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Evaluation Secondary Metabolite Extract Produced by *Aspergillus terreus* Isolated from Poultry Droppings as Anticancer Agent

Sanaa A. Ghali[1*], Furdos N. Jafer[2], Areej H. S. Aldhaher[3]

[1]Department of Biology, College of Science, University of Basrah, Iraq
[2,3]Department of Basic Science, College of Dentistry, Basrah University, Iraq

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***Corresponding author:** Sanaa A. Ghali
Department of Biology, College of Science, University of Basrah, Iraq

Abstract

Malignant diseases are considered one of the problems of our time, and cancer is defined as the abnormal growth of malignant cells. It is widely accepted as the leading cause of death. There is currently no proven cancer cure. As a result, scientists have concentrated on creating secure and efficient therapies. Research has been done on the effects of naturally occurring substances that have been extracted from living things, such fungus on cancer cells. This study sought to determine the natural products' efficacy against human cancer cell line MCF-7. After *A. terreus* was isolated from samples of chicken droppings, it was grown on potato and Sabouraud Dextrose Agars (SDA and PDA) with chloramphenicol media. It was then identified using the extracted genomic DNA, the internal transcribed spacer (ITS) region was amplified and sequenced. After 30 days of incubation at 27°C, natural metabolite products were also recovered from the fermentation medium using the ethyl acetate extraction technique. The effectiveness of the fungal extract against the human cancer cell line MCF-7 and the normal human cell line NHF cell was also determined after incubation for 27 hours with the natural extract. The treated human cancer cell line MCF-7 showed decrease of proliferation, whereas the normal human cell line NHF showed no effect. Significant inhibitor compared to cancer line. The IC50 values for MCF-7 cell lines and NHF normal human cell lines were 7.672 and 1431 µg/mL, respectively. In summary, MCF-7 was affected by the natural extract extracted from A. terreus, in contrast to the control. When these results were combined, they showed that the fungal extract is an effective anti-cancer treatment.

Keywords: Cancer, *A. terreus*, ITS, Natural products, Anticancer.

1. INTRODUCTION

A benign tumor results from unchecked cell proliferation, while cancer is classified as a malignant tumor. Since cancer is thought to be a common cause of death in modern times, researchers are working harder to come up with fresh solutions for this issue (Yang *et al*., 2022). One effective and secure method of treating cancer is to use natural materials to limit the size of lesions (Noman *et al*., 2021). A lot of work has gone into finding solutions for issues that arise during cancer treatment, like chemotherapy resistance. Recently, there has been a rise in expectations for the use of natural goods (Pardos *et al*., 2018) , Secondary metabolism (SM) in general, as Chemical transformations that occur inside living cells are mostly catalyzed by enzymes and are not necessary for the growth or survival of living organisms and also called natural products ,The secondary metabolism is represented by a wide range of compounds (Nielsen, 2017) ,the most common organisms that produce secondary metabolism are plants and filamentous fungi (Bérdy, 2005).

Fungi may produce dozens or perhaps hundreds of secondary metabolites in varying quantities and with different physical and chemical properties (Ettre, 2003). Secondary metabolites (SM) are naturally occurring organic compounds produced by fungi that have been shown to enhance certain biological functions (Aprilia *et al.*, 2022). An significant species of Aspergillus, Aspergillus terreus is used in the commercial manufacturing of lovastatin, a medication that lowers cholesterol, and itaconic acid, a biobased chemical. These industrial applications have proved the superior fermentation capability. The genome data showed that more research is needed to fully understand A. terreus' exceptional ability to synthesize natural products. The products of various cryptic biosynthetic gene clusters have recently been found thanks to advancements in

genome mining strategies. To further enhance the manufacturing procedures, a number of metabolic engineering investigations have been carried out on the commercial strains of itaconic acid and lovastatin (Huang *et al*., 2021).

These natural substances are thought to be a common source of pharmaceutical medications, including those that increase their effectiveness against cancer-causing tumors (anticancer), antibiotics, and medications that lower cholesterol. For instance, leptosins, a naturally occurring substance produced by Leptosphaeria sp., trigger an apoptotic pathway (Gomes *et al*., 2015).

2. MATERIALS AND METHODS

2.1. Sample Collection

At the university of Basra/Karma Ali, samples of poultry droppings were taken from backyard hens and commercial poultry. We scooped fresh droppings from chicken houses with antiseptic plastic forks. (Hostettmann.,1999), Any poultry litter that was too small to gather with a plastic spoon was removed from each sample by running a sterile swab over it until it was completely dark. Samples were collected, serially labeled, and transported in a nylon bag to the laboratory for processing within an hour.

2.2 . Handling of Samples

Ten milliliters of sterile water were used to homogenize one (1) gram of poultry excrement. Ten serial dilutions were applied to the mixes. 10-5, 10-6, 10-7, and 10-8 were plated using aseptic plating on Potato Dextrose Agar and Sabouraud Dextrose Agar (SDA) with Chloramphenicol. Five (5) days were spent incubating the culture plates (Maghraby *et al.*, 1991).

2.3. Isolation of Fungi

The pure culture was created by growing the fungal culture in fresh PDA media and then re-incubating it for another 5 days. After the colony grew, the dishes were covered with pouch and kept in the refrigerator. (Norhafizah., 2012).

2.4. Identification and Classification of Fungal Isolate

2.4.1. Microscopic Examination

A little portion of the cell and agar from each fungal isolate's culture were taken off and placed on a microscope slide. The slides were stained blue with lactophenol dye and then properly covered with a cover slip. At low power (X40), the slides were looked at under a light microscope. Microscopic features including the mycelial end, branching, hyphae structure, and presence of spore were seen and noted. The results of their physical and cultural characteristics were compared with other fungal isolates to identify the fungal isolates and identify the fungi using the known classification in the fungal atlas (Norhafizah, 2012 & Adegunloye and Adejumo,2014).

2.4.2. Characterization of molecular genetics

The Prestomini gDNA yeast kit (Geneaid) was used to isolate fungal genomic DNA. For further study, genomic DNA was stored at −80°C; the conserved ITS region was amplified using the universal primer pair technique. The quantity, purity, and titer of genomic DNA products were evaluated using NanoDrop, electrophoresis, and polymerase chain reaction (PCR) (Mirhendi *et al.*, 2006) **(Figure 3,2).**

2.5. Preparation of fungal extract

Fungal cultures represented by fermentation media were filtered separately if the fermentation media was decanted, the media were placed in centrifuge tubes at 6000 rpm for 10 minutes, filtered using Whatman No1 filter papers, and then the pH was adjusted to 3 By adding drops of hydrochloric acid HCL at a concentration of 0.1, then the active ingredients were extracted from the filtrate by adding a volume of the filtrate to an equal volume of ethyl acetate. Then the extracts were collected and placed in sealed containers for preservation in the refrigerator at a temperature of 4 °C (Kupski *et al.*, 2012).

2.5. Keeping cell cultures alive

In addition to trypsin-EDTA, which was utilized to transit the cells into single cells before re-cultivating them, the NHF cell line was maintained using media containing 10% conjugated fetal bovine conjugate and 100 µg/ml antibiotic. Raised to a temperature of 37 °C, this procedure is conducted twice a week (Adil *et al.,2020*).

2.5.1. Measurement of cytotoxicity of cell lines

Cell viability was measured using MTT stain on 96-well plates to confirm the cytotoxic effect (Al-Shammari *et al*., 2015). The cell lines were seeded at a density of 1 x 104 cells/well. The fungal extract was added to the cells once a confluent monolayer had formed, 24 h later. The medium was taken out, 28 µL of a 2 mg/mL MTT solution was added, and the cells were treated for 72 hours. After that, they were incubated for 1.5 hours at 37 °C to ascertain their viability. 130 µL of dimethyl sulphoxide (DMSO) was added to each well after the MTT solution was removed in order to dissolve any remaining crystals. The mixture was shaken for fifteen minutes at 37 °C to get this. (Adil *et al*., 2020).). At the test wavelength of 492 nm, the assay was performed in triplicate, and the absorbency was determined using a microplate reader. To calculate the percentage of cytotoxicity, or the rate at which cell development is inhibited, the following formula was utilized: (Abdullah *et al*., 2020):

- % Cell viability is calculated as follows: absorbance of treated cells / absorbance of non-treated cells * 100.
- % Cytotoxicity = 100 - Viability of Cells

Analytical statistics:

In GraphPad Prism 8, the collected data was statically evaluated using an unpaired t-test. (Al-Shammari *et al.*, 2020). The values were reported as the mean ± SD of triplicate measurements, per Mohammed *et al.* (2019). The data were compared, and the significance of the coefficients was evaluated, using the Tukey's multiple comparisons test.

3. RESULT

3.1. Fungal Isolate Identification from Poultry Droppings

The color, height, and capacity of the colony to produce reproductive spores were critical factors in the phenotypic diagnosis of the filamentous fungi isolated in this investigation. For the fungus, the differential ectophenol dye was employed. The diagnosis of various fungus relied on the conidia's form and kind Figure 1.

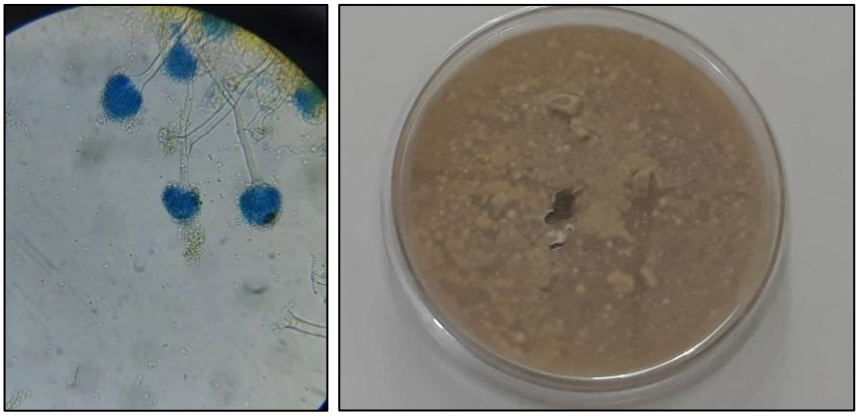

Figure (1): The phenotypic diagnosis of *A. terreus*

3.2 Molecular characterisation method

By amplifying the ITS region as a conserved region, roughly 500–600 bp in size, and then sequencing it for comparison, the *A. terreus* strain was identified by an advanced molecular characterization technique. The *A. terreus* isolate that was identified was deposited in GenBank with accession number MT558939.1, after homology was examined using BLAST on a public database. Figure 2.

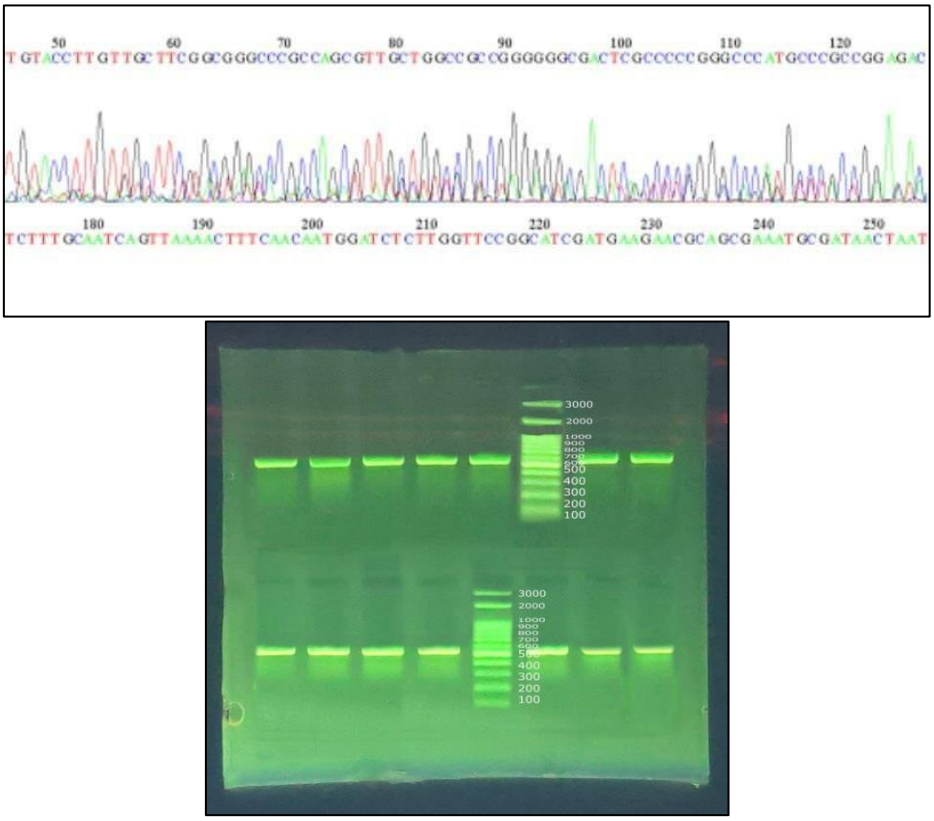

Figure (2): The molecular identification of *A. terreus* species

3.4 Production and extraction of bioactive metabolites from *A. terreus*

The secondary metabolites of the fungi produced in the fermentation medium were extracted after 30 days of incubation, and the organic acid ethyl acetate proved highly efficient in extraction, as shown in the following Figure. (3).

Figure (3): Production and extraction of secondary fresh metabolites of A. terreus.. A: Room-temperature dried organic solvent extract. B: Using ethyl acetate for bioactive metabolite extraction. C: Growth of *A. terreus* strain on PDB medium for 30 days at 27 °C.).

3.5 Cytotoxicity assay mediated by MTT stain of crude against human cell lines

We have studied the toxic effect of the fungal extract on human breast cancer cell line MCF-7 and normal cell line NHF, where it was observed that there was an inhibition in the reproduction of cancer cells, as it increased with increasing dose. If it was found that the percentage of cell toxicity at the highest concentration, which is 1000 ug/ml, reached, as 67.68% shown in Figure (4).

The half-lethal dose, lC50, was also measured 7.672. As shown in the following Figure (5). While the results showed that the rate of inhibition of the fungal extract towards the normal cell line was very low compared to the breast cancer cell line, as it reached the highest concentration1000 ug/ml , reached, as 42.67% .The lethal dose was measured as it reached 1431% The data was analyzed using the program GraphPad Prism 8 where the values of three replications were calculated as the mean ±SD, and the examination The comparison was made and the impact of the coefficients assessed using Tukey's multiple comparisons test., as shown in the following Tables (1, 2).

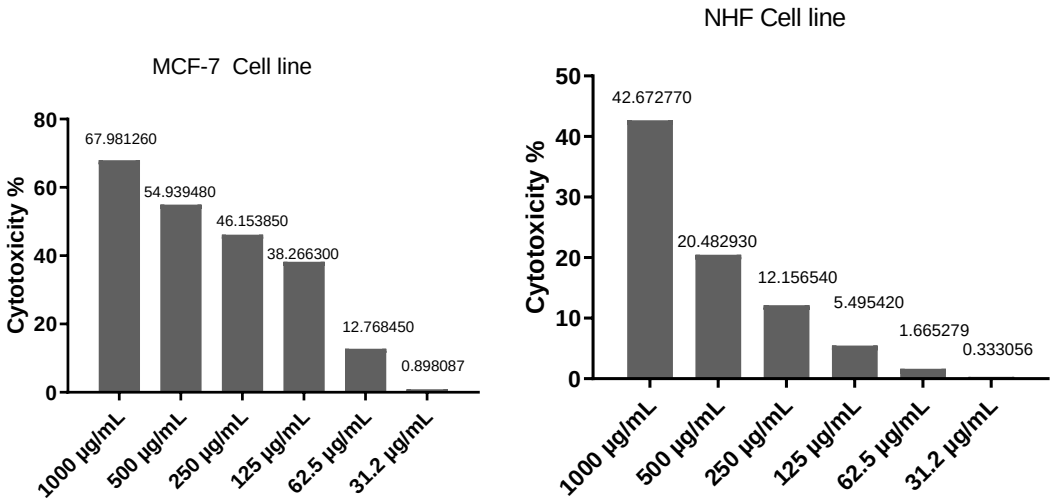

Figure (4), shows the toxic effect of the fungal extract against human cancer cell line MCF-7 and normal human cell line NHF

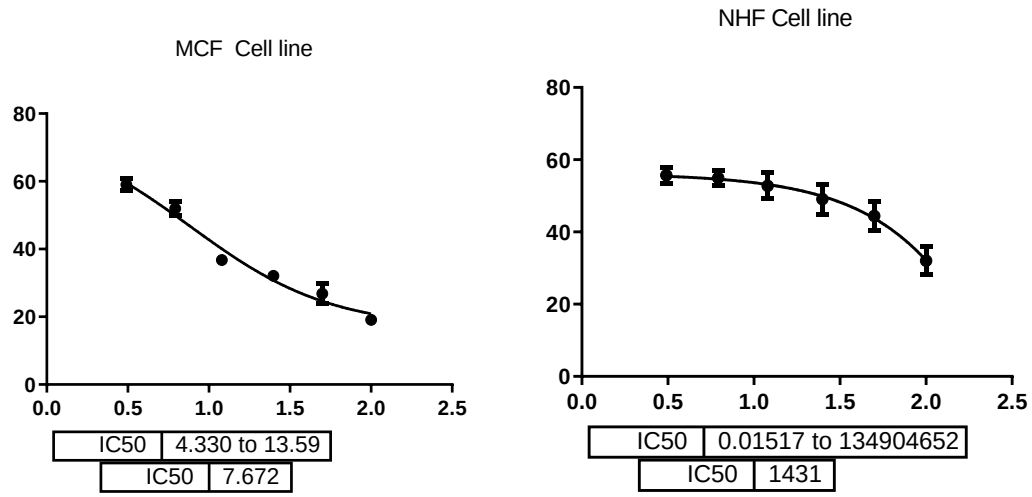

Figure (5), shows the lethal half dose of the fungal extract on a cell line NHF and the lethal half dose of the fungal extract on a cell line MCF-7

Table (1): Effect rating of the MCF-7 cell line treatments, where * stands for low effect, ** for medium effect, and* * for high effect, while NF stands for no cell killing**

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value
1000 µg/mL vs. 500 µg/mL	**-0.1113**	**-0.1833 to -0.03935**	+	**	**0.0017**
1000 µg/mL vs. 250 µg/mL	**-0.1863**	**-0.2583 to -0.1143**	+	****	**<0.0001**
1000 µg/mL vs. 125 µg/mL	**-0.2537**	**-0.3257 to -0.1817**	+	****	**<0.0001**
1000 µg/mL vs. 62 µg/mL	**-0.4713**	**-0.5433 to -0.3993**	+	****	**<0.0001**
1000 µg/mL vs. 31.2 µg/mL	**-0.5727**	**-0.6447 to -0.5007**	+	****	**<0.0001**
1000 µg/mL vs. control	**-0.5803**	**-0.6523 to -0.5083**	+	****	**<0.0001**
500 µg/mL vs. 250 µg/mL	**-0.07500**	**-0.1470 to -0.003014**	+	*	**0.0387**
500 µg/mL vs. 125 µg/mL	**-0.1423**	**-0.2143 to -0.07035**	+	***	**0.0001**
500 µg/mL vs. 62 µg/mL	**-0.3600**	**-0.4320 to -0.2880**	+	****	**<0.0001**
500 µg/mL vs. 31.2 µg/mL	**-0.4613**	**-0.5333 to -0.3893**	+	****	**<0.0001**
500 µg/mL vs. control	**-0.4690**	**-0.5410 to -0.3970**	+	****	**<0.0001**
250 µg/mL vs. 125 µg/mL	**-0.06733**	**-0.1393 to 0.004652**	_	**ns**	**0.0738**
250 µg/mL vs. 62 µg/mL	**-0.2850**	**-0.3570 to -0.2130**	+	****	**<0.0001**
250 µg/mL vs. 31.2 µg/mL	**-0.3863**	**-0.4583 to -0.3143**	+	****	**<0.0001**
250 µg/mL vs. control	**-0.3940**	**-0.4660 to -0.3220**	+	****	**<0.0001**
125 µg/mL vs. 62 µg/mL	**-0.2177**	**-0.2897 to -0.1457**	+	****	**<0.0001**
125 µg/mL vs. 31.2 µg/mL	**-0.3190**	**-0.3910 to -0.2470**	+	****	**<0.0001**
125 µg/mL vs. control	**-0.3267**	**-0.3987 to -0.2547**	+	****	**<0.0001**
62 µg/mL vs. 31.2 µg/mL	**-0.1013**	**-0.1733 to -0.02935**	+	**	**0.0040**
62 µg/mL vs. control	**-0.1090**	**-0.1810 to -0.03701**	+	**	**0.0021**
31.2 µg/mL vs. control	**-0.007667**	**-0.07965 to 0.06432**	_	**Ns**	**0.9997**

Table (2): Effect rating of the NHF cell line treatments, where * stands for low effect, ** for medium effect, and* * for high effect, while NF stands for no cell killing**

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
1000 µg/mL vs. 500 µg/mL	-0.1777	-0.3096 to -0.04575	+	**	0.0058
1000 µg/mL vs. 250 µg/mL	-0.2443	-0.3762 to -0.1124	+	***	0.0003
1000 µg/mL vs. 125 µg/mL	-0.2977	-0.4296 to -0.1658	+	****	<0.0001
1000 µg/mL vs. 62 µg/mL	-0.3283	-0.4602 to -0.1964	+	****	<0.0001
1000 µg/mL vs. 31.2 µg/mL	-0.3390	-0.4709 to -0.2071	+	****	<0.0001
1000 µg/mL vs. control	-0.3417	-0.4736 to -0.2098	+	****	<0.0001
500 µg/mL vs. 250 µg/mL	-0.06667	-0.1986 to 0.06525	_	Ns	0.6117

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
500 µg/mL vs. 125 µg/mL	-0.1200	-0.2519 to 0.01191	_	Ns	0.0859
500 µg/mL vs. 62 µg/mL	-0.1507	-0.2826 to -0.01875	+	*	0.0208
500 µg/mL vs. 31.2 µg/mL	-0.1613	-0.2932 to -0.02942	+	*	0.0126
500 µg/mL vs. control	-0.1640	-0.2959 to -0.03209	+	*	0.0111
250 µg/mL vs. 125 µg/mL	-0.05333	-0.1852 to 0.07858	_	Ns	0.8031
250 µg/mL vs. 62 µg/mL	-0.08400	-0.2159 to 0.04791	_	Ns	0.3657
250 µg/mL vs. 31.2 µg/mL	-0.09467	-0.2266 to 0.03725	_	Ns	0.2482
250 µg/mL vs. control	-0.09733	-0.2292 to 0.03458	_	Ns	0.2238
125 µg/mL vs. 62 µg/mL	-0.03067	-0.1626 to 0.1012	_	Ns	0.9818
125 µg/mL vs. 31.2 µg/mL	-0.04133	-0.1732 to 0.09058	_	Ns	0.9271
125 µg/mL vs. control	-0.04400	-0.1759 to 0.08791	_	Ns	0.9052
62 µg/mL vs. 31.2 µg/mL	-0.01067	-0.1426 to 0.1212	_	Ns	>0.9999
62 µg/mL vs. control	-0.01333	-0.1452 to 0.1186	_	Ns	0.9998
31.2 µg/mL vs. control	-0.002667	-0.1346 to 0.1292	_	Ns	>0.9999

4.5.1. Cytopathological changes

The pathological cellular changes of the cancerous line and the normal line treated with the fungal extract were studied. It was observed that changes occurred in the cells of the cancerous line, such as the presence of atrophic to dissolved cells, and the presence of clear voids or space between the cells. While no changes were observed in the normal cell line, As shown in the following Figure (6) and Figure (7).

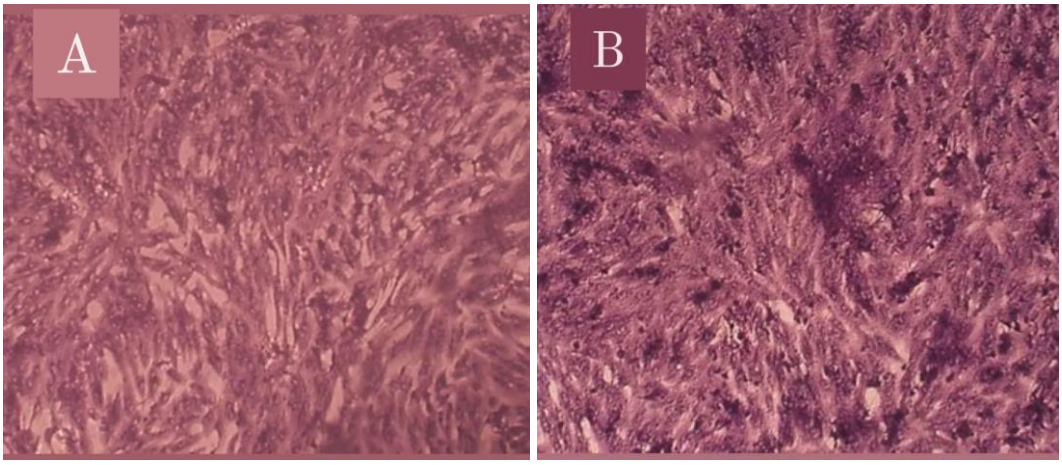

Figure (6): Shows the NHF cell line, where A represents the cells treated with the fungal extract and B represents the control

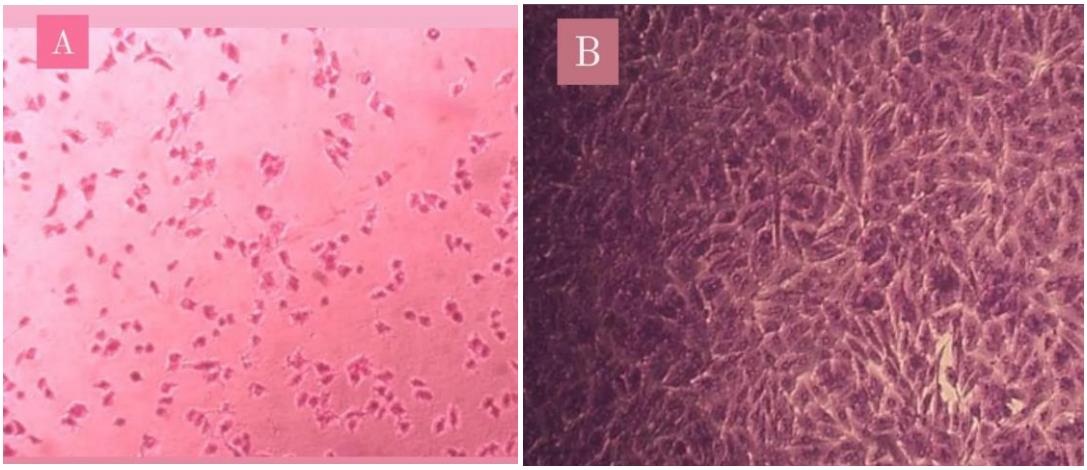

Figure (7): The cancerous MCF-7 cell line, where A represents the cells treated with the fungal extract and B represents the control

4. DISCUSSION

There is a need to search for new medicines to combat malignant diseases and their various health complications, because these incurable diseases remain a global problem due to the development and spread of drug-resistant pathogens. (Anaissie *et al.,* Citation 1996). In addition to cancerous diseases and their spread, there is a great demand for new anti-cancer drugs due to the rise in Mortality rates worldwide (Parkin *et al*. Citation1999).

The pharmaceutical and industrial sectors have long been interested in the eukaryotic organisms that make up the kingdom of fungus because they provide as important natural sources of bioactive secondary metabolites. Compared to other items made from plants. Biochemical from fungi are active agents with unexplored potential (Hung and Lin 2017). The group of filamentous fungi that belong to the phylum Ascomycota and its genera *Talarromyces*, *Fusarium*, *Aspergillus* and *Claviceps* are important fungal groups medically, environmentally and industrially, because they produce a wide variety of metabolites such as industrial enzymes, antibiotics, anticancer and other pharmaceuticals (Polli, 2017).

Many biologically active secondary metabolites have been investigated, although we can only produce them in limited concentrations. These fungi can be induced to improve their production of biologically active products in many ways, such as modifying growth conditions. (Miao & Kwong, 2006). *A. terreus* plays an important role in the synthesis of many new chemical compounds, many of which have interesting bioactivity. One such substance is bufferocin, an anti cancer agent (Deng *et al*., 2013).

The findings demonstrated the potential cytotoxicity of secondary metabolites isolated from Aspergillus terreus toward ovarian adenocarcinoma cells (SKOV3) and human prostate metastatic cell lines (PC-3). The activity of fungal metabolites against cancer cells is due to the activation of the apoptotic pathway with little to no necrotic pathway. It will offer significant data in this regard for the creation of possible medications derived from fungus. Utilizing secondary metabolites in the management of ovarian and prostate cancer in humans (Ghfar *et al.,* 2021).

This study aimed to isolate and characterize Aspergillus terreus species from chicken waste and study the activity of the natural metabolic product of the Aspergillus terreus fungus. Data indicated that the Aspergillus terreus extract suppressed the growth of the MCF-7 cancer cell line, prompting an examination of the fungus extract's action. Since secondary metabolites extracted from Aspergillus terreus were reported to be beneficial in reducing the proliferation of MCF-7 breast cancer cells in vitro, the current work seeks to assess the efficacy of Aspergillus terreus mushroom extract against a breast cancer cell line. (Vaidya *et al.* 2020).

This is what encouraged us to expand the scope of our work and search for new fungal species in other extreme environments and encourage them to reproduce in the laboratory by controlling environmental conditions to enhance their production, which enables us to discover new medicines that come from natural sources to fight cancer, especially breast cancer.

5. CONCLUSION

In this work *A. terreus* was isolated, and the biological effects of the natural product extract were examined in relation to human cell line for cancer MCF-7. The presence of antioxidants in the examined fungl extract may be related to this effect. These outcomes demonstrate that the product may be a potentially effective anticancer therapy.

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