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Cytotoxic potential of bioactive compounds from Aspergillus flavus, an endophytic fungus isolated from Cynodon dactylon: experimental and computational approach

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Abstract: Endophytic fungi are a diverse group of microorganisms that colonize the inter- or intracellular spaces of plants for mutual benefits. The interactions with a host plant and other microbiomes are multidimensional and play a crucial role in the production of secondary metabolites. We screened bioactive compounds present in the extracts of *Aspergillus flavus*, an endophytic fungus isolated from the roots of the medicinal grass *Cynodon dactylon*, for its anticancer potential. Ethyl acetate extract from isolated *A. flavus* showed significant cytostatic effects (IC_{50}: 16.25 μg mL^{-1}) against breast cancer cells (MCF-7). Morphology of cells and DAPI stained nuclei along with the results of flow cytometry annexin V/PI assay suggested apoptosis to be the main process leading to cells' death. While investigating the mechanism that triggers apoptosis, we found that the extract of *A. flavus* increased ROS generation and caused loss of mitochondrial membrane potential of MCF-7 cells. To identify the metabolites that might be responsible for the anticancer effect, the extract was examined using gas chromatography-mass spectrometry (GC-MS). Interestingly, nine phytochemicals were found to have potential inhibitory effects of anti-apoptotic protein (Bcl-2) in the breast cancer cells. In the *in silico* molecular docking and molecular dynamics simulation studies revealed that two compounds: 2,4,7-Trinitrofluorenone and 3alpha, 5alpha-Cyclo-ergosta-7,9(11),22t-triene-6beta-ol exhibited significant binding affinities (-9.20, and -9.50 Kcal mol^{-1}, respectively) against Bcl-2 along with binding stability and intermolecular interactions of its ligand-Bcl-2 complexes. Overall, the study found that the endophytic *A. flavus* from *C. dactylon* contains plant-like bioactive compounds that have a promising effect in breast cancer.

Keywords: *Cynodon dactylon; Aspergillus flavus*; endophytic fungus; secondary metabolites; anticancer; breast cancer; Bcl-2

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1. Introduction

Cancer is one of the major devastating diseases worldwide, with approximately 10 million deaths in 2020, or about one in every six deaths. It has been estimated that the number of cancer-related deaths may increase to 13.1 million by 2030 [1]. Each cancer case is different and unique, features like origin of the tissue, type of genetic mutations and surface protein markers are used to systematically analyze and classify cancers for better prevention, diagnostics and treatment [2]. Among the various types, breast cancer is one of the most frequent among women, with an estimated 1.38 million new cases every year [3]. Breast cancer ranks second in the world based on its incidence and mortality rates [4]. Although the exact mechanism that triggers cancer transformation is not recognised, several genetic mutations and epigenetic dysregulations affecting genes related to proliferation, cell differentiation and death are frequently observed in cancer cells [5]. Normal breast development depends on apoptosis, and the cell death process carried out by caspases. In healthy breast epithelial cells, pro-apoptotic and anti-apoptotic signals are closely controlled. The members of the B-cell lymphoma-2 (Bcl-2) family composed of pro-apoptotic and anti-apoptotic protein, plays a pivotal role in the mitochondria-mediated apoptosis. Dysregulation of this balance leads to breast tumorigenesis and raises acquired resistance to treatments, including molecularly targeted therapies, radiation and chemotherapies [6].

Last decades brought great improvement in the treatment of breast cancer patients. In many cases, if diagnosed early, breast cancer is considered curable. The current treatment strategies, however, still rely on surgery, radio- and chemotherapy. Targeted therapy using small drug inhibitors and immunotherapy is now used more often. The treatment regime includes: classic chemotherapeutic medicines, such as taxol (Paclitaxel), doxorubicin (Adriamycin) or vinblastine (Velbe), small-molecule targeted drugs like lapatinib, neratinib or imatinib mesylate (Gleevec/Glivec), a tyrosine kinase inhibitors, and monoclonal antibodies, particularly trastuzumab (Herceptin), are used in therapy [7]. Despite the undoubted progress in the treatment of breast cancer, in some cases the disease develops resistance to therapy. Another problem is the influence of treatment on a patient's life which is significantly affected by the adverse side effects of the therapy [8]. These disadvantages of the current mode of breast cancer treatment are addressed using two strategies: first is the optimization of treatment protocols (multiple therapies, algorithms of procedures, doses etc.) and second is searching for new, effective compounds that could selectively inhibit cancer cells survival pathways, minimizing the side effects. The overexpression of Bcl-2 in breast cancer cells leads cell proliferation and survival, and promotes therapy resistance due to the evasion of apoptosis [9]. Therefore, it is vital to seek novel effective therapeutic agents with limited side effects for treating breast cancer.

Natural compounds derived from plant, bacteria, fungi and algae from both terrestrial and marine sources have played a significant role in the development of cancer therapeutics as evidenced by the large number of drugs from these sources that have been approved for the cancer treatment [10-12]. The biodiversity of fungi has been estimated at 2.2 million to 3.8 million species and about 148,000 already described natural compounds from fungi could be a potent source of drugs [13]. Unfortunately, only around 5% of these fungi have been cultivated in the laboratory, thus only a small number of fungal-derived compounds have reached tests so far [14-15]. The huge variety of metabolites produced by fungi includes different structural types of compounds such as: amino acids, aromatic chemicals, anthracenones, butenolides, cytochalasans, macrolides, naphthalenones, terpenes and pyrones [16-18]. There are various factors that make fungi an attractive source of medicinal compounds; these include: their rapid growth, culture conditions, high cell density, ease of genetic modification [19]. Growing amount of research on fungal metabolites reveals their promising anticancer properties [20].

Endophytic fungi (phyla: fungi), live inter- or intracellularly in various plant species, contribute to their host plants by producing a plethora of substances that

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provide protection and ultimately enhance the survival value of the plant [21]. Endophytic fungi are auspicious resources of secondary metabolites, from which enzymes [22]. antimicrobials [23], and anticancer chemicals, such as bikaverine [20] and triterpenes [24], have been identified. Many unique natural compounds with antioxidant and anticancer properties are yet to be discovered in endophytes.

Cynodon dactylon, often known as "Durva Grass", "Bermuda grass", "Dog's Tooth grass", "Bahama grass", "Devil's grass", "Couch grass", "Indian Doab", "Scutch grass", "Dhub", and "Arugampul", is a plant of immense religious significance that is reportedly used in Ayurvedic, Siddha, Unani, Nepalese, and Chinese medical systems [25]. *C. dactylon* extract has been successfully tested in preclinical studies for its anti-tumor activity. For example, in vivo studies of *C. dactylon* roots extract in diethyl nitrosamine (DEN) inducing liver cancer in mice have shown its significant anticancer activity [26]. As we know that *C. dactylon* is a home for endophytic fungi that can be a source of unique substances, a present study aimed to investigate the potential antitumor properties of the bioactive compounds of endophytic fungi alone in breast cancer models. To achieve this goal, we used a wide range of research tools, from the isolation of fungi from the natural environment, cultivation and identification of fungi, through molecular biology methods, to computational analyzes at the level of single molecule interactions.

2. Materials and Methods

2.1. Materials and reagents

Dulbecco's Modified Eagles medium (DMEM), rhodamine-123, fetal bovine serum (FBS), Tris-EDTA buffer, propidium iodide (PI), ethidium bromide (EtBr), annexin-V, 4′,6′-diamino-2-phenylindole, fluorescein isothiocyanate (FITC), amphotericin B, phosphate buffered saline (PBS), 2′,7′-dichlorofluorescein diacetate (H2DCF-DA), penicillin G sodium, streptomycin sulphate, lactophenol were purchased from HiMedia Laboratories, Mumbai, India. Other organic solvents and fine chemicals were of analytical grade and were purchased from Merck and SD Fine Chemicals, Mumbai, India.

2.2. *Cell lines and culture media*

The MCF-7 cell line (human breast cancer epithelial cells) was obtained from the National Centre for Cell Science, Pune, India. For the culture and *in vitro* cytotoxicity experiment, MCF-7 cells were cultured in a culture flask or plates (Falcon, USA) in DMEM, supplemented with 20% heat inactivated FBS, 1% penicillin, streptomycin, and amphotericin B. In stable conditions of 37 °C and 5% CO_2 in a humidified atmosphere. Passage no higher than 20 was used (P18 in most of the experiments). The cells were frequently tested against mycoplasma according to a laboratory protocol.

2.3. Collection of plant and isolation of endophytic fungus

Nourishing free growth *C. dactylon* plants were gathered from Sundarapandiam village, Virudhunagar Dist., Tamil Nadu, India (latitude 9.59721°N, longitude 77.67405°E). The location is characterized by a high level of vegetation development and a large variety of plants, including several species of grass. Immediately after collecting the grass, samples were transported to the laboratory and carefully cleansed with distilled water. On the collection day, fungal endophytes were isolated as described by Tapfuma et al. [27]. The *C. dactylon* plant root was chopped into tiny (1 cm) pieces under sterile conditions. This was washed using running tap water for 2–5 min to remove dirt particles and undesired debris, followed by rinsing with deionized water. The chopped plant root was then sterilized by soaking in 70% ethanol for 1 min, followed by 1% sodium hypochlorite for 6 min. The root sample was cleaned again for 1 min in 70% ethanol before being rinsed with sterile deionized water. The sterilized root sample was

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coarsely crushed and serially diluted in sterile water using a sterile mortar and pestle. Dilutions ranging from 10^{-2} to 10^{-6} were plated on potato dextrose agar (PDA, pH 5.6) and incubated at 27 °C for 4–7 days. The plates were examined, after 48 h, for the formation of fungal colonies. The morphology, texture and color of the colonies were recorded. Lactophenol cotton blue (LCB) wet mount was used to observe microscopic morphology of the fungi.

2.4. Molecular identification of endophytic fungi

The endophytic fungus isolated from *C. dactylon* was identified using molecular gene sequencing using the methods used by Sette et al. 2006 [28]. The 200 mg of fungal mycelial mat were mashed with a pestle and mortar in 500 μL of extraction buffer (200-mM Tris–HCl (pH 8.0), 25-mM EDTA (pH 8.0), 250-M NaCl, 10% CTAB) containing [Cetyltrimethyl ammonium bromide (CTAB)-phenol–chloroform–isoamyl alcohol technique (CTABPCI)]. Transferred to a new tube, 3 μL proteinase K and 3 μL RNase were added, vortexed, and incubated at 37 °C for 1 h. After incubation, tubes were placed in a 65 °C water bath for 10 min. After adding one volume of phenol, chloroform, and isoamyl alcohol (25:24:1), the solution was well mixed for 5 min before being centrifuged at 12,000 rpm for 5 min. The clear aqueous phase was collected and combined with one volume of chloroform: isoamyl alcohol (24:1) mixture before centrifugation at 12,000 rpm for 5 min to recover the aqueous phase. One volume of ice-cold isopropanol was added and held overnight at -20 °C for DNA precipitation. The DNA was precipitated with 100% ethanol after being centrifuged at 10,000 rpm for 5 min and was then washed twice with 1 mL of 70% ethanol before being resuspended in 200 μL of deionized water or 1x TE [200 mM Tris–HCl (pH 8.0), 20 mM EDTA (pH 8.0)] buffer. A portion of the eluted fungal DNA was electrophoresed on a 0.8% agarose-EtBr gel, and the concentration was calculated using Qubit 3.0.

PCR with universal primers recognizing internal transcribed spacer (ITS) region of the fungal DNA: ITS 5 (TCCTCCGCTTATTGATATGC) forward and ITS 4 (GGAAGTAAAAGTCGTAACAAGG) reverse was used to identify species of isolated fungi. Amplified DNA fragments. was validated and purified with a GeneJET PCR purification kit (Thermo Scientific, EU-Lithuania) to exclude primer dimer and other carryover contaminants. Using a 2% agarose gel and a 100-bp DNA ladder as a size reference, the product's quality was evaluated and determined to be acceptable for sequencing. The Big Dye® Terminator 3.1 sequence kit was used to purify and prepare PCR-amplified products for sequencing (Applied Biosystems, Foster City, California, USA). According to the manufacturer's instructions, denatured products were sequenced in both the forward and reverse directions using a Genetic Analyzer 3500 (Life Technologies Corporation, Applied Biosystems®, California 94404, USA). To validate the species, sequences were aligned and modified using Mega software version 10. The annotated ITS rDNA contig was submitted to GenBank and assigned an entry number.

2.5. Evaluation of anticancer activity

2.5.1. MTT assay

The cytotoxic properties of endophytic fungal extract was assessed using metabolic MTT assay in MCF-7 cells. The test allows to assess cellular redox potential that gives information about viability of cells and indirectly estimate cells' number. It is widely used in the *in vitro* testing of anticancer properties of novel compounds and therapeutic strategies. Briefly, MCF -7 cells were seeded into 96-well microtiter plates and cultured at 37 °C with 5% CO_2 for 24 h to reach confluency. Then, the cells were treated with various concentrations of endophytic fungal extract (100, 50, 25, 12.5, 6.25, 3.125 μg mL^{-1}). After 24 h, MTT assay was performed according to standard procedure [29]. Untreated cells served as control. Absorbance was measured at 570 nm using a microplate reader (Bio-Rad, USA). The percentage viability was calculated using the following formula:

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Cell viability (%)* = (A (mean absorbance of extract treated cells) × A(control cells)$^{-1}$) × 100; *n = 3, where n is the number of independent experiments.

2.5.2. Detection of apoptotic morphology using dual staining assay

The MCF-7 cells were cultured in 24-well microtiter plates and treated with IC_{50} concentration of the endophytic fungal extract for 24 h. After 24 h treatment, the cells were rinsed in ice cold 1x PBS (phosphate buffered saline) and then stained with two fluorescent DNA binding stains [(10 μg mL^{-1}) (Acridine orange and Ethidium bromide)][30]. After 30 min of incubation, the cells were observed for apoptosis under fluorescent microscope (Labomed TCM 400 Inverted Binocular Microscope, USA, using Image aR Pro software) at 40× magnification. The untreated cells served as control for the study. The percentage of the apoptotic cells was calculated using the formula: Total number of apoptotic cells: Total number of normal cells^{-1}) × 100.

2.5.3. Apoptosis analysis using flow cytometry

MCF-7 cells were seeded on 24-well microtiter plates and cultivated overnight at 37 °C in a CO_2 incubator. Next, the cells were exposed to an IC_{50} concentration of the fungal extract for 48 h. Following incubation, the cells were washed in PBS and centrifuged for 5 min at 500 × g in 4 °C. The supernatant was then removed, and the cell pellets combined to a concentration of 1×10^5 mL^{-1} in an ice-cold 1x binding buffer. 1 μL of annexin V-fluorescein isothiocyanate reagent and 1 μ of propidium iodide (PI) were added to the tubes on ice. The tubes were then kept on ice and incubated in the dark for 15 min. It was meticulously mixed in 400 μL of ice-cold 1x binding buffer. Within 30 min, flow cytometry analysis was performed (BD FACS calibur-Becton Dickinson, CytExpert v 1.2.11.0) [30].

2.5.4. Assessment of mitochondrial transmembrane potential

The mitochondrial transmembrane potential was determined by using lipophilic dye, Rhodamine-123 [32]. MCF-7 cells were seeded in the 24-well and maintained for 24 h in a 5% CO_2, 37 °C conditions to reach exponential growth phase. Then, the cells were exposed to IC_{50} of the fungal extract. After 48-h incubation the cells were stained with Rhodamine-123 dye for 30 min. Subsequently, they were washed with PBS and fixed with paraformaldehyde (4%) for 30 min. The morphological alterations and the permeability of the membrane was examined under the fluorescent microscope. The untreated cells served as a control.

2.5.5. Nuclear integrity measurement

MCF-7 cells were treated with endophytic fungal extract for 48 h in a 24-well flat bottom microplate as described in previous sections. Then, the cells were washed with PBS and fixed in paraformaldehyde (4%) for 30 min. After this, the cells were washed in Triton X100 (0.4%) for 20 min and washed in PBS. Next, the cells were stained with DAPI (0.5 μg mL^{-1}) in the dark for 1 min and washed with PBS. A fluorescent microscope with an appropriate filter was used to capture the images of the cells [31].

2.5.6. Determination of reactive oxygen species (ROS)

The level of intracellular ROS was measured using 2′,7′-dichlorofluorescein diacetate. The MCF-7 cells were cultured for 24 and 48 h after being exposed to 10% FBS and the IC_{50} concentration of endophytic fungal extract. The cells were then washed twice with PBS before being tagged with 10 M H_2DCF-DA according to the manufacturer protocol. Fluorescence intensity was measured using a fluorescence spectrophotometer (SpectramaxM2 fluorescence, spectrophotometer, Molecular devices, USA) at 475 nm (λex) and 525 nm (λem) [32].

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2.6. Gas Chromatography-Mass Spectrum (GC-MS) Analysis

We used sonication to lyse and homogenise fungal mycelia for extraction of secondary compounds [33]. Around 500 mg of fungi (pure mycelial culture) was taken and dispersed in 10 mL of 80% ethyl acetate solution. The contents were subjected for sonication in the ultrasonic bath sonicator (Elma Ultrasonic Cleaner S100H, Mumbai, India) at 80 W cm^2 intensity for 0.5 pulse cycles at 40 °C temperature. After the process of sonication, the contents were filtered by using Whatman No. 1 paper filter to eliminate the mycelia and the remaining filter was centrifuged at 2500 rpm. The liquid containing secondary metabolic compounds of the endophytic fungi was determined by GC-MS analysis. A Shimadzu Make QP-2010 on non-polar 60 M RTX 5MS column was used for the GC-MS analysis with helium as the carrier gas, containing 15 psi as constant pressure.

2.7. In silico molecular docking

GC-MS spectrum provided a list of biologically active phytochemicals contained in the isolated endophytic fungal metabolic extract. The compounds were utilized for the molecular docking studies against over expressed anti-apoptotic protein receptor (Bcl-2, PDB id: 6O0K, Resolution: 1.62 Å) of breast cancer. The three-dimensional molecular structure of compounds (ligands) from the endophytic fungal extract was created using Chemsketch software. The Chemistry at Harvard Molecular Mechanics (CHARMm) force field was used to optimize ligands and minimize energy. The generated ligands structures were converted into the ".pdb" file format using BIOVIA|Discovery Studio Visualizer v20.1.0.19295 software (Accelrys Software Inc., San Diego, USA). The target protein model was retrieved from the RCSB's protein data repository [33].

2.8. Molecular dynamics (MD) simulation

The MD simulation studies were performed to investigate the binding stability of the top scored compounds of an endophytic fungal extract with anti-apoptotic protein receptor (Bcl-2) of breast cancer [34]. Using the Desmond dynamic package 2017 in Schrodinger (Academic version) in a Linux environment, the time-dependent change of the complexes was calculated over 200 ns [34]. The complex of identified phytocompounds with the Bcl-2 receptor was created using the OPLS (optimal potentials for liquid simulations)-2005 force field [35]. Additionally, a water model was created using the established SPC water model at distances of 10 Å units from an orthorhombic periodic boundary [36]. Additionally, the electric charges were neutralized by adding the required number of counter ions, and before the MD simulation process started, the system decreased its energies through heating and equilibrium processes. The Nose-Hoover approach with the NPT (isothermal-isobaric ensemble) was used to apply 300 K temperature and one atmospheric pressure (1.01325 bar) throughout the system's final manufacturing stage, which lasted up to 200 ns [37, 38]. The best confirmations were chosen with regard to the complex's interactions and dynamical characteristics.

2.9. Statistical analysis

SPSS Statistics version 20.0 was used for all statistical analyses. One-way ANOVA followed by Dunnett's multiple comparisons post hoc test was used, with significance level set at $p < 0.05$ or lower. Representative results of at least three independent repeats are shown in each experiment.

3. Results

3.1. Isolation of fungi and microscopic examination

Separate triplicate efforts were made for the selection of the endophytic fungus from the roots of *C. dactylon*. Final endophytic fungi were selected for the study based on

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the morphology of the colonies and antioxidant potentials of their extracts of four. In potato dextrose agar, the fungi colony looks powdery and has an olive-green conidial appearance. Later, the chosen fungus was sub-cultured and purified for further analysis. Figure 1(a) depicts the colony morphology of the endophytic fungi and Figure 1(b) illustrates the microscopic view of the mycelia and the fungal spores using the lactophenol cotton blue mounting (LCB) technique.

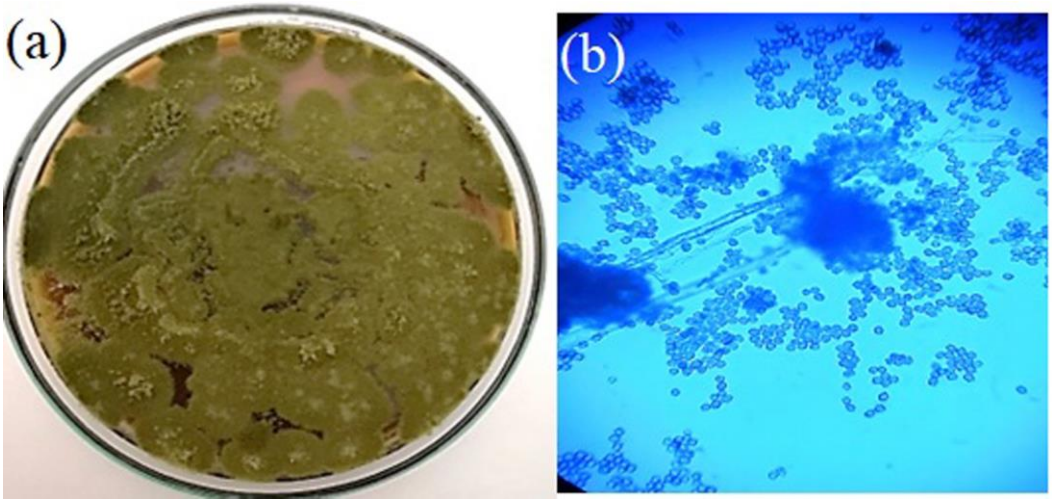

Figure 1. *Morphology of the colonies of the endophytic fungus: (a) image of the colonies in Potato Dextrose Agar (b), microscopic view of the fungal spores (LCB).*

3.2. Phylogenetic analysis

Four endophytic fungal isolates were successfully isolated from *C. dactylon*. One of these isolates, CD02, has shown higher anti-oxidant activity and hence was investigated further. Through the systematic selection of isolates, the fungal culture, CD02, was categorized according to their morphological parameters, such as shape and color of the colony, and reverse media colour. Each fungal colony was purified by transplanting sequential hyphal ends over 2–3 passes. The selected fungal isolate was identified using polymerase chain reaction (PCR) with ITS4 and ITS5 as forward and reverse primers respectively. The BLASTn analysis revealed that one of the fungal strains, CD02 belongs to the genus Aspergillus, and the species was identified as *Aspergillus flavus* (Genbank No: ON509999.1). The rRNA sequence of the isolate, CD02 has 96% identity with Aspergillus flavus. The 18S rRNA sequence of these fungal isolates was aligned with reference strains from GenBank and this formation was used to build a phylogenetic tree (Figure 2) showing relations of the newly identified fungal strain with other members of the genus Aspergillus. The Neighbour Joining tree demonstrated a tight link between the strains recovered in this investigation and Aspergillus flavus MT584825.

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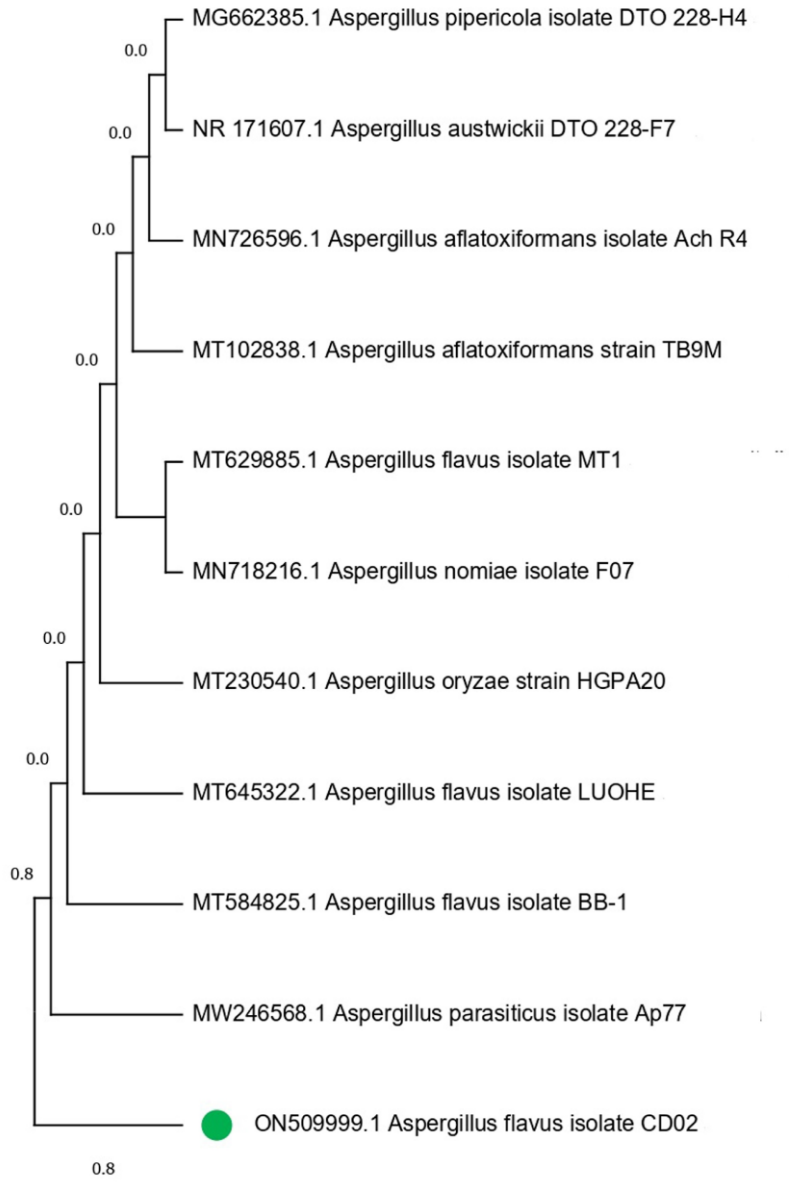

Figure 2. *Aspergillus* phylogeny tree based on analysis of ITS sequence. The isolate CD02 (with bootstrap index >50%) is marked with green dot.

3.3. Anticancer activity

3.3.1. Cytotoxicity assay

The cytotoxic efficacy of the *A. flavus* extract against MCF-7 cells was assessed using metabolic MTT assay, a viability test directly measuring cellular redox potential in the population of cells. It was found that the lowest studied concentration of *A. flavus* extract (3.125 μg mL^{-1}) reduced the viability of cells to 86.3 ± 1.097%. The IC_{50} value of the fungal extract was 16.25 μg mL^{-1}. Figure 3(a) illustrates the % of viability of cells at various concentrations (μg mL^{-1}) and Figure 3(b, c) shows the untreated control cells (Figure 3(b)) and changes in morphology of the MCF-7 cells after being exposed to *A. flavus* extract (Figure 3(c)). The IC_{50} concentration of *A. flavus* extract (16.25 μg mL^{-1}) was then selected for further anticancer activity studies. Doxorubicin (0.25 μM), a widely used chemotherapeutic, served as a positive control for this experiment. Statistical data was presented in supplemental Table S1.

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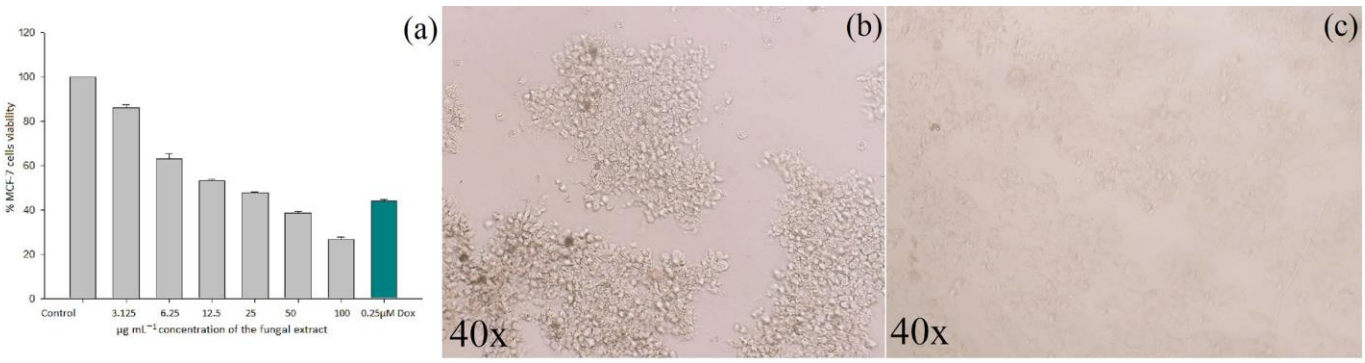

Figure 3. Cytostatic effects of 24-h treatment with serial dilutions of A. flavus extract on MCF-7 cells. (a) The percentage loss of viability of cells determined with MTT assay is shown. Values are mean ± standard deviation of triplicate measurements ($p < 0.05$); (b) morphology of the control cells; (c) morphology of cells treated with 16.25 μg mL^{-1}.

3.3.2. Analysis of apoptosis using dual staining

MCF-7 cells treated with the IC_{50} concentration of *A. flavus* extract were stained with AO/EtBr to visualize living and dead cells. Using fluorescent microscopy, apoptotic and necrotic morphology in MCF-7 cells treated with *A. flavus* extract were observed. Orange early apoptotic cells and late apoptotic cells displaying irregular shape of chromatin in the nucleus and deformed membrane (red) were seen in the extract-treated samples (Figure 4).

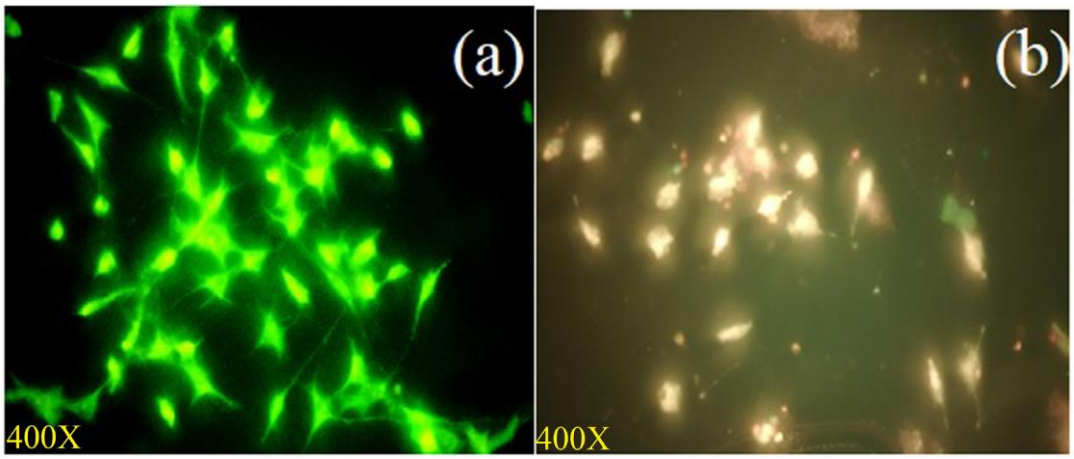

Figure 4. Fluorescent microscopy images of apoptotic structural changes of Acridine orange/ Ethidium bromide (AO/EtBr) stained MCF-7 cells upon *A. flavus* extract exposure: (a) Untreated control cells; (b) cells treated with 16.25 μg mL^{-1} of *A. flavus* extract for 48 h.

3.3.3. Assessment of apoptosis by flow cytometer

Apoptosis was also studied using annexin V and PI staining. Figure 5 shows the results of the flow cytometry analysis of *A. flavus* extract induced apoptosis in MCF-7 cells treated with 16.25 μg mL^{-1} of extract for 48 h. Early apoptosis was observed in 14% of treated cells, which was significantly higher than in the control cells (0.89%). The percentage of cells in late apoptosis induced by *A. flavus* extract was also significantly higher (38.9%) as compared to control cells (5.52%). Table 1 displays the percentage fraction of live, early apoptosis, late apoptosis, and dead cells.

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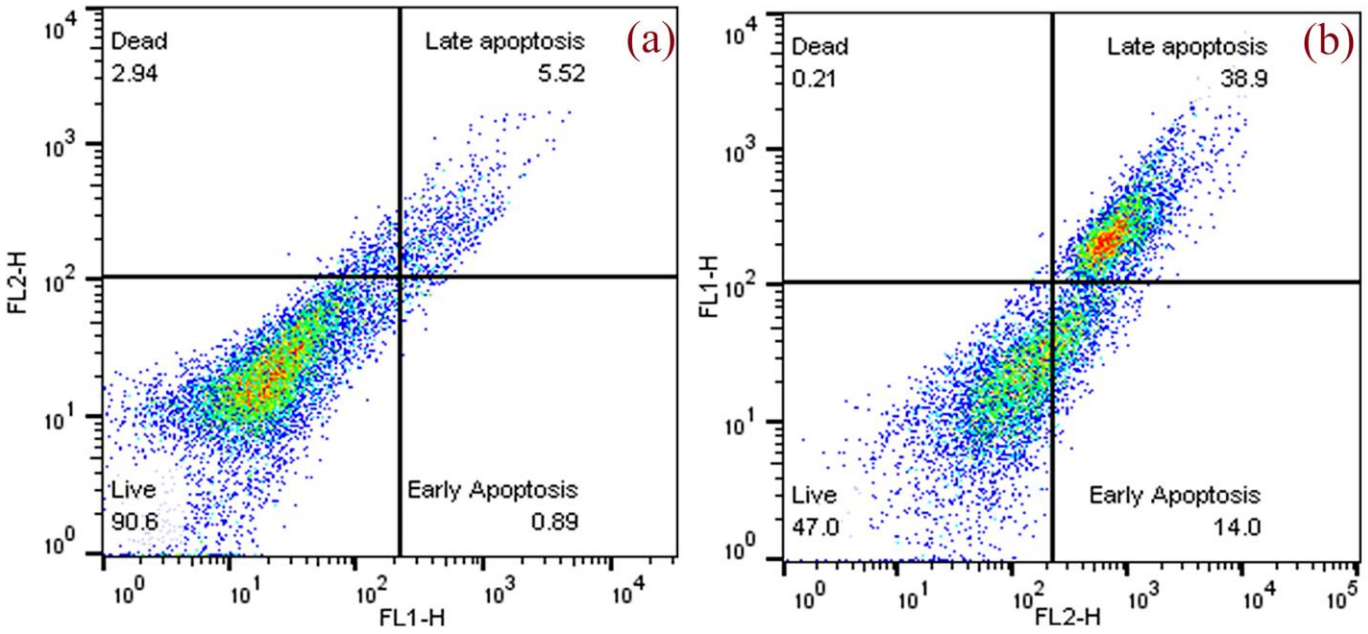

Figure 5. Flow cytometry Annexin V/Propidium Iodide (PI) analysis shows increased apoptosis of MCF-7 cells treated with *A. flavus* extract: (a) untreated control (b); MCF-7 cells treated with *A. flavus* extract.

Table 1: Flow cytometry analysis of annexin V/ PI apoptosis assay performed on MCF-7 cells treated with *A. flavus* extract

MCF-7 cells	Q4 Live (%)	Q3 Early apoptosis (%)	Q2 Late apoptosis (%)	Q1 Dead (%)
Control	90.6	0.89	5.52	2.94
EF extract	47.0	14.0	38.9	0.21

3.3.4. Measurement of mitochondrial transmembrane potential

Next, the changes in mitochondrial membrane potential were examined using rhodamine-123 dye following 24-h exposure with or without *A. flavus* extract (IC_{50}). After 24 h, the mitochondrial membrane potential in MCF-7 cells treated with *A. flavus* extract was significantly reduced (Figure 6 (a) and (b)). Some morphological changes in *A. flavus* extract treated cells and their mitochondria can also be observed.

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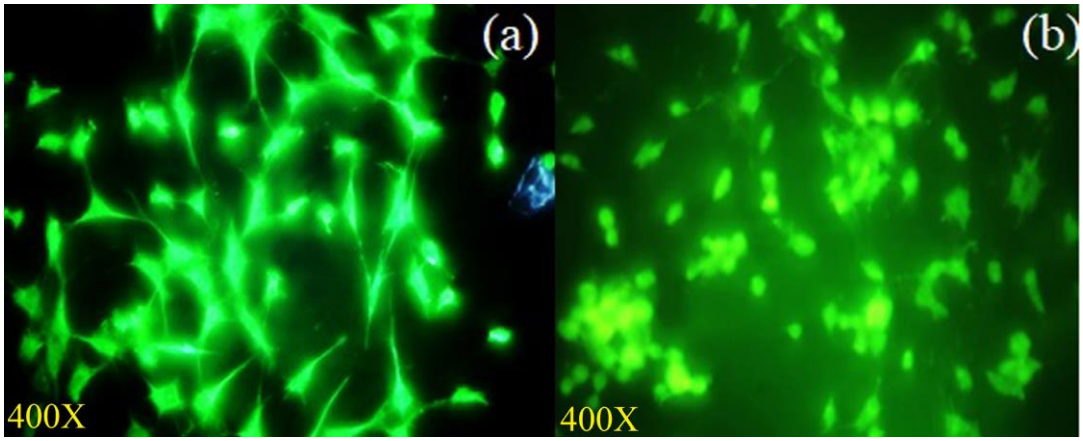

Figure 6. Fungal extract reduces the mitochondrial transmembrane potential as seen under fluorescent microscope using rhodamine-123 staining: (a) Untreated control cells; (b) depolarized mitochondrial membrane in MCF-7 cells treated with IC_{50} of *A. flavus* extract.

3.3.5. Assessment of nuclear integrity by DAPI staining

As apoptotic processes induced in MCF-7 cells treated with *A. flavus* extract were observed, DAPI staining was used to observe apoptosis associated morphological changes of cellular nuclei (Figure 7). DAPI stain binds to adenine and thymine regions of the nuclear DNA. After 48-h treatment of cells with *A. flavus* extract, we observed shrinkage of the cells, condensation of the chromatin, and damage to the nuclear DNA (Figure 7). In addition, compared to the control, the *A. flavus* extract-treated MCF-7 cells showed a decreased intensity of blue fluorescence. All these phenomena confirm the apoptotic nature of the cell death induced by *A. flavus* extract.

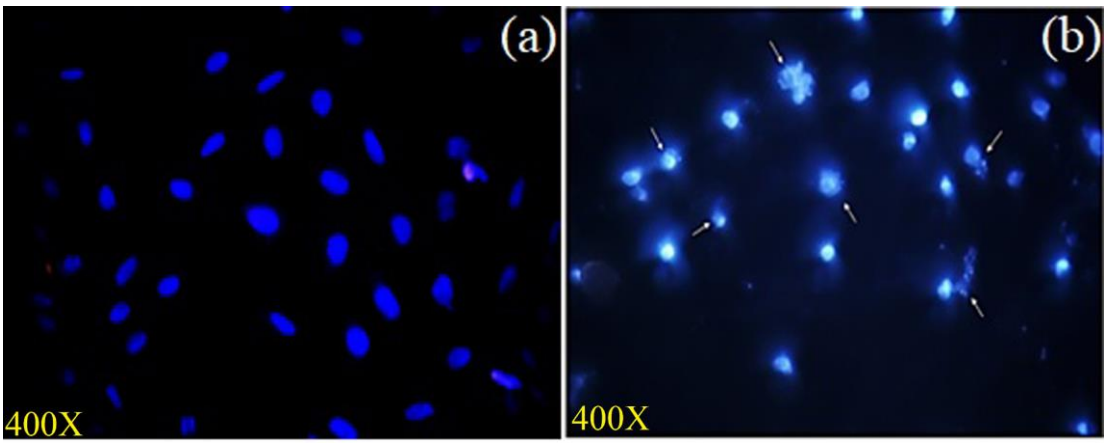

Figure 7. MCF-7 cells stained with DAPI: (a) untreated control; (b) MCF-7 cell treated with 16.25 μg mL^{-1} of extract Chromatin condensation and fragmentation (indicated by arrow), nucleus shrinkage and blebbing was also observed.

3.3.6. Assessment of intracellular reactive oxygen species

An important factor in the development and biology of cancer is the imbalance between levels of reactive oxygen species and antioxidants. Figure 8 (a) displays the results of the induction of ROS by *A. flavus* extract in MCF-7 cells using H_2DCF-DA staining method. Increased green fluorescence was seen in 16.25 μg mL^{-1} of *A. flavus* extract-treated cells as compared to the control (Figure 8 (b) and (c)), indicating the beginning of apoptosis in MCF-7 cells. Statistical data are presented in Table S2.

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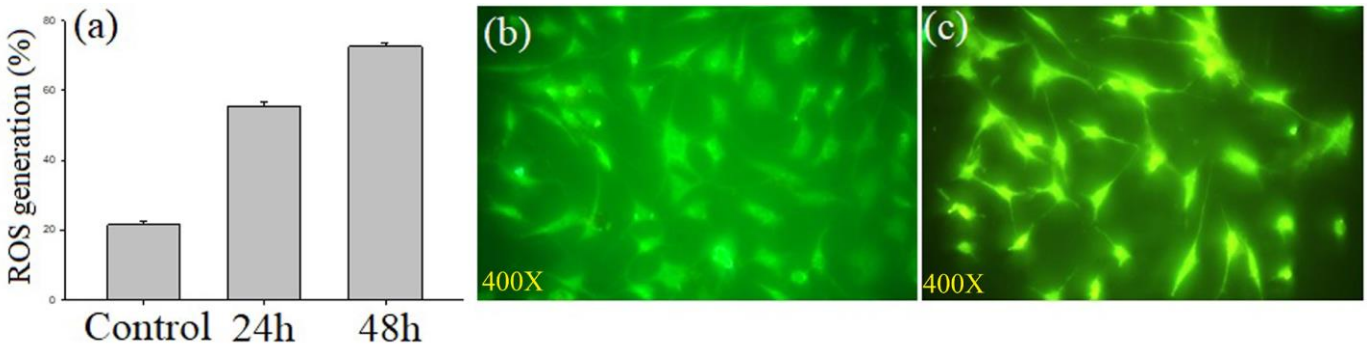

Figure 8. Effects of 16.25 μg mL^{-1} of *A. flavus* extract on ROS generation (percentage of control) in MCF-7 cancer cells. Results expressed as mean ± standard deviation of triplicate measurements ($p < 0.05$) (a); ROS generation measured as relative fluorescence intensity using fluorescence microscope. (b) Image of untreated control cells; (c) increased ROS in *A. flavus* extract treated cells.

3.4. GC-MS analysis

The CG-MS spectra of the *A. flavus* extract revealed the presence of bioactive phytochemicals with corresponding peaks at time of retention. In total, 9 bioactive compounds were identified from the GC-MS spectrum (Figure 9). The major bioactive phytochemicals present were Tritetracontane, Heptadecanoic acid, Methyl 2,8-dimethyltridecanoate, 2,3,4-Trimethyllevoglucosan, 2,4,7-Trinitrofluorenone, 1H-Thiopyrano[3,4-c] pyridine-5-carbonitrile, 3,6-Bis(N-formamido) carbazole, 3 alpha, 5 alpha,-Cyclo-ergosta-7,9(11), 22t-triene-6.beta.-ol, 1H-Isoindole-1, 3(2H)-dione. Table 2 displayed the identified phytochemicals and their structures.

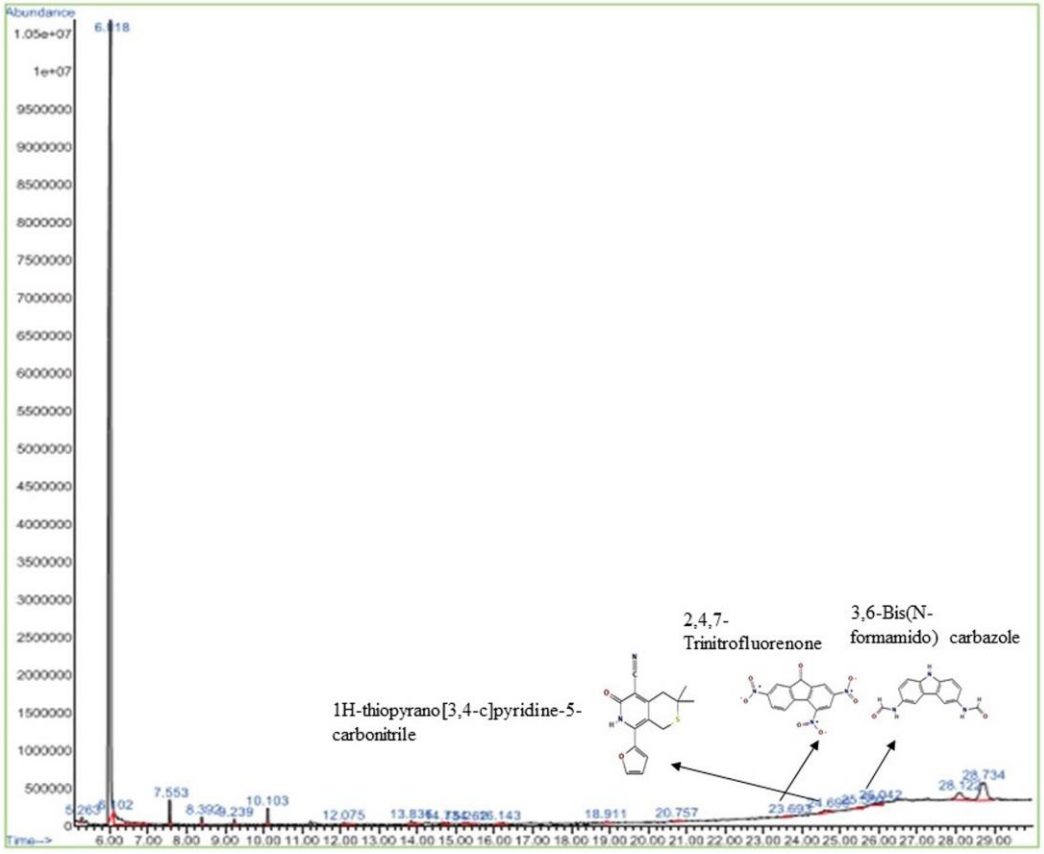

Figure 9. GC-MS based phytochemical profiling of *A. flavus* extract.

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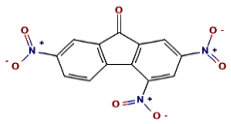
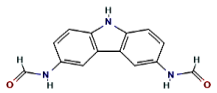
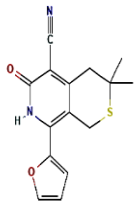
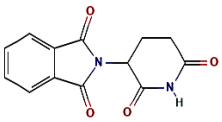
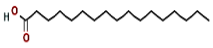
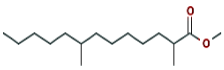
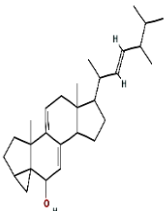
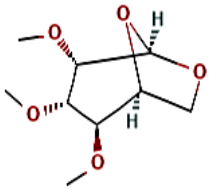
Table 2: Phytochemicals found in *A. flavus* extract using GC-MS.

S. No	Retention time	% Area of peak	Phytochemical compounds	Molecular formula	Molecular weight (in g mol^{-1})	Structure
1	23.693	0.25	2,4,7-Trinitrofluorenone	$C_{13}H_5N_3O_7$	315.19	
2	25.580	0.80	3,6-Bis(N-formamido) carbazole	$C_{14}H_{11}N_3O_2$	253.26	
3	24.699	0.86	1H-thiopyrano[3,4-c]pyridine-5-carbonitrile	$C_{15}H_{14}N_2O_2S$	286.4	
4	28.734	6.00	1H-Isoindole-1,3(2H)-dione	$C_{13}H_{10}N_2O_4$	258.23	
5	18.911	0.30	Heptadecanoic acid	$C_{17}H_{34}O_2$	270.5	
6	18.911	0.30	Methyl 2,8-dimethyltridecanoate	$C_{16}H_{32}O_2$	256.42	
7	26.042	1.15	3alpha,5alpha-Cyclo-ergosta-7,9(11),22t-triene-6beta-ol	$C_{28}H_{42}O$	394.6	
8	20.757	0.33	2,3,4-Trimethyllevoglucosan	$C_9H_{16}O_5$	204.22	

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9	14.734	0.21	Tritetracontane	$C_{43}H_{88}$	605.2	

3.5. Molecular docking

Molecular docking studies were used to examine the intermolecular interaction between the target protein (Bcl-2) and bioactive phytochemicals. The identified bioactive phytochemicals showed a strong intermolecular interaction and significant binding affinities against Bcl-2. According to molecular docking studies, the binding energies of the bioactive phytochemicals ranged from -4.50 to -9.50 kcal mol^{-1}, as shown in Table 3. Two bioactive compounds, 2,4,7-Trinitrofluorenone (-9.20 kcal mol^{-1}), and 3alpha, 5alpha-Cyclo-ergosta-7,9(11),22t-triene-6beta-ol (-9.50 kcal mol^{-1}), demonstrated better binding affinities against the anti-apoptotic protein receptor (Bcl-2) of breast cancer as compared to standard drug Venetoclax (-10.90 kcal mol^{-1}). Two top scoring compounds along with Venetoclax as a reference drug were taken for further MD simulation studies to confirm the stability of the complexes. Furthermore, the protein-ligand interaction profiler online tool (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index) was used to visualize the intermolecular interactions between ligand and target protein (Bcl-2). The visualized result indicates that the chosen compound, 2,4,7-Trinitrofluorenone, showed a docking score (-9.20 kcal mol^{-1}) against the anti-apoptotic protein Bcl-2, and it formed three hydrophobic interactions (PHE104A (3.28Å), ASP111A (3.93Å), LEU137A (3.55Å)) with the receptor, as depicted in Figure 10(a) and 10(b). The top scored bioactive phytocompound, 3alpha, 5alpha-Cyclo-ergosta-7,9(11),22t-triene-6beta-ol established contact with Bcl-2 through 7 hydrophobic bonds (PHE104A (3.41Å), TYR108A (3.82Å), TYR108A (3.97Å), LEU137A (3.57Å), ARG146A (3.91Å), PHE153A (3.76Å), VAL156A (3.69Å)), and 2 hydrogen bonds (ARG156A (2.20Å), ARG156A (1.98Å)) as presented in Figure 10(c) and 10(d). The standard drug Venetoclax formed eleven hydrophobic bonds (PHE104A (3.17Å), PHE104A (3.68Å), PHE104A (3.99Å), PHE104A (3.84Å), ASP111A (3.76Å), VAL133A (3.96Å), GLU136A (3.74Å), LEU137A (3.76Å), VAL148A (3.75Å), ALA149A (3.99Å), TYR202A (3.62Å)), three hydrogen bonds (ASP103A (1.93Å), TYR108A (3.25Å), ASN143A (2.03Å)), and one salt bridges (ASP111A (4.73Å)) with the target anti-apoptotic protein Bcl-2, as presented in Figure 10(e) and 10(f).

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Table 3: Bioactive phytochemicals from *A. flavus* extract and standard drug with their binding affinity against Bcl-2 receptor (PDB id: 6O0K).

S. No	CID	Compound	Binding affinity (Kcal mol^{-1})
1	8521	2,4,7-Trinitrofluorenone	-9.2
2	620086	3,6-Bis(N-formamido) carbazole	-6.2
3	6809	1H-Isoindole-1,3(2H)-dione	-6.1
4	658451	1H-thiopyrano[3,4-c]pyridine-5-carbonitrile	-6.2
5	10465	Heptadecanoic acid	-5.4
6	560473	Methyl 2,8-dimethyltridecanoate	-5.5
7	5363271	3alpha, 5alpha-Cyclo-ergosta-7,9(11),22t-triene-6beta-ol	-9.5
8	91699158	2,3,4-Trimethyllevoglucosan	-4.5
9	522398	Tritetracontane	-5.3
Standard drug			
10	49846579	Venetoclax	-10.9

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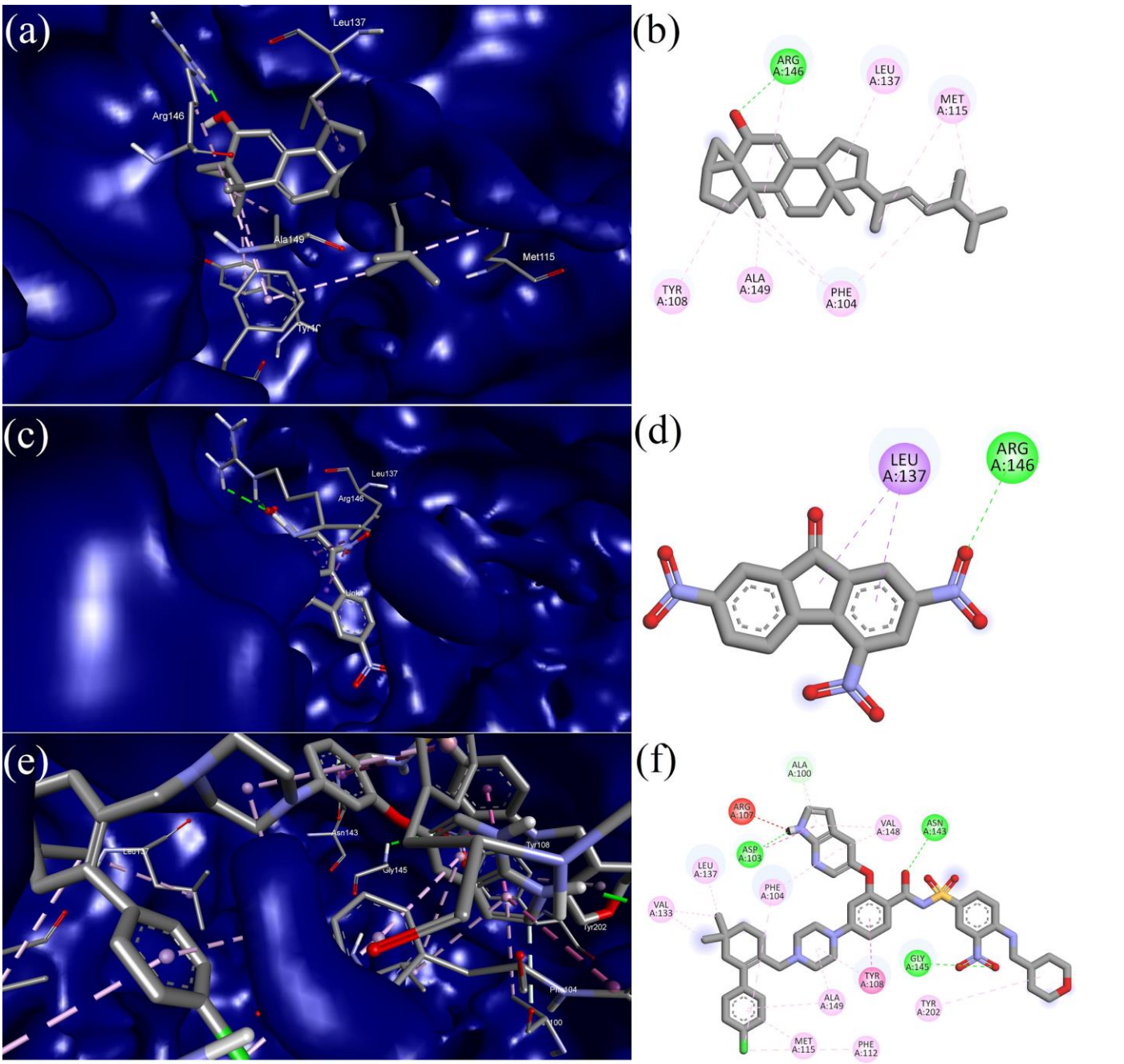

Figure 10. Images represents 3D (left) and 2D (right) maps of protein–ligand complex interaction between: (a, b) 3alpha, 5alpha-Cyclo-ergosta-7,9(11),22t-triene-6beta-ol and Bcl-2 receptor; (c, d) 2,4,7-Trinitrofluorenone and Bcl-2 receptor; and (e, f) Venetoclax and Bcl-2 receptor.

3.6. Molecular dynamics (MD) simulation

The molecular dynamic simulation was carried out for 3alpha,5alpha-Cyclo-ergosta-7,9(11),22t-triene-6beta-ol, 2,4,7-Trinitrofluorenone and standard Venetoclax complexed with Bcl-2 protein for 200 ns. The MD trajectory events of Apo protein Bcl-2 showed fluctuation to 3.2 Å and retained its stability to 3 Å throughout the simulation period with an average RMSD of 2.851 Å. The phytoconstituent 3alpha,5alpha-Cyclo-ergosta-7,9(11),22t-triene-6beta-ol, 2,4,7-Trinitrofluorenone complex showed fluctuated initially up to 3 Å and retain its stability thereafter till the end with 2.8 Å throughout the simulation period with an average RMSD of 2.14 Å, while the other selected phytoconstituent 2,4,7-Trinitrofluorenone showed 2.98 Å and retained its stability 2.6 Å throughout the simulation period with an average of 2.278 Å. The Venetoclax complex showed the fluctuation of 2.8 Å and retained its stability to 2.4 Å throughout the

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simulation period with an average RMSD of 2.368 Å (Figure 11). RMSF analysis of all complexes shows no major fluctuation due the binding of amino acids with the key functional groups of ligands (Figure 12).

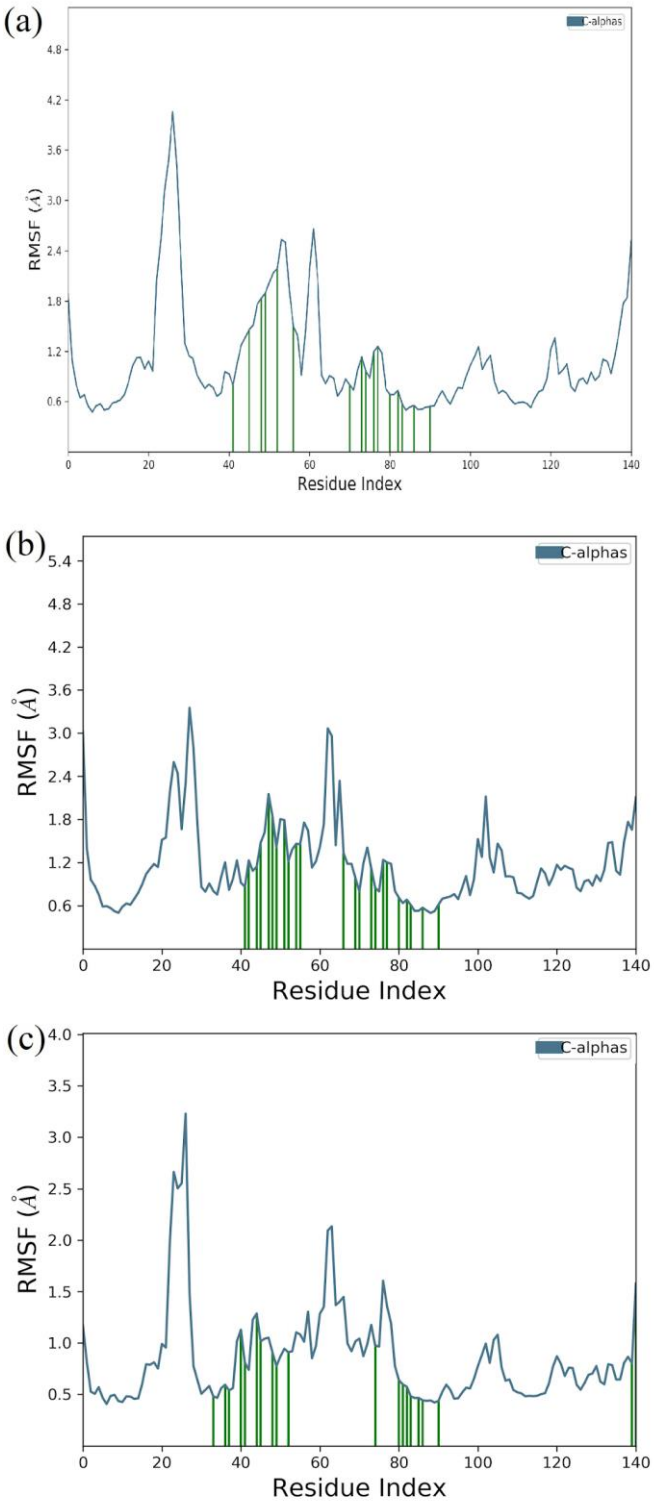

Figure 11: Root mean square fluctuation (RMSF) of 3alpha, 5alpha-Cyclo-ergosta-7,9(11),22t-triene-6beta-ol and Bcl-2 receptor complex; 2,4,7-Trinitrofluorenone and Bcl-2 receptor complex; and Venetoclax and Bcl-2 receptor complex.

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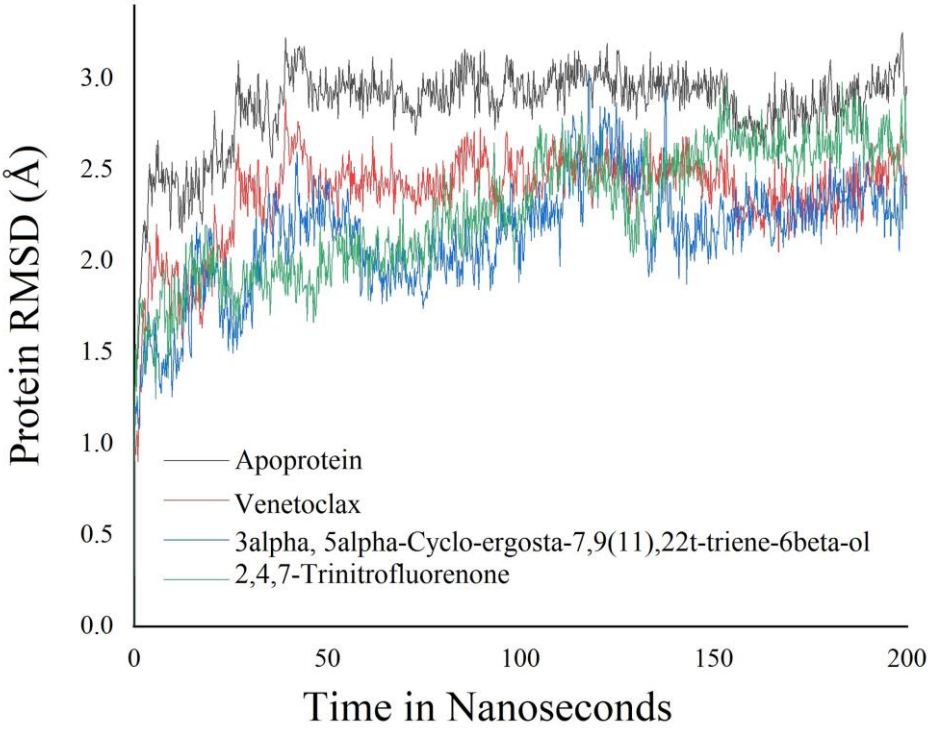

Figure 12: Root mean square deviation (RMSD) of backbone atoms of APO and its complex with Bcl-2 receptor; 2,4,7-Trinitrofluorenone and complex with Bcl-2 receptor; and Venetoclax and complex with Bcl-2 receptor.

4. Discussion

Despite undoubted progress in cancer treatment new approaches are still needed to overcome imperfectness of current therapies such as development of drug resistance, relapse, secondary cancers and adverse effects of therapeutic procedures. A potent source of new compounds for cancer treatment can be found in the greatest chemical laboratory on earth – nature. Sustainable drug discovery programs to find new therapeutic phytocompounds with anticancer potentials are run in parallel to chemical engineering of drug molecules [39]. In recent years, the use of phytomedicines for cancer treatment has noticeably increased [40]. Phytomedicines are already used for successful treatment of many diseases and overcome many harmful adverse effects of standard methods [41]. Plant derived medicines are also used more and more often in combination therapy with other medicines to reduce adverse effects and to increase effectiveness of the treatment [42-43]. Almost 60% of the anticancer drugs currently being used for anticancer therapy are obtained from natural sources[44-45]. In this study we found highly effective phytocompounds with anticancer potential from a fungus, *A. flavus*, that is found in medicinal plants.

Unexpectedly in recent years, materials and products with considerable medicinal values have been found to be produced by microorganisms associated with plants rather than by actual plants [46]. Endophytic bacteria and fungi that thrive in apparently healthy internal plant tissues are either facultative or obligate symbiotic microbes [47]. As mentioned earlier, *Aspergillus* species are an important resource of novel active phytochemicals with potential therapeutic values [48]. The biggest advantages of endophytic fungi are their fast renewal, relatively easy cultivation and economic and environmentally safety [49]. Numerous bioactive substances with a wide range of activities were found in endophytic fungi over the last 20 years [50]. These bioactive compounds could be classified as: flavonoids, alkaloids, terpenoids, diterpenes, sesquiterpenes, polyphenols, phenolic acids, indole derivatives, aliphatic compounds, pyridine, pyrazolidine, pyrimidine, and thiazole derivatives, etc., In this study, the bioactive compounds present in the isolated endophytic fungi, *Aspergillus flavus* from the

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root of *C. dactylon* have been determined to have anticancer properties against human breast cancer cells. *C. dactylon* (Family: Poaceae) was described to contain numerous bioactive compounds, such as vitexin, orientin, homoorientin, friedlein, beta-carotene, triterpenoids, ergonovine, luteolin, ergometrinine, phytosterols, 2''-O-glycosylisovitexin, arundoin, apigenin, tricin, beta-ionene, 2-coumarinate, triglochinin, ferulic acid, phenyl acetaldehyde, syringic acid, vanillic acid, l-ascorbic acid, phytol, palmitic acid, docosanoic acid, hexadecanal, tritriacontane, furfuryl alcohol, furfural, etc., which are involved in contributing to the therapeutic effects [51].

Lead compound extraction is one of the important steps to isolate desired bioactive compounds from natural sources [52]. Unfortunately, the lead compounds produced by plants and microorganisms are very minimal [53]. Thus, we need an ideal extraction technique for separation of these traces of lead compounds from natural resources. In this view, the selection of ideal solvent, solvent concentration, temperature, time, and suitable extraction technique might be increasing the quantity as well as quality of bioactive compounds from endophytic fungus [54]. In this study, fungal secondary metabolites (bioactive compounds) were successfully extracted through ultrasound-assisted extraction (UAE) method using 80% ethyl acetate as the extraction solvent. UAE has grown more and more popular, in recent years, due to its many benefits, including reduced energy consumption, shorter extraction time, less active component degradation, and higher extraction yield as compared to traditional extraction methods [32].

In order to elucidate the mechanism of the loss of MCF-7 cell viability under the influence of the *A. flavus* extract observed in the MTT assay, we performed assays for apoptotic cell death as a possible cause of this phenomenon. In this study, we investigated the anticancer activity and apoptosis-inducing properties of *A. flavus* extract against human adenoma breast cancer cells (MCF-7). For the cytostatic/ cytotoxic screening we used metabolic MTT assay and after 24 h of treatment we observed concentration-dependent drop of viability, where 100 μg mL^{-1} of *A. flavus* extract reduced cell viability by more than 75%. The IC_{50} value against MCF-7 cells was 16.25 μg mL^{-1}. This is similar to the observations made in endophyte, *Terminalia catappa,* wherein the IC_{50} against human cervical cancer, HeLa cells was estimated to be 33.35 μg mL^{-1} [55].

In order to evaluate the mechanism of the loss of MCF-7 cell viability under the influence of the *A. flavus* extract observed in MTT assay, we conducted assays for apoptotic cell death as a possible cause of this phenomenon. Apoptosis is a cell's ordered process of programmed cell death; and is the preferred type of cancer cell death in the treatment of cancer [56]. Therefore, a quantitative and qualitative apoptosis assays were performed using AO/EtBr double staining, flow cytometric analysis with Annexin V, PI, and FITC staining, along with DAPI staining assay and finally assays for changes in mitochondrial membrane potential, and measurement of ROS generation as a possible direct cause triggering apoptosis. The results of AO/EtBr stained MCF-7 cells showed that endophytic fungal extract induces intrinsic apoptosis. *A. flavus* extract treated cells were undergoing apoptosis, indicated with observation such as reduction in the number of cells, round or irregular shape, shrinkage, condensed nuclei and distorted membrane. Further, microscopic images of the *A. flavus* extract treated MCF-7 cells stained with DAPI, showed nuclear shrinkage, chromatin condensation, nuclear fragmentation and apoptotic morphology like cell shrinkage and swelling. These results confirmed that the extract's cytotoxicity was due to the induction of apoptosis in MCF-7 cells.

Mitochondria play a significant role in cellular functions, including the production of energy (ATP), maintenance of Ca^{2+} ions, cell signalling, cell cycle progression, and finally cell death as loss of mitochondrial membrane potential and Ca^{2+} release is among intrinsic signals triggering apoptosis. Mitochondrial membrane potential is generated by protons pumped into the inner membrane space of the mitochondria, an essential process to generate ATP. Mitochondrial membrane depolarization can be caused by excessive generation of ROS, high intracellular calcium concentrations or oxidative

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stress of endoplasmic reticulum [57]. In this study, 16.25 μg mL^{-1} (IC_{50}) *A. flavus* extract triggered mitochondrial membrane potential break down. This led to the respiratory chain uncoupling, excessive ROS production, followed by oxidative stress with oxidative damages of essential biomolecules and thus cell cycle arrest and finally induced apoptotic cancer cell death [58]. It has been shown before that *Aspergillus* extracts can lead to oxidative stress [59].

The observed cytotoxicity can be a result of activity of different compounds present in the *A. flavus* extract. Thus, to determine which components of *A. flavus* extract are responsible for observed anticancer effects, GC-MS analysis has been performed. GC-MS spectral data demonstrated the presence of bioactive compounds that might be responsible for anticancer activities. The obtained GC–MS spectra have shown the presence of 9 phytochemicals, and seven of them were considered as bioactive compounds. The cytotoxic activity of one of these bioactive compounds, i.e. 1H-Isoindol-1,3 (2H)-dione against the HeLa, C6 and A549 cancer cell lines has already been established [60]. Another one, heptadecanoic acid inhibited cell proliferation in PC9 non-small cell lung cancer cells [61]. Similarly, tritetracontane have also been already proven for its anticancer efficacies [62].

Computational drug discovery tools are helpful in identifying highly active molecules narrowing down the biological and synthetic research needs [63]. Moreover, these tools assist to predict pharmacokinetics and pharmacodynamic properties of the molecules, which are subsequently confirmed by *in vitro* and *in vivo* studies [64]. In addition to that, they provide information about how these molecules bind with, interact with, donating/accepting electrons and up or down regulate the protein/ enzyme activity. This may aid researchers in developing treatment alternatives for specific diseases. Surprisingly, some of the compounds identified in the extract exerted structural features that showed structural properties that predisposed them to possible interaction with the Bcl-2 receptor, which is overexpressed on cells of the breast cancer subtype called Bcl-2-enriched breast cancer [65]. Thus, we performed molecular docking analysis of bioactive compounds, 3alpha, 5alpha-Cyclo-ergosta-7,9(11),22t-triene-6beta-ol which showed highest binding affinity (-9.5 Kcal mol^{-1}) against over expressed anti-apoptotic protein receptor, Bcl-2 of breast cancer. The binding affinity score was close to the standard drug Venetoclax (-10.90 Kcal mol^{-1}), that targets Bcl-2 and some other receptor kinases and is clinically used in breast cancer treatment for that purpose. Recent evidence suggests Bcl-2 can directly influence apoptosis by translocation to the mitochondria to inhibit cytochrome c release [66]. Altogether, compounds identified in the endophytic fungal extract have a vast range of possible anticancer action against breast tumors. Not only can it hit the universal cellular energy metabolism system by uncoupling membranes of mitochondria and subsequently generate ROS but also potentially be effective against Bcl-2-enriched breast cancer subtype.

5. Conclusions

To sum up, *A. flavus* is an endophytic fungus isolated from the roots of *C. dactylon*, and their ethyl acetate extract showed a potent anticancer activity against breast cancer (MCF-7) cells. The profiling of bioactive compounds from ethyl acetate extract of endophytic fungus was detected through GC-MS analysis, and 9 bioactive compounds were identified. Among them, two phytocompounds, namely, 3alpha, 5alpha-Cyclo-ergosta-7,9(11),22t-triene-6beta-ol, and 2,4,7-Trinitrofluorenone exhibited highest binding affinities, -9.50, and -9.20 Kcal mol^{-1}, respectively, to the anti-apoptotic protein (Bcl-2) receptor. MD simulation studies confirmed stability of intermolecular interactions of ligands-receptor binding complexes of *A. flavus* extract exerted cytotoxic effects significantly reducing viability of MCF-7 cells with an IC_{50} of 16.25 μg mL^{-1}. The extract induced apoptosis in MCF-7 cells associated with high ROS generation, nuclear material damage and dissipation of mitochondrial transmembrane potential. To conclude, *A. flavus*, an EF, isolated from the roots of *C. dactylon* have a potential to be a

source of novel therapeutic drugs. Additional studies are required to purify the compounds and elucidate the mechanism of action and study its safety, so that these compounds could be developed as future drugs for therapy of cancer.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: MTT assay; Table S2: ROS generation assay;

Author Contributions: Conceptualization, P.M. (Piotr Maszczyk), K.S.; S.K.; methodology, A.K.K.; S.K.; software, P.M. (Piotr Maszczyk); V.D.; validation, E.B.; P.M. (Piotr Mrowka); P.M. (Piotr Maszczyk), K.S. and S.K.; formal analysis, A.K.K.; P.P.; T.P.; and G.R.; investigation, P.M. (Piotr Maszczyk), K.S.; S.K.; resources, S.K.; K.S.; E.B.; J.P.; and P.M. (Piotr Maszczyk); data curation, E.B.; S.K.; K.S.; P.M. (Piotr Maszczyk); writing-A.K.K.; S.K.; writing-review and editing, P.M. (Piotr Mrowka); P.M. (Piotr Maszczyk), K.S.; SK.; visualization, S.K.; K.S.; supervision, S.K.; K.S.; P.M. (Piotr Maszczyk); project administration, S.K.; K.S.; funding acquisition, P.M. (Piotr Maszczyk); J.P.; All authors have read and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Any data or material that support the findings of this study can be made available by the corresponding author upon request.

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Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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