Evaluation and isolation of anti-cancer compounds from the endophytic fungus *Penicillium funiculosum* isolated from *Persicaria salicifolia* (Brouss. Ex Willd.) seeds growing in Egypt

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Background
The endophytic fungus Penicillium is an important source of natural bioactive products. *Persicaria salicifolia* (Brouss. ex Willd) (family Polygonaceae) is a widely distributed plant on the Nile River and was reported to have several biological activities, such as antioxidative, antibacterial, and anti-inflammatory effects.

Objective
This study aimed to explore the potential activities (cytotoxic, anti-oxidant, and anti-microbial) of the endophytic fungus *Penicillium funiculosum* isolated from *Persicaria salicifolia* seeds growing in Egypt.

Materials and methods
The endophytic fungus *Penicillium funiculosum* was isolated from the seeds of the *Persicaria salicifolia* plant. The fungi were grown on Basmati rice as a solid media for the enhancement of pure fungi production. The collected fungi were extracted with ethyl acetate and fractionated using n-hexane and methanol. All fractions were examined for their cytotoxic, antioxidant, and antimicrobial activities. The secondary metabolites were isolated from the active fractions through column chromatography, and the isolated compounds were identified by spectroscopic technique. Molecular docking analysis was applied to the isolated compounds.

Results and conclusion
Ethyl acetate extract (Pf-2) of *P. funiculosum* was proved to have a highly potent antioxidant (IC_{50} 37.5±0.70 μg/ml) and cytotoxic effects. It was affected on hepatic cancer (HepG-2), human colon carcinoma (HCT-116), lung carcinoma (A-549), and human breast cancer (MCF-7) cells with IC_{50} values (μg/ml) 4.26±0.2, 6.66±0.9, 9.36±0.3, and 9.41±0.7, respectively. Pf 2 was subjected to fractionation, resulting in four fractions (Pf 2–1 to Pf 2–4). The most potent cytotoxic fraction, Pf 2–2, was further fractionated into six sub-fractions: Pf 2–2 A to Pf 2–2 F. Pf 2–2 A possessed the most potent cytotoxic activity. The ethyl acetate extract (Pf 2) also had antimicrobial activity against gram-positive *Bacillus subtilis* and gram-negative bacteria (*E. coli*). Four compounds were isolated from Pf 2–2 A and identified by spectroscopic methods: NMR (^{1}H and ^{13}C) and Mass as (1) (9E, 11E, 13Z, 15Z)-tetracosa-9, 11, 13, 15-tetraenoic acid, (2) 3-(1Z, 3E-hexa-1, 3-dienyl)-4b-methyl-tetradecahydrophenanthrene, (3) mannitol, (4) d-Cerebroside A-glucose. It is the first time to report the isolation of compound (2) from the genus *Penicillium* and compounds (1, 3, and 4) from the species *P. funiculosum*. Compound 2 was the most potent cytotoxic one. Molecular docking was studied for polar compounds 1, 3, and 4 using the COX-2 enzyme, which indicated that compound 4 was the most potent anti-inflammatory one.

Keywords:
antimicrobial activity, cytotoxicity, endophytes, *Penicillium funiculosum*, *Persicaria salicifolia*

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Introduction

Cancer is a major contributor to the disease burden worldwide. The prevalence is increasing every year around the world known for its mortality. New drugs are critically needed for the prevention and treatment of cancer. Endophytes are colonies of microorganisms inside plant tissues. Its kingdom includes more than 300 000 species on Earth. Endophytes are an excellent source of bioactive compounds [1], alkaloids, steroids, terpenoids, tannins, saponins, quinones, and phenolic acids. They possess numerous biological activities [2]; antiviral, anti-inflammatory, antidiabetic, anticancer, antimalarial, and immunosuppressive properties. Plants are represented as sources of endophytes,

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which generally hinder the livelihood of the host in a special environment [3].

The endophytic *Penicillium* genus is known to possess antibiotic potential, phytoremediators, plant growth promoters, biocatalysts, and enzyme producers [4]. *Penicillium* species represent a promising, rich source of novel natural products that have the ability to produce large quantities at low cost through the cultivation and fermentation of the organisms on a large scale. Several phytochemical compounds were isolated, such as alkaloids [5], meroterpenoids- polyketides [6], lipopeptides [7], and sterols [8]. Meanwhile, the impressively structurally diverse metabolites from this fungus exhibit extensive bioactivities, including anti-inflammatory [8], insecticidal [9], antitumor [10], antifungal [3], and immunosuppressive activities [11].

Medicinal plants are the major source of bioactive endophytic metabolites [12]. *Persicaria salicifolia* (Brouss. ex Willd) seeds (Syn. *Polygonum serrulatum*), family Polygonaceae, is one of ten *Persicaria* species that grow in the Nile Delta, Egypt [13]. It is a hydrophyte found along the borders of watercourses in canals, drains, and on river banks [14]. *Persicaria* species were reported to have antioxidative, anti-inflammatory, antibacterial, analgesic, hypothermic, and diuretic activities [15–21]. Several reports were found concerning the isolation and identification of phytoconstituents in *Persicaria salicifolia* seeds [22–24]. It is worth nothing to trace the endophytic fungi isolated from *P. salicifolia*.

Moreover, molecular modeling approaches present useful tools in biological and medicinal research. Certainly, molecular modeling is very important and necessary to understand the interaction between inhibitors and the disease's enzymes at the outset of new drugs; it helps to save time and financial spending [25]. The present work aimed to evaluate the biological effects of different extracts and fractions of the endophytic fungus *Penicillium funiculosum* isolated from *Persicaria salicifolia* seeds (F. Polygonaceae). Cytotoxicity against different cell lines was the main target, while antimicrobials and antioxidants were also screened for. Isolation and identification of the main phytoconstituents were achieved using chromatographic and spectroscopic techniques. Molecular docking, as well as cytotoxic evaluation for the isolated compounds, was also done.

Materials and methods

Sample collection; plant and fungal materials

The seeds of *Persicaria salicifolia* were collected from Tanta region (Akhnaway), Gharbia Governorate, and kindly authenticated by Prof. Abdo H. Marey, Botany Department, Faculty of Science, Al-Azhar University, based on mycological keys for the morphological and cultural characters using the method described [26].

The seeds were treated for the elimination of containing microbes by methods of Johnson and curl [27]. The seeds were cut, washed with sterilized distilled water, treated with ethanol (70%) for 1–2 min, and ultimately air-dried under a laminar flow hood. Under sterile conditions, the inner tissues were carefully dissected and placed onto malt agar (MA) plates containing antibiotics. After 3–4 weeks of incubation at room temperature, the hyphal tips of the fungi were removed and transferred to a fresh MA medium. A pure strain was isolated by repeated inoculation. After the isolation of pure fungal strains, it was identified as *Penicillium funiculosum* by Dr. Amal A. E. Mekawy, Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University.

Extraction and fractionation of *Penicillium funiculosum* fungi

Penicillium funiculosum fungi were grown on a solid medium; basmati rice, and treated under the conditions described by Nickles [28] for the mass production of pure fungi, extraction, isolation, and identification of secondary metabolites.

Ethyl acetate (EtOAc, 250 mL x3) was added to the cultures and left overnight for 3–5 days to allow complete extraction. The residue of the prepared EtOAc extract (Pf 2, 50 g) was fractionated using n-Hexane (n-Hx; 2×2 L) for purification, filtered, and concentrated at 40°C under vacuum till dryness (Pf 1, 26 g). The remaining culture media was dissolved in methanol and fractionated by the same way with n-Hx. Both methanol extract and its n-Hx fractions were filtered off and concentrated to give (Pf 4, 8.5 g) and (Pf 3, 5.1 g), respectively. The concentrated residues (Pf 1, Pf 2, Pf 3, and Pf 4) were subjected to biological evaluation including cytotoxic screening using different cancer cell lines, antioxidants, and antimicrobials to choose the most active extract and/or fractions for further phytochemical study (bio-guided fractionation).

Biological studies

Ethical approval

This work was done according to the ethical approval of the Medical Research Ethics Committee (MREC) of the National Research Centre (Egypt) under the approval number 06078092021.

Cytotoxic activities

The cytotoxic activities of all tested samples (*P. funiculosum* Pf 1–Pf 4) were evaluated on hepatic cancer (HepG-2), human colon carcinoma (HCT-116), lung cancer (A-549), and breast cancer (MCF-7) cell lines. The cultivation of the cells and the samples were tested according to the methods of Mosmann [29].

Antioxidant activity (free radical scavenging activity procedure)

The tested extracts (Pf 2 and Pf 4) and their n- Hx fractions (Pf 1 and Pf 3) were evaluated using an *in-vitro* assay, DPPH (2, 2-diphenyl-1-picryl-hydrazil) spectrophotometric method reported by Romano *et al.* and Oktay *et al.* [30,31].

Antimicrobial activity

The residues of the prepared samples were investigated for antimicrobial activity using the agar well diffusion assay method as described [32]. The tested organisms were fungi: *Aspergillus fumigatus* RCMB 002008, *Candida albicans* RCMB 005003 (1) ATCC 10251, Gram-Positive bacteria; *Staphylococcus aureus* (RCMB 010010); *Bacillus subtilis* RCMB 015 (1) NRRL B-543 and gram-negative bacteria: *Proteus vulgaris* ATCC 13315, and *E. Coli* ATCC 25955. The cultures were incubated at 25–30°C for 3–7 days and at 37°C (24 h) for fungi and bacteria, respectively. Sabaroud dextrose agar and nutrient agar medium (Oxford laboratory, UK) were used for the subculture of fungi and bacteria, respectively. Ketoconazole and gentamycin were used as positive controls.

Separation and identification of the major compounds

Pf 2 was chosen according to its cytotoxic activity for further isolation of the main phyto-constituents. It was fractionated using vacuum liquid column chromatography (VLC, silica gel) with different solvent systems (dichloromethane (DCM): methanol (MeOH), 100 : 0–0 : 100). Four fractions, Pf 2–1 (4.6 g), Pf 2–2 (10.2 g), Pf 2–3 (5.1 g), and Pf 2–4 (8.5 g), were obtained. They were subjected to cytotoxic screening using the aforementioned cells under the same conditions. The Pf 2–2 showed promising activity, so it was chosen for further fractionation by VLC (silica gel) using DCM: MeOH (100 : 0–0 : 100) affording 6 sub-fractions: Pf 2–2 A, Pf 2–2B, Pf 2–2C, Pf 2–2D, Pf 2–2E, and Pf 2–2 F. They were reevaluated for their cytotoxic activity in the same manner, and Pf 2-2 A was the most potent. The Pf 2–2 A was subjected to a normal solid phase extraction (SPE) column affording different fractions, monitored through thin layer chromatography (TLC, silica gel 60 F254, Merck) using n-Hx: EtOAc; 80 : 20, 70 : 30) and ($CHCl_3$: MeOH; 85 : 15). Similar fractions were collected and concentrated under vacuum where four compounds (1–4) were given. The isolated compounds were characterized by physical, chromatographic, and spectroscopic data (^{1}H-NMR, ^{13}C-NMR) (Bruker Bioapex).

Computational analysis (molecular docking)

The crystallographic structure of COX-2 was obtained from the Protein Data Bank [PDB ID: 3LN1 with resolution 2.4 Å] (https://www.rcsb.org), which was used for the docking study. The molecular operating environment, version 2016.08, was applied for the analysis of the docking study [33]. The interactions of compounds, amino acids, and hydrogen bond lengths were also detected [25,34].

Statistical analysis

All results were expressed as the mean±standard deviations (SD) of three replicates in each group. Statistical analysis was performed using one-way analysis of variance (ANOVA), accompanied by a *post–hoc* test [Least Significant Difference (LSD) test] using Costate software computer program. Results of *P* less than or equal to 0.05 were considered to be statistically significant.

Results and discussion

Biological activities

The biological activity of ethyl acetate (Pf 2) and methanol (Pf 4) extracts and their n-Hx fractions (Pf 1, Pf 3) of the endophytic fungus *P. funiculosum* were evaluated. The most active sample was chosen to isolate the phytoconstituents. The activity of isolated compounds was tested using cytotoxicity and molecular docking.

Cytotoxic activity

Pf 2, Pf 4 extracts, and Pf 1, Pf 3 fractions were assessed for their cytotoxicity (IC_{50} values, μg/mL) on HepG-2, HCT-116, A-549, and MCF-7 cancer cells (Table 1). Significant potent activity was revealed by Pf 2 against all tested cells, 4.26±0.2, 6.66±0.9, 9.36±0.3, and 9.41 ±0.7 μg/ml, respectively. Accordingly, Pf 2 extract was the chosen sample for the isolation and identification of the main phytoconstituents of *P. funiculosum*. Four fractions (Pf 2–1- Pf 2–4) were obtained by VLC and examined on the same human cell lines (Table 2).

Pf 2-2 fraction exhibited a significantly lower IC_{50} value when examined against human cells; A-549 (IC_{50} 9.36±0.3), HepG-2 (IC_{50} 14.26±0.2), and HCT-116

Table 1 Cytotoxicity (IC_{50} values, µg/mL) of the main extract and its fraction of *Penicillium funiculosum* against hepatic cancer-2, human colon carcinoma-116, A-549 and MCF-7 Cell lines

	IC_{50} values (µg/ml)			
Sample Code	Hepatic cancer –2	Human colon carcinoma –116	A-549	MCF-7
Pf 1	27.40±0.5[a]	38.60±0.4[a]	32.80±1.30[a]	58.50±0.80[a]
Pf 2	**4.26±0.2[b]**	**6.66±0.9[b]**	**9.36±0.30[b]**	**9.41±0.70[b]**
Pf 3	90.30±1.70[c]	112.00±1.30[c]	121.00±1.40[c]	183.00±1.90[c]
Pf 4	190.00±1.80[d]	224.00±2.30[d]	165.00±1.70[d]	374.00±1.80[d]
*Vinblastine sulphate	3.48±0.22[e]	3.50±0.20[e]	24.60±0.70[e]	5.90±0.90[e]

All data are presented as the mean±S.D of three replicates in each group. Statistical analysis was performed using one-way analysis of variance (ANOVA), accompanied by a *post–hoc* test [Least Significant Difference (LSD) test] using Costate software computer program. Different letters are significant at *P* less than or equal to 0.05. Pf 1= N-hexane of ethyl acetate extract Pf 2= Ethyl acetate extract. Pf 3= N-hexane of methanol extract Pf 4=Methanol extract. ***Vinblastine sulphate** is a standard reference drug. All data are presented as the mean±S.D of three replicates in each group. Statistical analysis was performed using one-way analysis of variance (ANOVA), accompanied by a *post–hoc* test [Least Significant Difference (LSD) test] using Costate software computer program. Different letters are significant at *P* less than or equal to 0.05.

Table 2 Cytotoxicity of the sub- fractions of *Penicillium funiculosum* (Pf 2) against hepatic cancerG-2, human colon carcinoma-116, A-549 and MCF-7 Cells, respectively)

	IC_{50} values (µg/mL)			
Sample Code	Hepatic cancer-2	Human colon carcinoma –116	A-549	MCF-7
Pf 2–1	26.40±0.50[a]	84.6 0±0.40[a]	39.80±1.30[a]	88.50±0.80[a]
Pf 2–2	**14.26±0.20[b]**	**16. 60±0.91[b]**	**9.36±0.30[b]**	**19.41±0.71[b]**
Pf 2–3	90.30±1.70[c]	112.00±1.30[c]	121.00±1.40[c]	183.00±1.90[c]
Pf 2–4	190.00±1.80[d]	224.00±2.3[d]	165.00±1.70[d]	374.00±1.80[d]
*Vinblastine sulphate	3.48±0.22[e]	3.50±0.20[e]	24.60±0.70[e]	5.90±0.90[e]

Pf 1= N-hexane of ethyl acetate extract Pf 2= Ethyl acetate extract. Pf 3= N-hexane of methanol extract Pf 4=Methanol extract. ***Vinblastine sulphate** is a standard reference drug.

cells (IC_{50}16. 6±0.91) µg/ml. Pf 2–2 was subjected to sub-fractionation by VLC, resulting in six sub-fractions (Pf 2–2 A - Pf 2–2 F), and all were examined in the same manner (Table 3). Pf 2–2 A was the most significant biologically active sub-fraction in A-549 and HepG-2 cells. It was used for the isolation and identification of the main compounds, which were then tested against the same cell lines. From the reported data, it was found that the ethyl acetate extract of other species, *P. chrysogenum,* showed strong cytotoxic activity on HEP-2 (IC_{50} 30.8±1.3), and HCT-116 (IC_{50} 22.6±0.8) [35].

Table 3 Cytotoxicity of different sub-fractions of the ethyl acetate extract of *Penicillium funiculosum* against most effective human colon carcinoma-116 and HepG-2Cells

	IC_{50} values (µg/ml)	
Sample Code	Hepatic cancer –2 Cells	A-549 Cells
Pf 2–2 A	**9.27±0.97[a]**	**14.30±0.90[a]**
Pf 2–2B	20.3±1.00[b]	24.30±1.02[b]
Pf 2–2 C	30.4±1.10[c]	41.00±1.23[c]
Pf 2–2D	66.9±1.04[d]	105.00±2.08[d]
Pf 2–2E	141.00±3.40[e]	180.00±3.01[e]
Pf 2–2 F	99.30±0.92[f]	124.00±3.10[f]
*Vinblastine sulphate	3.48±0.22[g]	24.60±0.70[g]

All data are presented as the mean±S.D of three replicates in each group. Statistical analysis was performed using one-way analysis of variance (ANOVA), accompanied by a *post–hoc* test [Least Significant Difference (LSD) test] using Costate software computer program. Different letters are significant at *P* less than or equal to 0.05. ***Vinblastine sulphate** is a standard reference drug

Antioxidant activity

The anti-oxidant activity of all samples prepared from *P. funiculosum* was evaluated *in-vitro* using the DPPH assay (1, 1-diphenyl-2-picrylhydrazyl). Ascorbic acid was used as a positive control (IC_{50} 14.30±1.00 µg/ml). Pf 2 showed a significant antioxidant effect with IC_{50} 37.5±0.70 µg/ml in comparison to other samples (Pf 1; 142.20±3.10, Pf 3; 292.40±6.70, Pf 4; 495.90±6.00 µg/ml) (Table 4). The lower the IC_{50} value, the more potent of the sample at scavenging DPPH and this indicates a higher antioxidant activity [36]. The obtained results were compatible with the reported data of Jakovljević *et al.*, who indicated that *P. funiculosum* ethanol extract may serve as an effective radical scavenging agent with DPPH free radical followed by *P. chrysogenum,* converting them to stable products. This antioxidant activity may be attributed to the total phenolic content in the *P. chrysogenum and P. funiculosum* ethanol extract, which were 2.859 mg GAE/g and 2.109 mg GAE/g, respectively [37]. The redox properties of the phenolic

Table 4 Antioxidant (IC_{50} values (μg/ml) of different fractions of *Penicillium funiculosum* against ascorbic acid using DPPH assay

Sample code	Antioxidant IC_{50}(μg/ml)
Pf 1	142.20±3.10[a]
Pf 2	**37.50±0.70[b]**
Pf 3	292.40±6.70[c]
Pf 4	495.90±6.00[d]
*Ascorbic acid	**14.30±1.00[e]**

All data are presented as the mean±S.D of three replicates in each group. Statistical analysis was performed using one-way analysis of variance (ANOVA), accompanied by a *post-hoc* test [Least Significant Difference (LSD) test] using Costate software computer program. Different letters are significant at *P* less than or equal to 0.05. Pf 1= N-hexane of ethyl acetate extract Pf 2= Ethyl acetate extract. Pf 3= N-hexane of methanol extract Pf 4=Methanol extract. ***Ascorbic acid** is a standard reference drug

constituents are responsible for the antioxidant effect in biological systems. These properties play a vital role in absorbing and neutralizing free radicals, as well as in quenching singlet and triplet oxygen or decomposing peroxides [38]. The reducing power of the ethanol extract *P. funiculosum* in the current and other reported studies suggests that this extract is a promising resource of natural antioxidants.

Anti-microbial activity

Pf 2 and Pf 4 extracts and fractions (Pf 1, Pf 3) were investigated for their *in vitro* antifungal activity against the pathogenic fungal strains *Candida albicans* and *Aspergillus fumigatus. In vitro* antibacterial potential was also evaluated against G-positive bacteria*, e.g.*, *Staphylococcus aureus* and *Bacillus subtilis* [RCMB 015 (1), NRRL B-543], and G-negative bacteria, *Escherichia coli* (*E. coli*) ATCC 25955 and *Proteus vulgaris* [39].

The modified agar-well diffusion method was used for evaluating the sensitivity of the organisms against the activity of tested samples (10 mg/mL concentration). All tested samples indicated no activity against all tested fungi in comparison with the positive control, ketoconazole. Meanwhile, Pf 2 extract, Pf 1, and Pf 3 fractions showed significant effects on G-positive bacteria, *Bacillus subtilis*; (20±0.91, 19±0.84 and 17 ±0.998, respectively) in comparison with the positive control gentamycin (33.1±1.9). While Pf 2 and Pf 4 extracts and Pf 1 and Pf 3 fractions showed significant activity on G-negative bacteria*, E. coli* (15±0.84, 14 ±0.9, 13±0.77, and 11±1.02, respectively) with respect to the positive control, gentamycin (29.5±1.3**)** (Table 5). This is the first report about the effect of ethanol extract of *P. funiculosum* on the selected fungi, G-positive and G-negative bacteria as well as on the selected yeast. Meanwhile, the obtained results were in agreement with the published data by Rančić *et al.* as the DMSO extract of *P. ochrochloron,* which had moderate activity against G-positive *B. subtilis* (14.7 ±0.6), and G-negative *E. coli* (12.3±0.6) [40]. Furthermore, it was reported that compounds isolated from *P. chrysogenum* separated from different origins have antimicrobial effects on *MRSA, Staphylococcus aureus, C. albicans, E. coli,* and *Bacillus licheniformis* with different ratios according to the origin of the fungi [41].

Isolation and identification of the secondary metabolites of *Penicillium funiculosum*

In our study, the potential activity of the endophytic fungi *Penicillium funiculosum*, isolated from *Persicaria salicifolia* seeds growing in Egypt against HepG-2, HCT–116, A-549 and MCF-7 cells encouraged the assessment of further phytochemical investigation.

Table 5 *In-vitro* antimicrobial activities of the *Penicillium funiculosum* extracts and fractions tested at 10 mg/mL (100 μl) using modified well diffusion agar method and expressed as mean inhibition zone diameter (mm)

Samples	Tested microorganisms[1]					
	Fungi		Gram-positive bacteria		Gram-negative bacteria	
	A. fumigatus [2]RCMB 002008	*C. albicans* [1]RCMB 005003 (1) [2]ATCC 10231	*B. subtilis* RCMB 015 (1) NRRL-B-543	*S. aureus* RCMB010010	*P. vulgaris* [2]ATCC 13315	*E. coli* [2]ATCC 25955
Pf 1	NA	NA	19.00±0.84[a]	NA	NA	13.00±0.77[a]
Pf 2	NA	NA	20.00±0.91[a]	NA	NA	15.00 ±0.84[cb]
Pf 3	NA	NA	17.00±0.99[b]	NA	NA	11.00±1.02[c]
Pf 4	NA	NA	NA	NA	NA	14.00 ±0.90[ab]
[3]Ketoconazole	26.2±1.60	25.7±1.50	–	–	–	–
[3]Gentamycin	–	–	33.10±1.9[c]	31.90±1.70	28.8±1.6	29.5±1.3[e]

All data are presented as the mean±S.D of three replicates in each group. Statistical analysis was performed using one-way analysis of variance (ANOVA), accompanied by a *post–hoc* test [Least Significant Difference (LSD) test] using Costate software computer program. Results of *P* less than or equal to 0.05 were considered to be statistically significant. Different letters are significant at *P* less than or

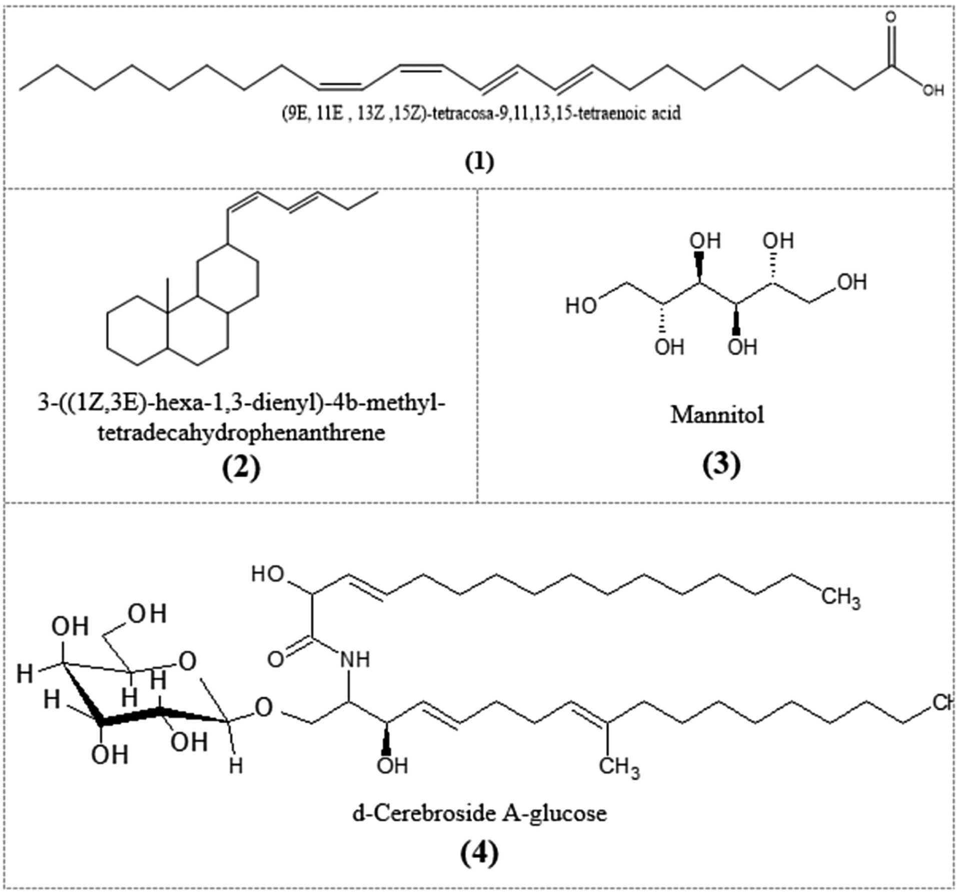
Pf 2 was subjected to extensive fractionation using silica gel chromatography followed by purification on a sephadex LH20 column according to their cytotoxic activity using the described method. Pf 2–2 A sub-fraction was selected to isolate the major secondary metabolites produced by that strain according to its significant high potency against the tested cell lines. Four compounds were isolated, purified and identified using physical, chromatographic and different spectroscopic techniques; ^{1}H NMR, ^{13}C NMR, and mass spectroscopy. The structure of these compounds (1-4) is showed in Fig. 1. The identification of the established compounds was confirmed by comparing their spectral data to those given in the literature [42–44].

Compound (1): white color powder; ^{1}H NMR (500 MHz, DMSO-d$_6$) δ: 2.4 (t, H3), 1.6 (m, H4), 1.33 (m, H4), 1.34 (m, H5), 1.35 (m, H6), 1.36 (m, H7), 2.09 (m, H8), 5.34 (m, H9), 5.36 (m, H10), 5.37 (m, H11), 5.38 (m, H12), 5.40 (m, H13), 5.42 (m, H14), 5.43 (m, H15), 5.46 (m, H16), 2.09 (m,H17), 1.33 (m,H18), 1.33–137 (m, H19-H23), 0.93 (t, H24). ^{13}C NMR (100 MHz, DMSO-d$_6$): 180.4 (C-1), 34.16 (C-2), 29.8 (C-3), 29.74 (C-4), 29.44 (C-5), 29.31 (C-6), 29.21 (C-7), 31.56 (C-8), 126.99 (C-9), 127.36 (C-10), 127.9 (C-11), 128.15 (C-12), 128.25(C-13), 128.74(C-14), 129.99(C-15), 130.2 (C-16), 32.01 (C-17), 29.4 (C-18), 27.23(C-19), 27.23 (C-20), 27.19 (C-21), 24.69 (C-22), 22.73(C-23), 14.09 (C-24). It was identified as (9E, 11E, 13Z, 15Z)-tetracosa-9, 11, 13, 15-tetraenoic acid.

Compound (2): white amorphous powder, on TLC; R_f =0.79 in solvent system [$CHCl_3$: MeOH; 85:15]. The molecular formula was set to be $C_{21}H_{35}$ on the basis of EI-MS, which showed the most important peak at m/z 288.

^{1}H NMR(500 MHz, DMSO-d$_6$) δ ppm: 1.98, dd (J=13.7, 3.3 Hz H-1), 1.47, dd (J=13.7, 3.8, Hz H-1), 2.4 (t, H-2), 1.6 (m, H-3), 1.49, dd (J=13.7, 3.8 Hz, H-4),1.33 (m, H-5), 0.9 (m, H-6), 0.9(m, H-7), 2.09 (m, H-8), 2.44 (m, H-9), 1.46, (dd, J=13.7, 3.8 Hz H-11), 1.37 (m, H-11), 2.38 (m, H-12), 1.42 (m, J =11.8, Hz,H-14), 5.43 (dd, J=6.5, 2.5 Hz, H-15), 5.61 (dd,

Figure 1



The isolated secondary metabolites compounds of *Penicillium funiculosum*.

J= 6.5, 2.5 Hz, H-16), 5.24 (dd, *J*= 7.5, 10.7 Hz, H-17), 5.24 (dd, *J*=7.5, 10.7 Hz, H-18), 2.7 (q, H-19), 0.88 (t, H-20), 0.67 (s, H-21). ^{13}CNMR (100 MHz, DMSO-d_6) δppm: 38.21 (C-1), 22.66 (C-2), 27.67(C-3), 29.64 (C-4), 46 (C-5), 29.61 (C-6), 29.61 (C-7), 31.2 (C-8), 37.41 (C-9), 37.31 (C-10), 36.71 (C-11), 39.74 (C-12), 36.58 (C-13), 29.65 (C-14), 128.5 (C-15), 127.75 (C-16), 126.92 (C-17), 129.65 (C-18), 36.95 (C-19), 23.41 (C-20), 14.37 (C-21).

^{1}H NMR showed olefinic protons at δ ppm: 5.43, 5.61, 5.24, and 5.24 (H- 15, 16, 17 and 18). A triplet of a doublet of a doublet was observed at δ: 1.66 (H-3). The presence of thirteen methylene protons with their signals between δ: 0.92 and 2.44. These signals assignments are in accordance with the reported values of steroids [45–49].

While at ^{13}C NMR; one quaternary carbon was observed at C10 (δ ppm: 39.31). Carbon 15, 16, 17 and 18 were confirmed by the presence of four alkene carbons with double bonds revealing distinct signals at δ ppm: 128, 125,126 and 127 and two carbons with them (CH_2 - CH_3) were also recognized at δ ppm: 14.41 and 22.06. The latter carbons were also confirmed by the Ms Fragment at M/Z at 208 (M-C_6H_9).

The assignments of values for ^{1}H NMR, ^{13}C NMR and mass spectroscopy indicated that it was a diterpene. It was identified as 3-(1Z, 3E-hexa-1,3-dienyl)-4b-methyl-tetradecahydrophenanthrene. It was considered to be firstly isolated from the genus *Penicillium.*

Compound (3). It was white amorphous powder with m.p. 167-169. The EI-MS analysis showed important peak at m/z 181 and the molecular formula was determined to be $C_6H_{14}O_6$. It was identified as mannitol. Mannitol was previously screened for their production from eleven strains of *Penicillium*. It was highly produced from *P. scabrosum* IBT JTER 4, and *P. aethiopicum* IBT MILA 4 on YES medium (sucrose and yeast extract) [50]. It was also isolated from the mycelium of *P. hirsutum, P. commune* [50], and *P. verruculosum* [51]. It is the first report to be isolated from this species.

Compound (4): white amorphous powder, on TLC R_f 0.49 in solvent system [$CHCl_3$: MeOH; 85:15]. The molecular formula was determined to be $C_{41}H_{75}NO_9$ on the basis of EI-MS which showed an important peak at m/z 546. It was identified as (d-Cerebroside A-glucose). It is the first report to be isolated from this species. A- cerebroside molecular, LAMA 1, was previously isolated from the ethyl acetate extract of *Penicillium oxalicum* and Penicillium chrysogenum and the reported data of ^{1}H-NMR and ^{13}C- NMR were similar to the isolated compound [52,53]. Penicillosides A and B (Cerebroside nature with glucose) were also isolated from the ethyl acetate extract of *Pencillium* species (marine-derived fungi, isolated from *Didemnum* species). Penicillosides A showed antifungal activity against *C. albicans*, while Penicilloside B showed antibacterial activity against *S. aureus* and *E. coli*. Our results about the ethyl acetate extract effect were in agreement with these compounds against *E. coli* [54].

Sphingosine: ^{1}H NMR (500 MHz, DMSO-d_6) H1A: 4.12 (m),d6) δ: 4.12 (m) H1B: 3.73 (dd, *J*=10.4, 3.6), 3.99 (dd, *J*=5.2, 8.4 H-2), 4.17 (m, H3), 5.47 (dd, *J* =14.4, 7, H-4), 5.74 (m, H5), 2.02 (m, H6), 2.07 (m, H7), 5.15 (t, 6.6, H8), 1.98 (m, H10), 1.42 (m, H11) 1.31 (H12–17), 0.92 (t, 6.5, H18), 1.62 (s, H-19). ^{13}C-NMR (100 MHz, DMSO-d_6): 68.3 (C-1), 53.2 (C-2), 71.5 (C-3), 133.1 (C-4), 129.6 (C-5), 32.4 (C-6), 27.4 (C-7), 123.5 (C-8), 135.3 (C-9), 39.4 (C-10), 27.8 (C-11), 28.9–31.7 (C-12-15), 32.1 (C-16), 22.4 (C-17), 13.1 (C-18), 14.8 (C-19).

Fatty acid: ^{1}H NMR, δ: 4.45 (d, 6, H2'), 5.52 (dd, 14.8, 6.4, H-3'), 5.86 (dd, 14.8, 6.4, 1.2, H4'), 2.02 (m, H5'), 1.31(H 6',13',14',15'), 0.92 (t, 6.5, H16'). ^{13}C-NMR: 174.1 (C-1'), 72.7 (2'), 133.3 (3'), 127.6 (C4'), 32.3 (5'), 28.9-31.7 (6'–14'), 22.4 (15'), 13.1 (16').

Sugar, glucose: ^{1}H NMR δ: 4.29 (d, 7.7, H1'), 3.22 (dd, *J*=8, 7.8, H2'), 3.37 (m, H3'), 3.31 (m,H4'), 3.31 (m, H5'), H6A: 3.67 (dd, *J* =12, 4.4), H6B: 3.88 (dd, *J* =10.8, 2.8). ^{13}C-NMR: 103.3 (C-1'), 73.6 (C-2'), 76.5 (3'), 70.2 (4'), 76.6 (5'), 61.3 (6').

Cytotoxic activity of isolated compounds

The cytotoxicity of four isolated compounds from active sub-fraction Pf 2–2 A was assessed against HepG-2 and HCT-116 cells. Compound 2, 3-((1Z,3E)-hexa-1,3-dienyl)-4b-methyl tetradecahydrophenanthrene, was found to be the most active against both cells, followed by compounds 4 (d-Cerebroside A-glucose) and 1 (9E, 11E, 13Z, 15Z)-tetracosa-9, 11, 13, 15-tetraenoic acid), respectively. Compound 3 (mannitol) did not show any activity (Table 6). There is not any reported data about the cytotoxic activity of the isolated compounds against HCT-116 cell line. While, A Cerebroside previously isolated from the *Penicillium chrysogenum* strain S003, LAMA (1), showed weak cytotoxic activity against hepatocellular carcinoma (HepG2), lung cancer (A-

Table 6 Cytotoxicity (IC_{50} values μg/ml) of the four isolated compounds from Pf 2-2 A sub-fraction of *Penicillium funiculosum* PF2 extract against human colon carcinoma-116 and hepatic cancer-2 cells

Compound	IC_{50} (μg/ml)	
	Hepatic cancer-2	Human colon carcinoma –116
1	29.00±1.30^{a}	34.00±1.07^{a}
2	**5.80±0.50^{b}**	**4.30±0.20^{b}**
3	-	-
4	17.40±1.00^{c}	10.50±0.40^{c}
*Vinblastine Sulphate	3.48±0.22^{d}	**3.50±0.70^{d}**

All data are presented as the mean±S.D of three replicates in each group. Statistical analysis was performed using one-way analysis of variance (ANOVA), accompanied by a *post–hoc* test [Least Significant Difference (LSD) test] using Costate software computer program. Different letters are significant at *P* less than or equal to 0.05. ***Vinblastine sulphate** is a standard reference drug. Compound 1= **(1)** 9E,11E,13Z,15Z)-tetracosa-9, 11, 13, 15-tetraenoic acid, **compound (2)** 3-(1Z,3E-hexa-1,3-dienyl)-4b-methyl-tetradecahydrophenanthrene**,** **Compound (3)** mannitol, **Compound (4)** d-Cerebroside **A-glucose)**.

549), breast adenocarcinoma (MCF-7), and prostate (DU-145) cell lines using SRB assay [53]. Meanwhile, Penicillosides A and B which were isolated from the ethyl acetate extract of marine-derived *Pencillium* possessed weak activity on cervical cancer (HeLa) cell line [54] and human leukemia (HL-60) cell line [55].

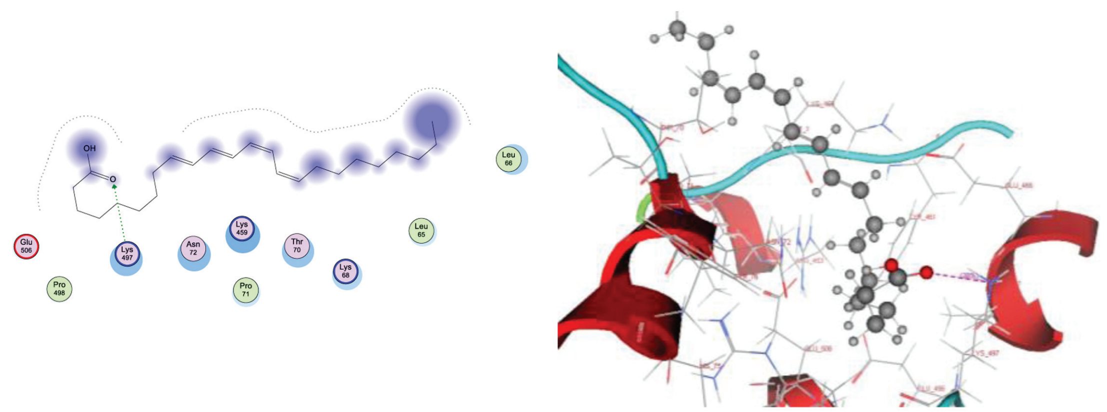
Molecular docking study

The detected interactions of the compounds with amino acids and hydrogen bond lengths are shown in Figs. 2–4. Virtual screening of the COX-2 inhibitory properties of all compounds was evaluated through molecular operating environment in an *in silico* molecular docking study. The results represented promising and valid potential binding modes with the same co-crystalline ligands.

The molecular docking study revealed that compound 4 was sitting deeply in the binding site of COX-2 with a binding free energy −22.8200 kcal/mol. It participates in hydrogen bonding interactions with Glu465 and Lys497 (Fig. 2a and b). The study also displayed the high affinity of compound 1 with a score energy= −19.2858 kcal/mol, and its binding modes showed interaction with Lys497 residue (Fig. 3a and b). Furthermore, compound 3 displayed a moderate affinity with a score energy= −10.5507 kcal/mol, and its binding mode showed hydrogen bonding interactions with Glu466 and Lys459 residues (Fig. 4a and b). There isn't any reported data about the molecular docking study of the isolated compounds.

Generally, the anti-inflammatory activity of the compounds isolated from the ethyl acetate extract of *P. funiculosum* is in agreement with the reported anti-inflammatory activity of compounds separated from *P. chrysogenum.* This species was isolated from different origins, examined using HEK293 cells, and showed a powerful inhibitory effect on TNF-α-stimulated NF-κB activation [56]. One of these previously reported

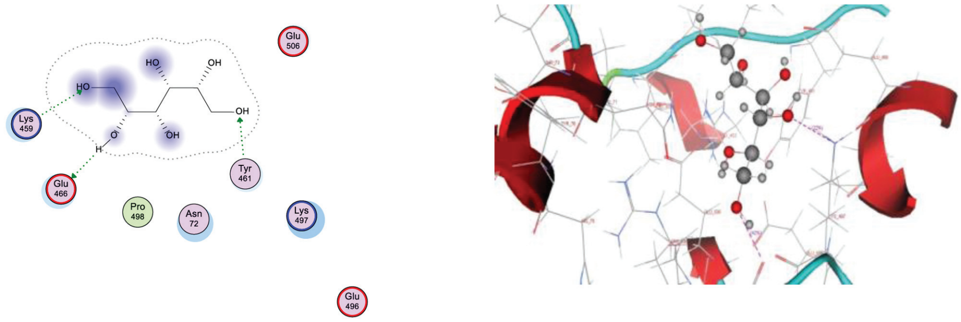
Figure 2



(a) **(b)**

Molecular docking of compound 1 (a) two-dimensional; binding modes and (b) three-dimensional; mapping surface of compound 1 with COX-2 with Score Energy= −19.2858 kcal/mol.

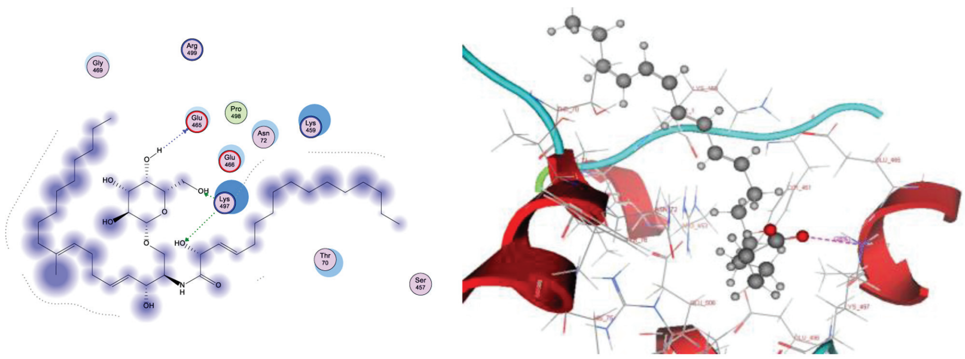
Figure 3



(a) **(b)**

Molecular docking of compound 3 (a) two-dimensional; binding modes and (b) three-dimensional; mapping surface of compound 3 with COX-2 with Score Energy= −10.5507 kcal/mol.

Figure 4



(a) **(b)**

Molecular docking of compound 4 (a) two-dimensional; binding modes and (b); three-dimensional mapping surface of compound 4 with COX-2 with Score Energy= −19.2858 kcal/mol

compounds isolated from *P. chrysogenum* was HPABA (benzoic acid derivative), which possesses a significant anti-inflammatory effect [57,58].

Conclusion

The ethyl acetate extract (Pf 2) of the endophytic fungi *Penicillium funiculosum*, isolated from the *Persicaria salicifolia* plant growing in Egypt was found to possess significant cytotoxic activity against HepG-2, HCT-116, A-549, and MCF-7 cell lines. Meanwhile, its antioxidant and antimicrobial activities were found to be moderate. Four compounds were isolated from Pf 2. Compound (2) was identified as 3-((1Z,3E)-hexa-1,3-dienyl)-4b-methyl tetradecahydrophenanthrene, which is considered to be firstly isolated from the genus *Penicillium* and from the nature and shows the most potent cytotoxic effect. Compounds 1, 3 and 4 were identified as (9E, 11E, 13Z, 15Z)-tetracosa-9, 11, 13, 15-tetraenoic acid, mannitol, and d-Cerebroside A-glucose, respectively. It is the first report of their isolation from this species *P. funiculosum*. They have significant cytotoxic activity against HepG-2 and A-549 cells. Compound 4 showed potent anti-inflammatory when studied by Molecular docking using COX-2 enzyme. These findings help in the recommendation of using the Pf 2 of endophyte *Penicillium* as a cytotoxic and an antioxidant agent.

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Nil.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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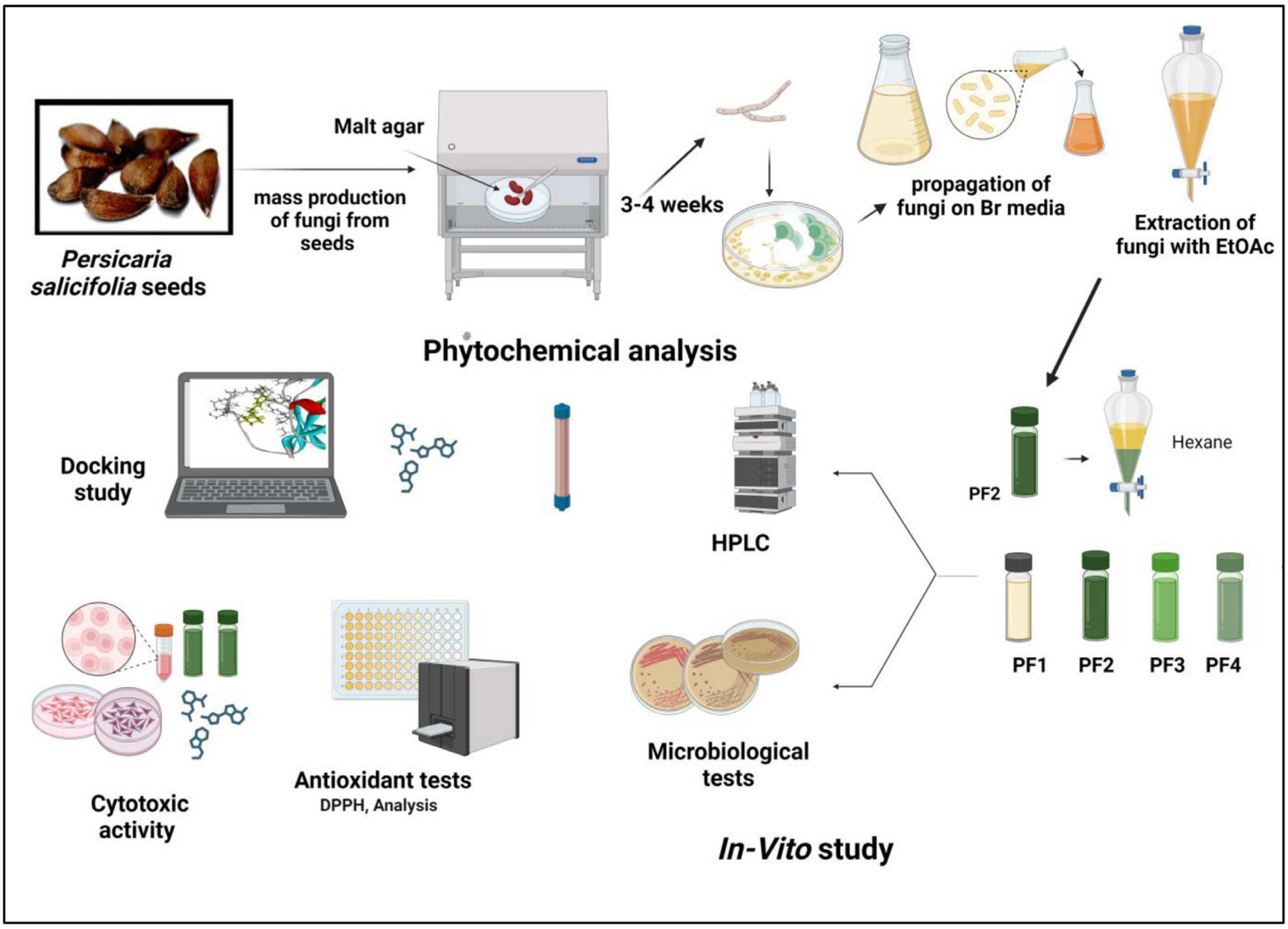

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