{34}O_2$
74738	11-Octadecenoic acid, methyl ester	27.485	1269088	$C_{19}H_{36}O_2$
8201	Methyl stearate	27.896	809007	$C_{19}H_{38}O_2$
14122972	Methyl 11-docosenoate	32.727	193852	$C_{23}H_{44}O_2$
5364423	13-Docosenoic acid, methyl ester, (Z)-	32.849	181477	$C_{23}H_{44}O_2$
8343	Bis(2-ethylhexyl) phthalate	33.051	7239136	$C_{24}H_{38}O_4$
8346	Di-n-octyl phthalate	33.182	29513129	$C_{24}H_{38}O_4$
6787	1,2-Benzenedicarboxylic acid, dinonyl ester	35.142	386713	$C_{26}H_{42}O_4$
590836	Phthalic acid, bis(7-methyloctyl) ester	35.206	233430	$C_{26}H_{42}O_4$
8496	Isophthalic acid	35.417	11348825	$C_8H_6O_4$
590836	Phthalic acid, bis(7-methyloctyl) ester	35.776	146946	$C_{26}H_{42}O_4$
5364492	9-Octadecenal, (Z)-	35.826	135933	$C_{18}H_{34}O$
6420241	Oleonitrile	35.970	9402151	$C_{18}H_{33}N$
98566	Nonadecanenitrile	36.259	224991	$C_{19}H_{37}N$

constituents have previously been found in the secondary metabolites of other *Aspergillus* species (47, 48). Moreover, many are well known for their diverse biological activities, including antimicrobial, antioxidant, cytotoxic, anthelmintic effects etc. (49-53).

Among the compounds identified in the GC-MS analysis, phthalates occupied a significant portion, including di-n-octyl phthalate, bis (2-ethylhexyl) phthalate and methyl 2-ethylhexyl phthalate. Although phthalates are typically regarded as synthetic, recent studies have reported their occurrence in natural sources, where they may exhibit diverse bioactivities such as antimicrobial, antioxidant, cytotoxic, antitumor and anti-inflammatory effects (54). Previous researchers isolated twenty-four phthalide monomers from the roots of the medicinal plant *Angelica sinensis* (Oliv.) (55). Bis(2-ethylhexyl) phthalate was isolated from *Capparis spinosa* leaves (56) and di-n-octyl phthalate was extracted from the brown seaweed *Ishige okamurae* with antifouling properties (57). However, the potential application of these phthalates requires careful purification of each component and further *in vivo* studies, which could validate their bioactivities and ensure the absence of possible health-related risks.

In silico studies

Drug likeness and toxicity predictions

The pharmacokinetic properties of the identified compounds were predicted based on Lipinski's rules using the SwissADME predictor (Table 3). According to Lipinski's criteria, a molecule is likely to be orally active if it satisfies some key features: molecular weight (MW) ≤ 500 (g/mol), octanol/water partition coefficient (Log P) ≤ 5, no more than five hydrogen bond donors (HBD) and not more than ten hydrogen bond acceptors (HBA) (25). Since each of the four rules is a multiple of five, it is referred to as the "rule of five" (58). Additionally, molar refractivity (MR) of orally active molecules should fall within the range of 40-130 (59). Out of the twenty-two compounds detected by GC-MS in our study, six compounds satisfied all rules. Notably, Lipinski's rule allows for one violation without excluding a compound from being considered orally active. Consequently, all compounds qualified as potential oral drug candidates for further application.

Drug solubility (log S) is a crucial parameter in the drug development process, as it influences absorption (oral bioavailability), distribution and elimination. Generally, most orally administered drugs have log S values between -1 and -5 and virtually none have

Table 3. Pharmacokinetic and drug-likeness properties of compounds from the ethyl acetate extract of *Aspergillus welwitschiae* imrd1

CID	MW (g/mol)	HBD	HBA	Log P	MR	Rules violations
7720	130.23	1	1	2.39	41.73	0
5370526	148.20	0	1	1.96	45.62	0
8815	148.20	0	1	2.78	47.04	0
70825	206.32	1	1	3.89	67.01	0
12403	296.57	0	0	8.26	103.06	1
8181	270.45	0	2	5.54	85.12	1
12592	338.65	0	0	9.35	117.48	1
596027	292.37	0	4	3.91	82.65	0
8203	294.47	0	2	5.69	93.78	1
74738	296.49	0	2	5.95	94.26	1
8201	298.50	0	2	6.24	94.73	1
14122972	352.59	0	2	7.34	113.49	1
5364423	352.60	0	2	7.40	113.49	1
8343	390.56	0	4	6.17	116.30	1
8346	390.56	0	4	6.30	116.30	1
6787	418.61	0	4	7.02	125.91	1
590836	418.61	0	4	6.70	125.91	1
8496	166.13	2	4	1.06	40.36	0
590836	418.61	0	4	6.70	125.91	1
5364492	266.46	0	1	5.90	88.37	1
6420241	263.46	0	1	5.99	87.91	1
98566	279.50	0	1	6.54	93.20	1

values below -6 (60). In the present study of twenty-two compounds, log S values of 17 compounds ranged from -1.64 to -5.76, indicating acceptable solubility. However, five compounds had log S values below -6, a range rarely observed in effective oral drugs.

Additionally, the drug score combines the overall potential quality of a compound as a drug, integrating parameters such as drug-likeness, Log P, log S, MW and toxicity risks. A drug score of zero or less denotes the compound is unlikely to be a drug, whereas a positive value indicates the potential as a drug candidate (58). Again, toxicity classification is an important parameter for assessing whether a compound exhibits toxic potential, with categories ranging from highly toxic (LD50 ≤ 50 mg/kg) to non-toxic (LD50 > 5000 mg/kg). Here, the LD50 is defined as the median lethal dose at which 50 % of test subjects die upon exposure to a compound (28, 61). To predict drug scores and toxicity factors, the compounds were evaluated using OSIRIS Property Explorer and the ProTox 3.0 server. The predicted drug scores ranged from 0.05 to 0.75, with all compounds showing positive values. The predicted LD50 values varied between 42 and 20000 mg/kg, with four compounds classified into classes 1-3, nine into class 4 and another nine into classes 5-6. On the other hand, eleven compounds were predicted to have no risk of tumorigenicity (TUM), mutagenicity (MUT), irritation (IRR), reproductive development (REP), carcinogenicity (CARC), cytotoxicity (CYT) and immunotoxicity (IMM), whereas the remaining compounds showed one or more such toxicological concerns (Table 4).

Considering drug-scores (> 0.25), toxicity profiles (class 5 or 6 and LD50 > 2000 mg/kg) and evidence from the literature, four compounds were selected for subsequent in-silico docking studies.

Molecular docking studies

Molecular docking is a structure-based drug design approach to predict the binding interactions between receptors and ligands, making the early stages of drug discovery faster and cost-effective (62). In this study, the molecular docking was carried out to determine the binding pattern of four selected compounds (Fig. 4) with NADPH oxidase (PDB ID: 2CDU) and the results were compared with the standard compound (ascorbic acid). NADPH oxidase is an enzyme that catalyzes the production of superoxide from oxygen and NADPH and plays a vital role in the generation of reactive oxygen species and immune defence against microorganisms; however, its over-expression has been linked to various neurological disorders and cancers (63, 64). Therefore, suppressing over-expression of this enzyme is essential to maintain the equivalence of body.

All four selected ligand molecules produced significant binding energy values compared to the standard compound, ascorbic acid. The binding affinities ranged from -5.0 to -5.7 Kcal/mol and ascorbic acid scored -6.1 Kcal/mol (Table 5).

Notably, all compounds exhibited hydrogen bond interactions, which are crucial for the activity of drug lead molecules. Compound 8203 reflected the highest binding affinity with a value of -5.7 Kcal/mol. This compound formed one conventional hydrogen bond with TYR159 (2.11 Å) and one carbon hydrogen bond with GLY341 (3.33 Å). Additionally, there was pi-alkyl interaction with TYR188 and TYR159 and multiple alkyl interactions with ILE297, PRO298, LEU299, LEU346, LYS187, ALA349 (Fig. 5(a)). Compound 8201 demonstrated a binding score of -5.5 Kcal/mol, forming two hydrogen bonds with SER326 (2.50 and 3.49 Å) and hydrophobic interactions (alkyl and pi-alkyl) with ILE297, LEU299, LEU346, PRO298, TYR159, TYR188 and PHE245 (Fig. 5(b)). Ligand 74738 showed a binding affinity of -5.4 Kcal/mol, interacting with SER326 (2.54 and 3.78 Å) and ASN343 (2.69 Å) via hydrogen bonds and with ILE297, LEU299, TYR159 and TYR188 through hydrophobic (alkyl and pi-alkyl) bonds (Fig. 5(c)). The binding affinity for compound 8181 was -5 Kcal/mol. Catalytic residues HIS10 (2.87 Å), ALA11 (1.93 Å) and ASP282 (2.27 Å) contributed to developing three conventional hydrogen bonds, while THR112 (3.47Å) and GLY281 (3.49 Å) were involved in forming two carbon hydrogen bonds. In addition, the complex was stabilized by hydrophobic interactions with amino acid residues, including ALA300, LYS134 and PHE245 (Fig. 5(d)).

The standard ligand (ascorbic acid) formed conventional hydrogen bonds with TYR159 (1.97 Å), LYS187 (2.73 Å), TYR188 (2.28 and 2.29 Å), SER326 (2.51 and 1.94 Å), SER328 (1.81 Å) and ASN343 (2.26 Å), as well as carbon hydrogen bonds with SER339 (3.46 Å) and GLY341 (3.79 Å) in the cavity of NADPH oxidase, consistent with previous findings (Fig. 5e) (63). Collectively, the docking scores and binding interactions reflect that these molecules could optimally occupy the pocket of NADPH oxidase and possess notable antioxidant potential.

Table 4. Toxicological properties of compounds from *Aspergillus welwitschiae* imrd1 extract

CID	log S (mol/L)	Drug score	Predicted Toxicity Class	Predicted LD50 (mg/kg)	Toxicity						
					REP	MUT	TUM	IRR	CARC	IMM	CYT
7720	-1.99	0.17	Class: 4	1000	Yes	No	No	Yes	No	No	No
5370526	-1.87	0.75	Class: 2	42	No	No	No	No	Yes	No	No
8815	-2.35	0.11	Class: 4	1230	No	Yes	Yes	Yes	Yes	No	No
70825	-3.64	0.4	Class: 4	800	No	No	No	No	No	No	No
12403	-6.11	0.24	Class: 3	750	No	No	No	No	No	No	No
8181	-4.37	0.29	Class: 5	5000	No	No	No	No	No	No	No
12592	-6.92	0.23	Class: 3	750	No	No	No	No	No	No	No
596027	-3.71	0.07	Class: 4	1340	Yes	Yes	Yes	Yes	Yes	No	No
8203	-4.45	0.27	Class: 6	20000	No	No	No	No	No	No	No
74738	-4.68	0.27	Class: 5	3000	No	No	No	No	No	No	No
8201	-4.91	0.26	Class: 5	5000	No	No	No	No	No	No	No
14122972	-5.76	0.24	Class: 5	3000	No	No	No	No	No	No	No
5364423	-5.76	0.24	Class: 5	3000	No	No	No	No	No	No	No
8343	-5.52	0.04	Class: 4	1340	Yes	Yes	Yes	Yes	Yes	No	No
8346	-5.74	0.05	Class: 4	1340	Yes	Yes	Yes	Yes	Yes	No	No
6787	-6.28	0.05	Class: 4	1340	Yes	Yes	Yes	Yes	Yes	No	No
590836	-6.06	0.05	Class: 4	1340	Yes	Yes	Yes	Yes	Yes	No	No
8496	-1.64	0.3	Class: 5	5000	No	No	No	Yes	No	No	No
590836	-6.06	0.05	Class: 4	1340	Yes	Yes	Yes	Yes	Yes	No	No
5364492	-4.78	0.06	Class: 5	5000	Yes	Yes	No	Yes	No	No	No
6420241	-5.22	0.27	Class: 5	5000	No	No	No	No	No	No	No
98566	-5.72	0.25	Class: 3	165	No	No	No	No	No	No	No

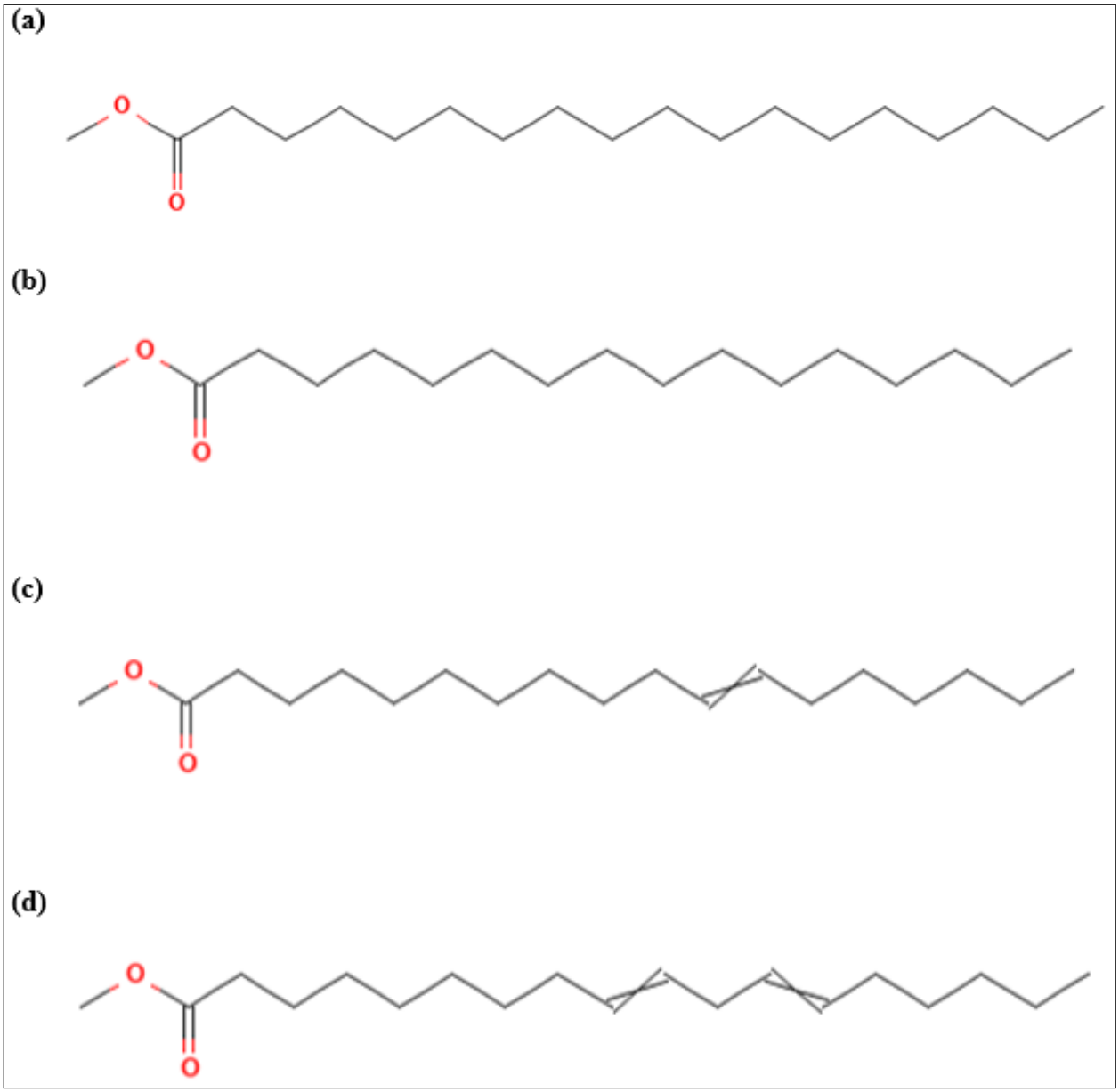

Fig. 4. 2D structures of the compounds selected for docking studies. (a) Methyl stearate (CID: 8201), (b) Hexadecanoic acid, methyl ester/ Methyl palmitate (CID: 8181) (c) 11-Octadecenoic acid, methyl ester (CID: 74738) (d) 9,12-Octadecadienoic acid, methyl ester (CID: 8203).

Table 5. Molecular docking results of screened ligands against NADPH oxidase

Ligands	Binding energy (Kcal/mol)	Hydrogen Bond and Distances (Å)		Hydrophobic bond and Distances (Å)	
		Conventional Hydrogen Bond	Carbon Hydrogen Bond	Alkyl	Pi-Alkyl
8203	-5.7	TYR159 (2.11)	GLY341 (3.33)	ILE297 (4.91), ILE297 (5.04), PRO298 (4.41), LEU299 (4.88), LEU299 (4.78), LEU346 (5.41), LEU346 (4.06), LYS187 (4.07), LYS187 (4.29), ALA349 (3.89)	TYR159 (4.67), TYR188 (4.65), TYR188 (5.30)
8201	-5.5	SER326 (2.50)	SER326 (3.49)	ILE297 (4.23), LEU299 (5.35), LEU299 (4.69), LEU299 (5.29), LEU346 (5.04), PRO298 (5.32)	TYR159 (5.05), TYR159 (4.18), TYR188 (4.63), PHE245 (5.18)
74738	-5.4	SER326 (2.54), ASN343 (2.69)	SER326 (3.78)	ILE297 (4.38), LEU299 (4.55), LEU299 (5.43)	TYR159 (4.19), TYR188 (5.11), TYR188 (5.41)
8181	-5	HIS10 (2.87), ALA11 (1.93), ASP282 (2.27)	THR112 (3.47), GLY281 (3.49)	ALA300 (4.38), ALA300 (4.83), LYS134 (4.65)	PHE245 (5.21), PHE245 (4.25)
54670067	-6.1	TYR159 (1.97), LYS187 (2.73), TYR188 (2.28), TYR188 (2.29), SER326 (2.51), SER326 (1.94), SER328 (1.81), ASN343 (2.26)	SER339 (3.46), GLY341 (3.79)	-	-

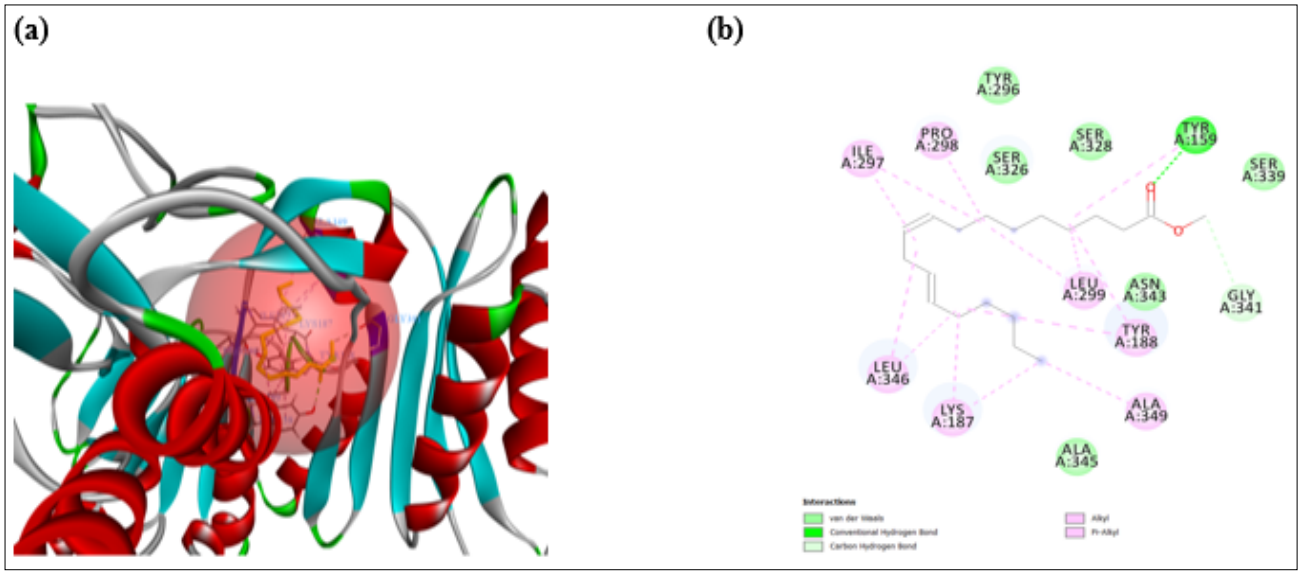

Fig. 5(a). Binding interactions of compound 8203 with NADPH oxidase (PDB ID: 2CDU). (a) The 3D representation illustrates the ligand's position within the binding pocket. (b) The 2D interaction diagram represents key interactions with the respective amino acid residues.

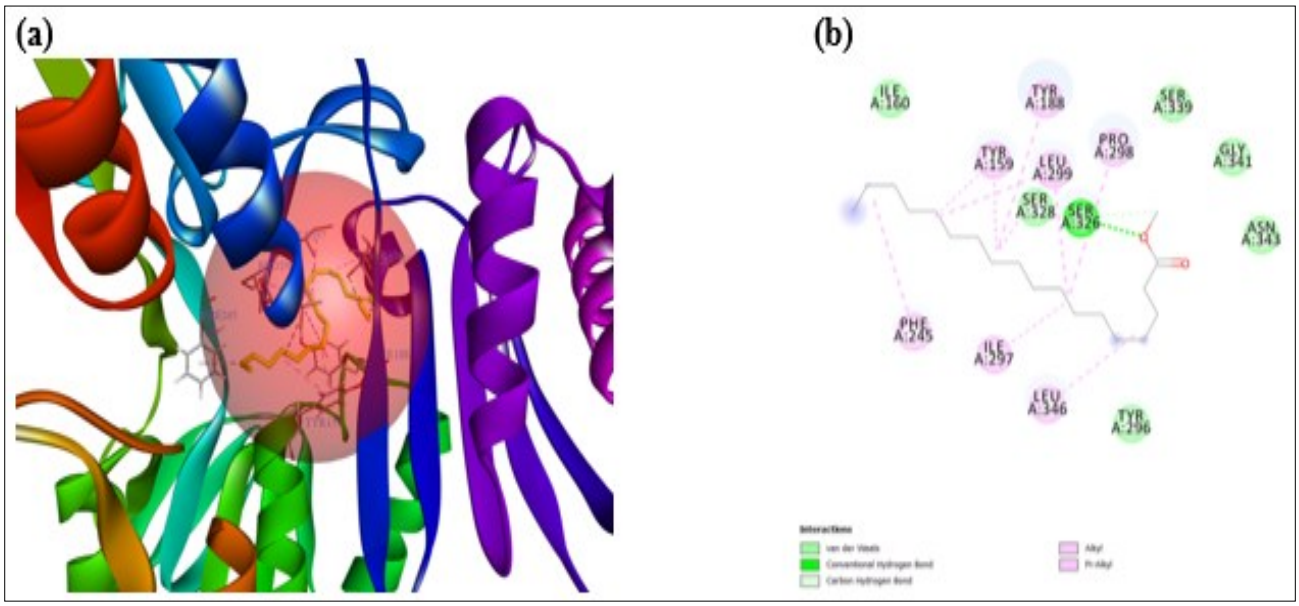

Fig. 5(b). Binding interactions of compound 8201 with NADPH oxidase (PDB ID: 2CDU). (a) The 3D representation illustrates the ligand's position within the binding pocket. (b) The 2D interaction diagram represents key interactions with the respective amino acid residues.

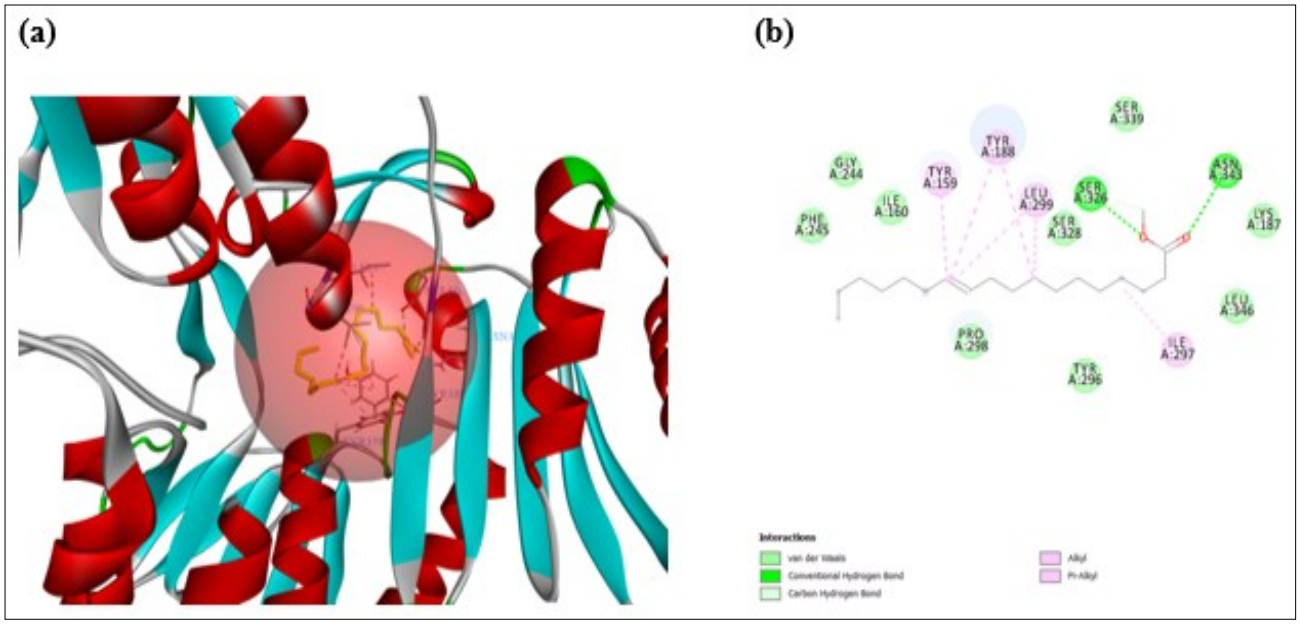

Fig. 5(c). Binding interactions of compound 74738 with NADPH oxidase (PDB ID: 2CDU). (a) The 3D representation illustrates the ligand's position within the binding pocket. (b) The 2D interaction diagram represents key interactions with the respective amino acid residues.

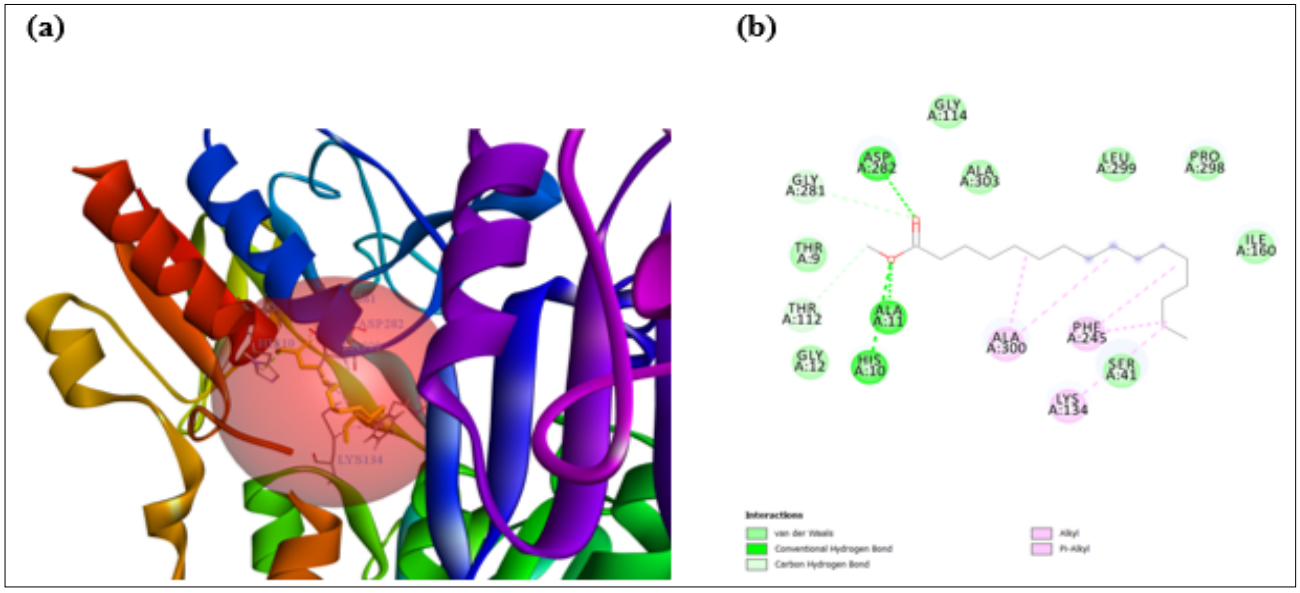

Fig. 5(d). Binding interactions of compound 8181 with NADPH oxidase (PDB ID: 2CDU). (a) The 3D representation illustrates the ligand's position within the binding pocket. (b) The 2D interaction diagram represents key interactions with the respective amino acid residues.

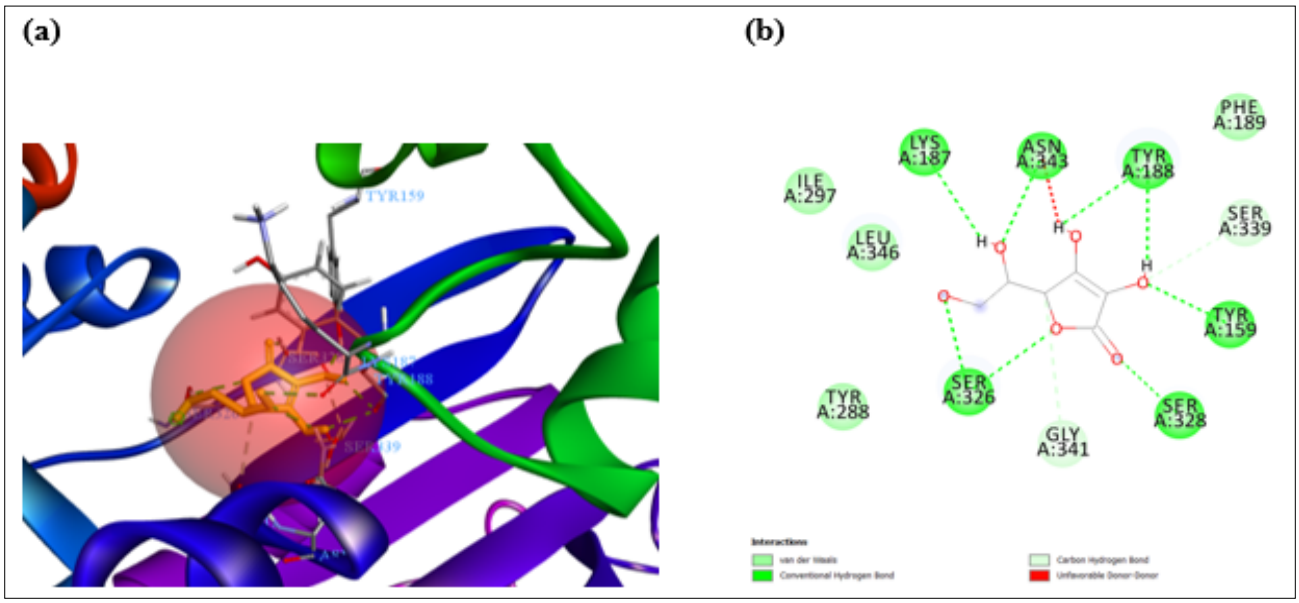

Fig. 5(e). Binding interactions of compound 54670067 with NADPH oxidase (PDB ID: 2CDU). (a) The 3D representation illustrates the ligand's position within the binding pocket. (b) The 2D interaction diagram represents key interactions with the respective amino acid residues.

Conclusion

Aspergillus welwitschiae imrd1 was identified as a prolific source of secondary metabolites. The crude extract from this fungus revealed accepted level of TPC and TFC reflected by its significant antioxidant activity. GC-MS profiling confirmed the presence of twenty-two constituents. Four of these were screened as potential antioxidant agents based on literature and in silico physicochemical and toxicity assessments. Molecular docking against NADPH oxidase showed favourable binding affinities and interactions compared to standard compound. This suggests their potential role as NADPH oxidase inhibitors. These findings provide a groundwork for further research, especially focusing on isolating, characterizing and validating components derived from *Aspergillus welwitschiae* imrd1 as potential therapeutic candidates.

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Authors' contributions

FAK contributed to methodology, formal analysis, investigation and writing of the original draft. FZN and OB contributed to methodology. AMW, SIC, JF, MJH and RS contributed to formal analysis, review and editing of the manuscript. SI contributed to conceptualization, acquired funding and supervised the work. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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