Egypt. J. Chem. **Vol. 77**, No. 2 pp. 339 - 355 (2024)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Evaluation and Enhancement of Bioactive Compounds of *Aspergillus terreus* Endophyte Isolated from Neem by Gamma Irradiation

Eman E. Abd Eltawab[1]*, Yousseria M. Shetaia[2], Tarek Mahmoud El-Mongy[1], Amany Badr El-Deen Abd El Aziz[1]

[1] *Radiation Microbiology Dept., National Centre for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (EAEA), Cairo,13759, Egypt.*
[2] *Department of Microbiology, Faculty of Science, Ain Shams University, Cairo,11566, Egypt.*

Abstract

The quest for new bioactive compounds from natural origins is continuously growing. In this study, Nine endophytic fungi were isolated and identified morphologically from healthy parts of the *Azadirachta indica* (neem) medicinal plant, and their crude extracts were evaluated for antimicrobial effectiveness against 12 pathogenic microorganisms, including bacteria, unicellular yeasts, and multicellular fungi. The cell-free filtrates were recovered using chloroform, diethyl ether, and ethyl acetate solvents. Ethyl acetate extraction of endophytic fungus E3 exhibited the highest antimicrobial activities using the response surface approach. The isolate was molecularly identified as *Aspergillus terreus* and deposited in the gene bank under accession number ON532722. In addition, the GC-MS analysis of normal and Gamma Irradiated *A. terreus* ethyl acetate crude extracts indicated the presence of 32 main bioactive components with diverse medicinal properties that demonstrate antioxidant scavenging activities of the DPPH free radical with an IC_{50} of 85.5 ± 3.62 µg/ml and 37.5 ±1.48 µg/ml for the normal and gamma-irradiated isolate respectively. Moreover, the bioactive substances demonstrated promising anticancer efficacy against human hepatocellular carcinoma (HepG-2) and lung carcinoma (A-549**)** with IC_{50} values of 20.74± 1.08 µg/ml, 26.89 ± 1.63 µg/ml, 48.91 ± 3.85µg/ml and 30.95 ± 1.97 µg/ml towards normal and irradiated fungal extract, respectively.

Keywords: Neem, Endophytic fungi, Optimization, Antimicrobial, Antioxidant, Anticancer, GC-MS

1. Introduction

The demand for novel, secure ecological niches that could serve as sources of natural bioactive chemicals for various medicinal, agricultural, and industrial applications, which should be conveniently accessible, renewable, and eco-friendly, is constantly rising. The issues facing microbiologists include the emergence of new diseases; the development of various drug-resistant pathogenic bacteria; the arrival of life-threatening viruses, and complications in patients undergoing organ transplantation. To address the demands of the developed world, this condition has led researchers to investigate several natural sources for safe and effective agents [1, 2].

A significant section of the world's population relies heavily on medicinal plants for their wellness, especially in underdeveloped nations where herbal medicine has a long history of use [3]. More than half of the world's population still solely relies on plants for all of their medical needs according to estimates from the World Health Organization (WHO) that 80% of the population living in underdeveloped nations. Neem (*Azadirachta indica*), a multifunctional medicinal tree of the family Meliaceae, is considered a heavenly tree [4, 5]. The tree is well-known for its pharmacological qualities, which include effects that are anti-inflammatory, hepatoprotective, antipyretic, hypoglycemic,

*Corresponding author e-mail: niesseria_em@yahoo.com ; (Eman Elsayed Abd Eltawab).
Received date 22 June 2023; revised date 31 July 2023; accepted date 02 August 2023
DOI: 10.21608/EJCHEM.2023.219348.8175

insecticidal, antifertility, nematocidal, antiulcer, neuroprotective, cardioprotective, and anti-leishmaniasis. These effects can be found in the tree's leaves, bark, and stems. *A. indica* is also abundant in a variety of phytochemicals, including fatty acids, carbohydrates, flavonoids, terpenoids, steroids, alkaloids, and flavonoids [6]. All microorganisms including fungi, bacteria and actinomycetes that were colonized inside plant tissues for all or a portion of their existence without manifestly infecting those tissues are referred to as "endophytes" [7, 8, 9]. Due to their enormous potential to aid in the discovery of novel bioactive chemicals, endophytes have recently attracted a great deal of interest in the microbial chemistry community. Contrary to epiphytes or soil-associated microorganisms, it has been proposed that endophytes and their plant hosts have a close biological relationship that leads to the synthesis of a greater variety and quantity of biologically active chemicals. Neem is a significant medicinal plant that has a wide variety of endophytes. The endophytes *Periconia sp*., *Stenella sp*., and *Drechslera sp*. were discovered in the neem plant [10]. Also, *Phomopsis oblonga*, *Trichoderma sp*., *Pestalotiopsis sp*., and *Aspergillus sp*. were reported to be dominant fungal endophytes of the neem plant [11]. They represented the main source of the interesting and useful bioactive compounds associated with *Azadirachta indica* such as the azadirachtins and related tetranortriterpenoids. The age of the plant parts may serve as a proxy for the diversity of fungal endophytes [12]. On that basis, there is a great attention towards the production of novel antimicrobial compounds from endophytic fungi [13]. This work aimed to isolate fungal endophytes from the medicinal plant *Azadirachta indica* and increase the bioactivity of the endophytic fungal strain by improving the fermentation conditions using the experimental design approach [14]. Moreover, using gamma radiation to enhance the bioactive components of fungal endophyte production was approved by previous studies [15, 14, 16]. Gas chromatography–mass spectrometry (GC-MS) was also a necessity to analyze the secondary active metabolites [17]. This research aims to extract and identify fungal endophytes from the medicinal plant *Azadirachta indica* and boost the bioactivity of the most powerful endophytic fungal strain by enhancing the fermentation conditions using the experimental design technique. In addition, employing Gamma radiation to enhance the bioactive components production by fungal endophyte. Further assess the antimicrobial, antioxidant activity, and cytotoxicity of extracted bioactive components and analyse the secondary active metabolites using Gas chromatography–mass spectrometry (GC-MS).

2. Materials and Methods

2.1. Collection of plant samples

For isolation of different endophytic fungi, Healthy and mature *Azadirachta. indica* (neem) plant parts (leaves, twigs, bark, and fruits) were collected. The duration period was extended from October to December in 2016, for sampling from different governorates in Egypt (The faculty of women, Ain shams university, Cairo; Qalyub agricultural unit, El-Qalyubia, and Nuclear Research Centre, Inshas, El-Sharkeya). The plant was identified at Botany and microbiology department, faculty of science, Al-Azhar University. All samples were then excised with a sterile scalpel and placed in sterile plastic bags to avoid moisture, stored at 4 °C, and were further used to screen for endophytic fungi within 48 h of collection.

2.2. Isolation of the endophytic fungi

Isolation of endophytic fungi from various parts of neem was done according to [18]. All samples were surface sterilized with 70% ethanol for 2 min and after that plant parts were immersed in 4% sodium hypochlorite (NaOCl) solution for a period from 30 sec to 1 min. The plant samples were rinsed in sterile distilled water for 1 min about three times and then allowed to surface dry on sterile filter paper under a laminar flow hood. After proper drying; (which is ensured by placing a few drops from the water used for the last wash of the samples on the PDA plate and examined for the absence of growth) surface disinfected plant parts were cut into small pieces (about 5 x 5 mm) with a sterilized blade and were cultured on Potato Dextrose Agar (PDA) plates supplemented with chloramphenicol (50 mg/ml) to suppress bacterial growth. The plates were incubated at 28 ±2 °C and were checked daily for 5 to 7 days. Hyphal tips of the developing fungal colonies were transferred to fresh PDA plates to get pure fungal cultures. The fungal isolates in the pure cultures were stored on PDA slants at 4 °C with proper label for identification and further experimental work.

2.3. Identification of endophytic fungi

The endophytic fungi were identified based on macroscopic and microscopic characterization using common taxonomic guides [19, 20]. Identification of the most potent endophytic fungal isolate which showed a significant effect at the preliminary screening was confirmed by molecular characterization based on the sequence of Polymerase Chain Reaction ,PCR-amplified fungal internal transcribed spacer (ITS) region using the universal primers ITS1 and ITS4 according to Kjer's protocol [21] where Genomic DNA isolation, amplification, and sequencing of the 26S rRNA gene, PCR purification and DNA sequencing were performed

using the fungal specific primers, (ITS-1) 5′-TCCGTAGGTGAACCTGCGG -3′, (ITS-4) 5′-TCCTCCGCTTATTGATATGC-3′.To determine taxonomy classification, the resulting sequence was compared to the NCBI's NT database using Blast's default settings (http://blast.ncbi.nlm.nih.gov/). The phylogenetic analysis was conducted using MEGA version 11.0. Using the neighbour-joining (NJ) algorithm and the Kimura 20-parameter distance, the phylogenetic tree was created [22] using bootstrap and the inferred tree's resilience was assessed (1000 replications).

2.4. Extraction of fungal metabolites

Endophytic fungal isolates of various morphologies were inoculated into PDA for 3-5 days at 28 °C. 250 ml Erlenmeyer flasks containing 100 ml of sterilized PD broth medium were inoculated with about 2-3 discs (6 mm in diameter) of mycelium from an active growing fungal culture. The inoculated flasks were incubated in a shaking incubator at 28 °C, 150 rpm for two weeks [23]. Preliminary screening of all fungal isolates was done. Following incubation, the cheesecloth filter was used to separate the broth from the mycelia. The mycelia that had been gathered in this way were overnight dried at 50 °C to estimate their dry weight. At a separating funnel, the filtrate was extracted with ethyl acetate in a 1:1 ratio 2-3 times by vigorous mixing for about 10 min, followed by waiting for 15–30 min. The organic phase was separated and the extract containing the fungal metabolites was dried over anhydrous sodium sulfate (Na_2SO_4) and was evaporated till dryness at 45 °C under reduced pressure using a rotary vacuum evaporator for obtaining the crude extract. The crude extract was then dissolved in Dimethyl sulphoxide (DMSO) at 1 mg/ml of concentration and then stored at − 20°C for further analysis [24].

2.5. Screening of fungal endophytes for antimicrobial activity

The antimicrobial activity test was carried out by disc diffusion method, against the following pathogens: (four were human pathogenic fungi from the genus *Aspergillus; A. flavus, A. niger, A. fumigatus* and *A. parasitics* and three yeasts of the genus *Candida* (*C. albicans, C. guilliermondii,* and *C. tropicalis*). The other five microorganisms were bacteria, one is gram-positive (*Staphylococcus aureus*), and the others were gram-negative (*Pseudomonas aeruginosa, Klebsiella sp., E. coli*, and *Acinetobacter sp.*). By measuring the diameter of the inhibition zone surrounding a tested component on an agar plate, this approach was used to determine a substance's potential to impede the development of microbes. According to the Bauer-Kirby-Test, the disc diffusion assay was completed [25]. On the surface of the nutrient agar (NA) medium, 100 µl of bacterial liquid culture in the exponential development phase was dispersed for antibacterial testing. Immediate loading of 50 µl of crude extracts onto the disc paper (5 mm diameter). The impregnated discs were transferred to the surface of the NA plates that had already been seeded with the test organisms of interest. The culture was then incubated for 24-48 hours at 37 °C. For antifungal testing, PD agar medium was used to inoculate fungal species, and the plates were incubated at 28±2 °C for 5-7 days. Ciprofloxacin and Nystatin were used as positive controls for the bacterial and fungal isolates, respectively.

2.6. Experimental design for optimization of the most significant parameters affecting the bioactivity of the selected endophytic fungus.

Screening of the most significant parameters affecting the bioactivity of the isolated endophytic fungus of neem was employed using various designs of response surface methodology (RSM) which were performed using Minitab software v.18.Each experiment was run in duplicates and the final response value was calculated as the mean of duplicate.

2.6.1. Plackett–Burman (PB) design

Plackett- Burman design was used for screening the most vital factors affecting the production of the bioactive metabolites by optimization of the media components [26]. A total of 10 factors including physical and chemical factors were studied as follows, temperature, pH, medium volume, incubation period, inoculum size, inoculum age, glucose, magnesium sulfate, dihydrogen potassium phosphate, and sodium chloride. The factors were tested at low (-1), and high (+1) levels as shown in **Table, (1)**. Based on the first-order model assumption, the obtained results were analyzed to determine the coefficient value for each chosen constituent using the following equation:

$$\boxed{Y = \beta 0 + \Sigma \beta i\, xi}$$

Where Y refers to the response, β0 is the model intercept. For each studied factor, βi is the linear coefficient; xi is the level of the factor.

2.6.2. Response surface methodology

To enhance the activity of fungal endophyte metabolites, the most significant affecting variables in the PB design are employed using central composite design (CCD) to study their interacted effect. The behavior of the system and the relationship between the response and the represented factors were explained by a 2nd order polynomial equation (quadratic equation) as follows:

$$Y = \beta o + \Sigma \beta i\, xi + \Sigma \beta ii\, xi2 + \Sigma \beta ij\, xixj$$

Where Y is the predicted response, βo is the interception coefficient; βi, βii, and βij refer to the linear, quadratic, and interaction coefficients respectively [27].

Table 1. Factors with their codes were investigated at PB design.

Code	Variables	Low level	High level	Unit
A	Temperature	20	30	°C
B	pH	5	7	-
C	Medium volume	50	100	ml
D	Incubation Period	10	14	Days
E	Inoculum size	2	6	Fungal disc
F	Inoculum age	5	7	Days
G	Glucose	20	30	g/l
H	$MgSO_4$	0.3	0.5	g/l
J	KH_2PO_4	1	2	g/l
K	NaCl	0.5	0.7	g/l

2.7. Gas Chromatography-Mass Spectroscopy (GC-MS) analysis

The GC/MS assessment was performed using a fused silica capillary column with a 30 m, 0.251 mm, and 0.1 mm film thickness Thermo Scientific Trace GC Ultra/ISQ Single Quadrupole MS, TG-5MS. With an electron ionization system and ionization energy of 70 eV, the carrier gas used was helium gas for GC/MS detection at a constant flow rate of 1 ml/min. The temperature settings for the MS transfer line and injector were changed to 280 °C. The oven's temperature was set to increase from 50 °C to 150 °C at intervals of 7 °C per minute, followed by 5 °C per minute to 270 °C (hold for 2 min), and then 3.5 °C per minute to 310 °C as the final temperature (hold for 10 min). Applying a relative peak area expressed as a portion of the total peak area, all determined components were quantified. The retention times and mass spectra of the components were compared to the data in the NIST and WILLY libraries of the GC/MS system in an initial attempt to identify the constituents.

2.8. Antioxidant assay

2.8. 1. DPPH Radical Scavenging Activity

The antioxidant activity of the crude extract of the endophytic fungal isolate (E3) was carried as follows:

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was the main compound in this assay. A UV-visible spectrophotometer was used to take immediate absorbance measurements (Milton Roy, Spectronic 1201). Data were recorded at intervals of one minute to measure the decline in absorbance at15 nm until the absorbance stabilized (16 min). Ascorbic acid was used as a reference compound. The Formula used to determine the percentage inhibition (PI) of the DPPH radical was:

$$PI = [(AC - AT)/\, AC\} x\, 100]\ (1)$$

Where AC = absorbance of the control at t = 0 min and AT = absorbance of the sample + DPPH at t = 16 min.

The 50% inhibitory concentration (IC_{50}), the concentration required for 50% DPPH radical scavenging activity was estimated from graphic plots of the dose-response curve using Graph pad Prism software (San Diego, CA. USA).

2.9. Evaluation of cytotoxicity using viability assay

The anticancer activity of endophytic fungal bioactive compounds was evaluated against human Hepatocellular carcinoma (HepG-2) and lung carcinoma (A-549**)** (VACSERA Tissue Culture Unit, Cairo, Egypt) by the MTT assay [28, 29]. In a 96-well plate, the cells of a concentration (10^4 cells /well) were added; incubated overnight, fresh media having different tested fungal extract concentrations was added. 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) were filled with confluent cell monolayers using a multichannel pipette. A two-fold dilution of the examined chemical component was applied in series. The microtitre plates were incubated for 24 hours at 37°C in a humidified incubator with 5% CO_2. Following the incubation

step, a crystal violet solution with a concentration of 1% was put there for at least half an hour. After the discoloration was removed, the plates were washed to remove any remaining residue. After that, glacial acetic acid with a concentration of 30% was added to each well, and everything was thoroughly mixed before the absorbance of the plates was measured on a microplate reader after they had been gently shaken. The test wavelength used was 490 nm (TECAN, Inc.). Using a microplate reader, an optical density reading was taken to carry out the task of determining the total number of viable cells (Sunrise, TECAN, Inc., USA). The percentage of viability was indicated by the following equation:

$$\boxed{[(ODt/\,ODc)]\,x100\%}$$

ODt (the mean optical density of wells treated with the tested sample), ODc (the mean optical density of untreated cells. The IC_{50} was determined using the GraphPad Prism software (San Diego, CA. USA). The standard which was used as a reference was doxorubicin.

2.10. Gamma irradiation treatment

The selected fungal isolate was exposed to gamma-irradiation to study its influence on antimicrobial proficiency. The fungal endophyte was irradiated with ^{60}Cobalt sources (Gamma cell 4000-A-India) at various dosages for gamma therapy (0.25-4.0 kGy). Under usual culture conditions, the irradiated spores were introduced into the optimum medium. Cultures that had not been exposed to radiation served as controls. After GC-MS analysis the irradiated crude extract of the chosen dose was evaluated for its antimicrobial, antioxidant and cytotoxicity activities after irradiation.

2.11. Statistical analysis

The results were expressed as the mean in three triplicates ± the standard deviation (SD). Statistical significance was studied by the one-way ANOVA test using SPSS software (v. 22).

3. Results

3.1. Isolation of the endophytic fungi

In this study, the sterilized leaves, fruits, and stems of neem plants grown on PDA media showed signs of fungal development. Pure fungal cultures that had grown around the samples were subcultured on brand-new PDA material. Nine different fungal genus isolates were chosen based on morphological and phenotypic traits.

3.2. Identification of the selected endophytic fungi.

An endophytic fungal isolate was molecularly characterized using ITS sequencing. An internal transcribed spacer polymerase chain reaction was used to amplify genomic DNA obtained from an endophytic fungal culture (ITS PCR). The primer enhanced the entire ITS region of interest. After electrophoresis of the PCR result, a single band at 600 bp was found. The ITS sequence was 99.49% similarity to *A. terreus* with accession number (MN429223.1) in a non-redundant blast search on the National Centre for Biotechnology Information, NCBI database, with zero E values and 96% query coverage. The target isolate has been identified as *A. terreus* (E3) and has been deposited in the gene bank under the accession number ON532722.1 (**Figure 1).**

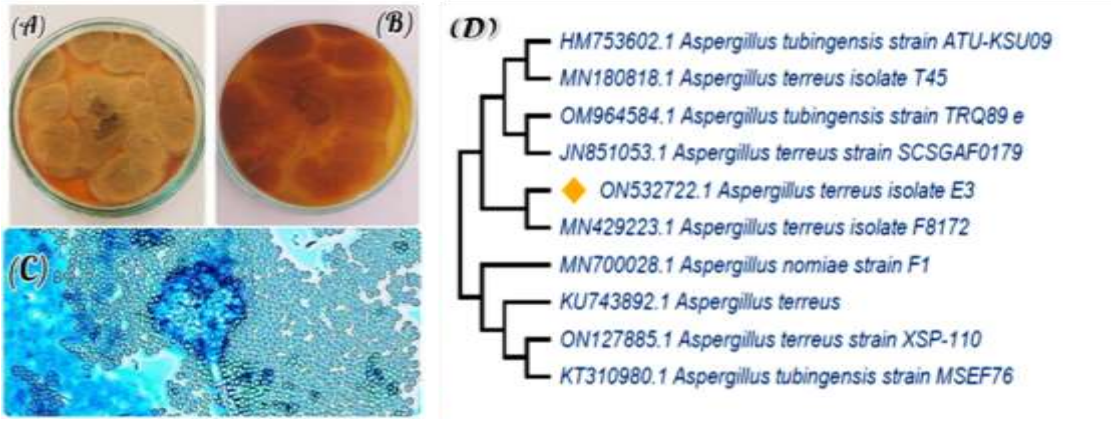

Fig. 1: Identification of *Aspergillus terreus* E3 isolate based on (A, B) cultural properties on PDA medium (c) morphological shape of conidia under light microscopic photos at magnification power (600X). (D) The phylogenetic tree elucidates the position of *Aspergillus terreus* E3 and the closely related strains in the gene bank, using MEGA 11.0 software (The selected isolate of rhombus symbol).

3.3.**Preliminary screening of endophytic fungi from Azadirachta indica (neem) for antimicrobial activity**

Table 2. Preliminary screening of endophytic fungi antimicrobial effect

Pathogenic microorganisms	*Alternaria sp*	*Cladosporium sp*	*Penicillium sp*	*Aspergillus sp.1*	*Aspergillus sp.2*	*Aspergillus sp.3*	*Mucor sp.*	*Fusarium sp.*	*Trichoderma sp*
Gram+ve Bacteria									
S. aureus	-	+	-	-	++	++	-	-	-
Gram-ve Bacteria									
E. coli	-	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	+	+	++	++	-	-	-
Klebsiella sp.	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	-	-	-	-	-	-	-	-	-
Fungi									
C.albics	-	+	+	+	++	+	-	-	-
C. guilliermondii	-	-	-	-	+	+	-	-	-
C.tropicalis	-	++	+	+	+	+++	-	-	-
A. flavus	-	+	-	-	-	-	-	-	-
A. fumigatus	-	-	+	+	++	-	-	-	-
A. niger	-	+	+	+	-	+	+	-	-
A. parasiticus	-	-	-	-	-	+	-	-	-

(-): no inhibition zone, (+): inhibition zone less than 5.00 mm,): inhibition zone is from 5.00 to 10.00 mm, (+++): inhibition zone is above 10.00 mm.

3.4. Screening of the isolated endophytic fungi for antimicrobial activity using ethyl acetate extract

After preliminary screening of the different 7 isolated fungal endophytes, five isolated ones were screened by different 3 solvents, and ethyl acetate exhibited more effect than the other two solvents (chloroform and diethyl ether), **Table (3).**

Table 3. Antimicrobial activity of ethyl acetate extract of fungal endophytes

Tested pathogens	Diameter of inhibition zone (mm)				
	Endophytic fungal isolates				
	E1	**E2**	**E3**	**E4**	**E5**
C.albicans	10.0 ± 0.5^{c}	11.0 ± 0.2^{a}	10.6 ± 0.34^{b}	9.0 ± 0.12^{d}	7.2 ± 0.15^{e}
C. guilliermondii	7.0 ± 0.8^{c}	11.5 ± 0.2^{a}	11.5 ± 0.15^{a}	8.0 ± 0.6^{b}	0.0 ± 0.0^{d}
C. tropicalis	11.3 ± 0.37^{c}	12.3 ± 0.55^{b}	18.0 ± 0.6^{a}	11.0 ± 0.2^{c}	8.0 ± 0.26^{d}
A .flavus	6.7 ± 0.2^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
A. niger	9.3 ± 0.47^{b}	11.6 ± 0.79^{a}	11.8 ± 0.26^{a}	7.6 ± 0.30^{c}	6.1 ± 0.1^{d}
A. fumigatus	0.0 ± 0.0^{c}	13.1 ± 0.70^{a}	13.3 ± 0.2^{a}	9.7 ± 0.21^{b}	0.0 ± 0.0^{c}
A. parasiticus	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	8.3 ± 0.40^{a}
S. aureus	10.3 ± 0.41^{c}	13.0 ± 0.40^{b}	14.3 ± 0.32^{a}	10.6 ± 0.37^{c}	9.0 ± 0.30^{d}
P. aeruginosa	8.0 ± 0.7^{c}	12.2 ± 0.20^{a}	12.5 ± 0.50^{a}	9.6 ± 0.35^{b}	0.0 ± 0.0^{d}
Klebsiella sp.	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E. coli	6.3 ± 0.21^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
Acinetobacter sp.	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

The values of average zones of inhibitions are expressed as mean ± SD ($n = 3$) within each column, a, b-means with small letters followed by different letters are significantly different at $p < 0.05$ level

3.5. Experimental design for optimization of the selected endophytic fungus Plackett–Burman (PB) design

The antimicrobial activity (mm) of the fungal isolate was considered the response as represented in the PB matrix, **table (4)**. The model trials were analyzed, **table (5)**. Fifteen runs were examined and between them, only four factors are shown to be significant which are temperature, medium volume, incubation period and inoculum size. These factors affected the production of the bioactive metabolites **Fig. 2**.

Table 4. Plackett-Burman design matrix for evaluating significant factors affecting the bioactivity by *Aspergillus terreus* ON532722 endophyte where IZ= inhibition zone in mm.

Trial no.	A	B	C	D	E	F	G	H	J	K	IZ
1	1	-1	1	-1	-1	-1	1	1	1	-1	15.0
2	1	1	-1	1	-1	-1	-1	1	1	1	13.0
3	-1	1	1	-1	1	-1	-1	-1	1	1	17.0
4	1	-1	1	1	-1	1	-1	-1	-1	1	15.0
5	1	1	-1	1	1	-1	1	-1	-1	-1	13.0
6	1	1	1	-1	1	1	-1	1	-1	-1	18.0
7	-1	1	1	1	-1	1	1	-1	1	-1	12.5
8	-1	-1	1	1	1	-1	1	1	-1	1	13.0
9	-1	-1	-1	1	1	1	-1	1	1	-1	10.0
10	1	-1	-1	-1	1	1	1	-1	1	1	15.0
11	-1	1	-1	-1	-1	1	1	1	-1	1	10.0
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	11.0
13	0	0	0	0	0	0	0	0	0	0	15.5
14	0	0	0	0	0	0	0	0	0	0	15.0
15	0	0	0	0	0	0	0	0	0	0	16.0

-1(low level), +1(high level), runs were duplicated and the mean of IZ was calculated

Table 5. Statistical analysis for the main effect of each variable on the bioactivity by *Aspergillus terreus* ON532722 in the PB design

Term	Effect	Coef	SE Coef	T-Value	P-Value	VIF
Constant		13.542	0.168	80.42	0.000	
Temperature	2.583	1.292	0.168	7.67	0.005	1.00
PH	0.750	0.375	0.168	2.23	0.112	1.00
Medium volume	3.083	1.542	0.168	9.16	0.003	1.00
Incubation period	-1.583	-0.792	0.168	-4.70	0.018	1.00
Inoculum size	1.583	0.792	0.168	4.70	0.018	1.00
Inoculum age	-0.250	-0.125	0.168	-0.74	0.512	1.00
Glucose	-0.917	-0.458	0.168	-2.72	0.072	1.00
$MgSO_4$	-0.750	-0.375	0.168	-2.23	0.112	1.00
KH_2PO_4	0.417	0.208	0.168	1.24	0.304	1.00
NaCl	0.583	0.292	0.168	1.73	0.182	1.00

The analysis of variance (ANOVA) at 95% confidence intervals, p-value <0.05 represent significance, R2=0.9875, R2 adjusted= 0.9415, R2 (coefficient of determination) ,(-) T- value indicates negative effect, (+) T-value indicates positive effect , SE Coef (standard error coefficient), P-Value (corresponding level of significance.

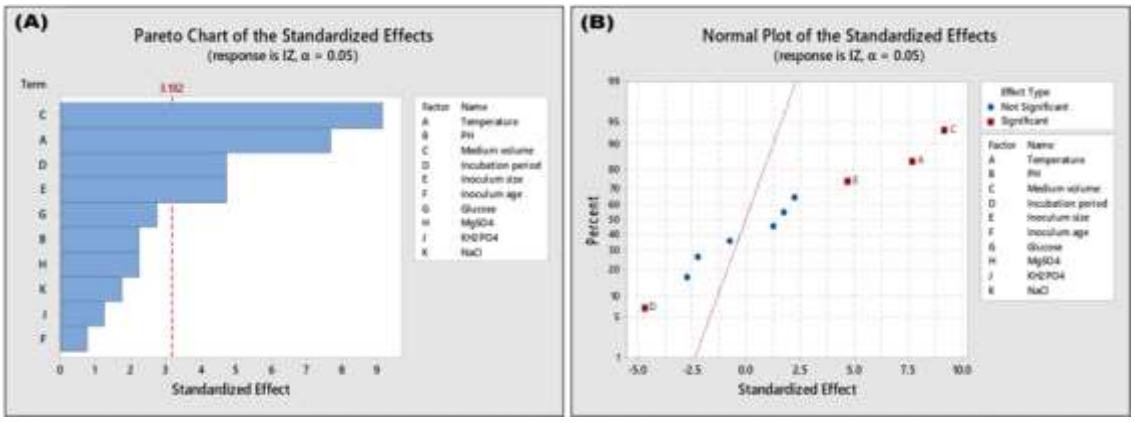

Fig. 2: Graphical design for inhibition zone results from the PB Model, (A) is the Pareto chart displaying the significance of effect for each tested variable on bioactivity from *A. terreus* ON532722., (B) is the effect plot displaying the positively and negatively affecting variables

The PB model gives a linear polynomial equation describing the relation between the 10 factors and the response (IZ) as follows:

IZ= 5.54 + 0.2583 Temperature + 0.375 PH + 0.06167 Medium volume - 0.3958 Incubation period + 0.3958 Inoculum size - 0.125 Inoculum age - 0.0917 Glucose - 3.75 MgSO4 + 0.417 KH2PO4 + 2.92 NaCl

Central composite design (CCD)

The central composite design was used to show the significant variables based on the PB design. These variables are (temperature, medium volume; incubation period and inoculum size) which were further optimized using CCD. **Table (6)** shows the significant effect of CCD by studying the interaction between the selected factors. Surface plots illustrated in **Fig. 3** are for visual observation of the interactive effects between each two factors.

Table 6: Statistical analysis and regression statistics in the CCD

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	15.143	0.435	34.83	0.000	
Temperature	1.135	0.246	4.61	0.000	1.05
Medium volume	1.302	0.246	5.29	0.000	1.05
Incubation period	-0.531	0.246	-2.16	0.048	1.05
Inoculum size	2.844	0.246	11.55	0.000	1.05
Temperature*Temperature	-1.559	0.216	-7.22	0.000	1.03
Medium volume*Medium volume	1.128	0.216	5.23	0.000	1.03
Incubation period*Incubation period	-0.247	0.216	-1.14	0.271	1.03
Inoculum size*Inoculum size	-2.184	0.216	-10.12	0.000	1.03
Temperature*Medium volume	0.547	0.308	1.77	0.097	1.08
Temperature*Incubation period	-0.078	0.308	-0.25	0.803	1.08
Temperature*Inoculum size	0.547	0.308	1.77	0.097	1.08
Medium volume*Incubation period	0.359	0.308	1.17	0.262	1.08
Medium volume*Inoculum size	0.984	0.308	3.19	0.006	1.08
Incubation period*Inoculum size	-1.141	0.308	-3.70	0.002	1.08

The analysis of variance (ANOVA) was applied at 95% confidence intervals. Variables and models would be statistically considerable at levels of significance, P value < 0.05. (−) T value indicates a negative effect, and (+) T value indicates positive effect.

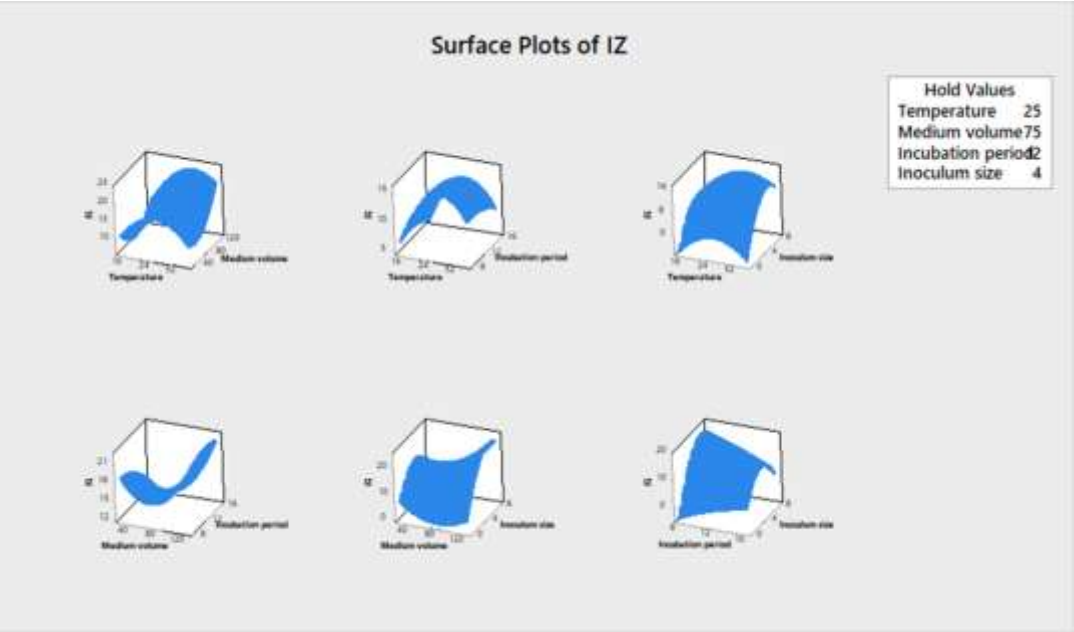

Fig.3: Three-dimensional response surface curve shows the interaction effect and the optimum levels of the four selected factors, IZ (inhibition zone in mm).

3.6. Gas Chromatography-Mass Spectroscopy (GC-MS) analysis

The ethyl acetate extract of the endophyte fungus (*A. terreus* ON532722) was subsequently analyzed using GC-MS to identify the bioactive compounds which showed that it contains a wide variety of biological compounds. The active substances' retention times (Rt), molecular formula (MF), molecular weights (MW), and concentration percentages (areas %) are displayed in (**Table 7**). The extract was then subjected to gamma radiation and analyzed after irradiation to study the effect after being irradiated (**Table 8**). The major compounds of the fungal extract were Orcinol, Z-10-Pentadecen-1-ol, E-15-Heptadecenal, n-Tetracosanol-1, (cis)-2-nonadecene and Cyclotetracosane. After irradiation the major compounds were 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R- [R*, R*-(E)]] , á-LAVANDULOL, 3-Methoxy-5-methyl phenol, 1-Methylcyclohex-1-en-4- carboxylic acid and Terrien. The Percentage of Total area of identified compounds is 96.67% and for unidentified compounds is 3.33% and is 93.37% for identified compounds after irradiation and 6.63% unidentified compounds.

Table 7: GC-MS analysis of the ethyl acetate extract of *A. terreus* ON532722.

S. No.	Rt (min.)	Compound name	Area (%)	MF	MW
1	**9.04**	2,5-Cyclohexadiene-1,4-dione, 2- methyl	**0.75**	$C_7H_6O_2$	**122**
2	**14.40**	2(3H)-Furanone, 5-butyldihydro (CAS)	**0.59**	$C_8H_{14}O_2$	**142**
3	**14.92**	5-Heptyl-3-deuteriotetrahy Drofuran-2-one	**0.94**	$C_{11}H_{19}DO_2$	**184**

4	**15.04**	Oxirane, (methoxymethyl)	**1.41**	**$C_4H_8O_2$**	**88**
5	**15.42**	2-Ethylnon-1-en-3-ol	**0.57**	**$C_{11}H_{22}O$**	**170**
6	**15.55**	4-(1-hydroxy-ethyl) ç butanolactone	**0.79**	**$C_6H_{10}O_3$**	**130**
7	**15.73**	4-Chloro-3-n-hexyltetrahydropyran	**0.50**	**$C_{11}H_{21}C_{lO}$**	**204**
8	**16.17**	Pentanoic acid,10-undecenyl ester	**0.43**	**$C_{16}H_{30}O_2$**	**254**
9	**18.85**	Orcinol	**50.10**	**$C_7H_8O_2$**	**124**
10	**19.24**	3-Tetradecene, (Z)	**0.86**	**$C_{14}H_{28}$**	**196**
11	**19.42**	Tetradecane	**0.50**	**$C_{14}H_{30}$**	**198**
12	**20.54**	1-Hydroxycyclododecane carbonitrile	**0.63**	**$C_{13}H_{23}NO$**	**209**
13	**20.98**	1-Hydroxycyclododecane carbonitrile	**1.05**	**$C_{13}H_{23}N_O$**	**209**
14	**21.32**	1(2H)-Naphthalenone, Octahydr-4-hydroxy-, trans-	**0.65**	**$C_{10}H_{16}O_2$**	**168**
15	**22.46**	Phenol,2,4-bis (1,1-dimethyl ethyl)	**0.76**	**$C_{14}H_{22}O$**	**206**
16	**22.71**	Dimethyl{bis[(2Z)-pent-2-en-1-yloxy]} silane	**0.68**	**$C_{12}H_{24}O_2Si$**	**228**
17	**24.01**	Z-10-Pentadecen-1-ol	**8.42**	**$C_{15}H_{30}O$**	**226**
18	**24.17**	Hexadecane	**1.18**	**$C_{16}H_{34}$**	**226**
19	**25.12**	2,7-Nonadienoic acid, 3,8-dimethyl-, methyl ester, (Z)	**0.73**	**$C_{12}H_{20}O_2$**	**169**
20	**26.73**	8,11,14-Eicosatrienoic acid, (Z, Z, Z)	**0.40**	**$C_{20}H_{34}O_2$**	**306**
21	**28.32**	E-15-Heptadecenal	**4.84**	**$C_{17}H_{32}O$**	**252**
22	**28.44**	Octadecane	**1.21**	**$C_{18}H_{38}$**	**254**
23	**28.61**	Pluchidiol	**0.49**	**$C_{13}H_{20}O_2$**	**208**
24	**31.03**	7,9-di-tert-butyl-1-oxaspiro [4.5] deca-6,9-diene-2,8 dione	**0.42**	**$C_{17}H_{24}O_3$**	**276**
25	**31.68**	1H-2-Benzopyran-1-one, 3,4 dihydro-3,8-dihydroxy-3-methyl-, (-)-	**1.76**	**$C_{10}H_{10}O_4$**	**194**
26	**32.22**	(cis)-2-nonadecene	**4.06**	**$C_{19}H_{38}$**	**266**
27	**32.33**	Eicosane	**0.85**	**$C_{20}H_{42}$**	**282**
28	**33.84**	Methyl 4,7,10,13-hexadecatetraen oate	**1.81**	**$C_{17}H_{26}O_2$**	**262**
29	**35.80**	n-Tetracosanol-1	**4.91**	**$C_{24}H_{50}O$**	**354**
30	**35.88**	Pentacosane	**0.95**	**$C_{25}H_{52}$**	**352**
31	**39.08**	Cyclotetracosane	**2.86**	**$C_{24}H_{48}$**	**336**
32	**42.12**	1-Hexacosanol	**0.57**	**$C_{26}H_{54}O$**	**382**

Table 8: GC-MS analysis of the irradiated ethyl acetate extract of *A. terreus* ON532722

S.No.	RT	Compound name	Area (%)	MF	MW
1	**5.17**	1,2-Propadiene	**1.78**	C_3H_4	**40**
2	**5.61**	Cyclopropene	**0.22**	C_3H_4	**40**
3	**8.56**	4-Cyano-1-cycloheptano ne	**0.21**	$C_8H_{11}NO$	**137**
4	**8.94**	Trimethyl cyclohexanone	**0.43**	$C_9H_{16}O$	**140**
5	**11.00**	Heptanonitrile	**0.32**	$C_7H_{13}N$	**111**
6	**11.13**	Tetradecane	**0.22**	$C_{14}H_{30}$	**198**
7	**13.27**	1-(1-Propynyl) cyclohexanol	**0.37**	$C_9H_{14}O$	**138**
8	**15.27**	2-(N-Methylanilino) Penta-2,4-di-enenitrile	**0.78**	$C_{12}H_{12}N_2$	**184**
9	**15.73**	á-LAVANDULOL	**8.80**	$C_{10}H_{18}O$	**154**
10	**16.19**	n-Octylidencyclohexane	**2.29**	$C_{14}H_{26}$	**194**
11	**17.84**	3-Methoxy-5-methyl phenol	**4.87**	$C_8H_{10}O_2$	**138**
12	**19.10**	1-Tetradecene	**0.37**	$C_{14}H_{28}$	**196**
13	**21.61**	Terrein	**0.22**	$C_8H_{10}O_3$	**154**
14	**21.84**	2,7,12,17-Tetraethyl-3,5:8,10:13,15:18,20-tetrakis (2,2 diethylpropion) porphyrin	**0.93**	$C_{48}H_{62}N_4$	**694**
15	**21.89**	(S)-(-)-7-Methylhexyhydropyrrolizin-3-one	**0.35**	$C_8H_{13}NO$	**139**
16	**22.00**	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethy l)	**1.60**	$C_{10}H_{16}O$	**152**
17	**22.26**	1-Methylcyclohex-1-en-4- carboxylic acid	**4.04**	$C_8H_{12}O_2$	**140**
18	**22.34**	Terrein	**3.44**	$C_8H_{10}O_3$	**154**
19	**23.87**	5-Methyl-1-heptanol	**0.45**	$C_8H_{18}O$	**130**
20	**25.09**	2-(2-fluorophenyl) bicyclo [2.2.1] heptane-2-carboxamide	**0.39**	$C_{14}H_{16}FNO$	**233**
21	**26.57**	Cyclandelate	**0.56**	$C_{17}H_{24}O_3$	**276**
22	**28.17**	4,4,7,7-Tetramethyldeca1,9-diene	**0.35**	$C_{14}H_{26}$	**194**
23	**31.77**	2-Methyl-5-hexen-3-ol	**0.26**	$C_7H_{14}O$	**114**
24	**32.08**	Silane, trichloroeicosyl-	**0.34**	$C_{20}H_{41}Cl_3Si$	**414**
25	**34.44**	3,8-dibromo-1-cyclooctene	**0.20**	$C_8H_{12}Br_2$	**266**
26	**41.60**	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R- [R*, R*-(E)]]	**60.12**	$C_{20}H_{40}O$	**296**

3.7. Antioxidant activity of the fungal extract

Since it is a free radical that is stable, DPPH has been extensively employed to assess the antioxidant capacities of various biological samples. This approach relies on the synthesis of DPPH-H, a non-radical form of DPPH, which causes DPPH to be reduced in the presence of a radical scavenger or hydrogen donors. The endophytic fungal culture *Aspergillus terreus* capacity to scavenge DPPH free radicals served as a measure of its antioxidant activity. The decolorizing result after DPPH's unpaired electrons are trapped is a good indicator of the compounds' capacity to scavenge free radicals. **Figure 4** showed the IC_{50} value of fungal extract, which was used to indicate antioxidant activity. Ethyl acetate fungal extract showed significant DPPH inhibition activity. IC_{50} of the extract was recorded as85.5 ± 3.62 µg/ ml in comparison to positive control ascorbic acid as 10.6 ± 0.8 µg/ ml,**Figure(5).**

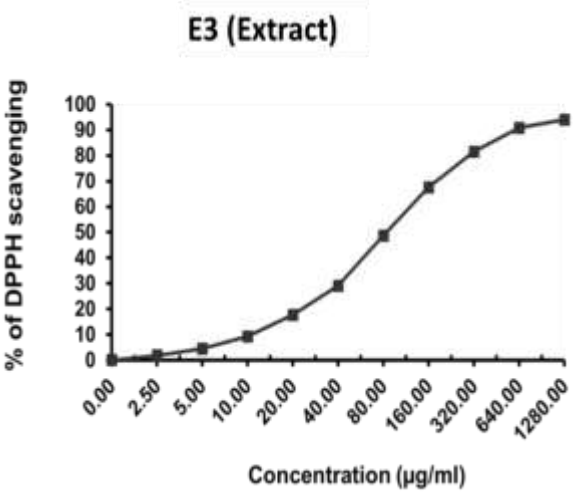

Fig.4: Antioxidant activity from ethyl acetate extract of endophytic fungi

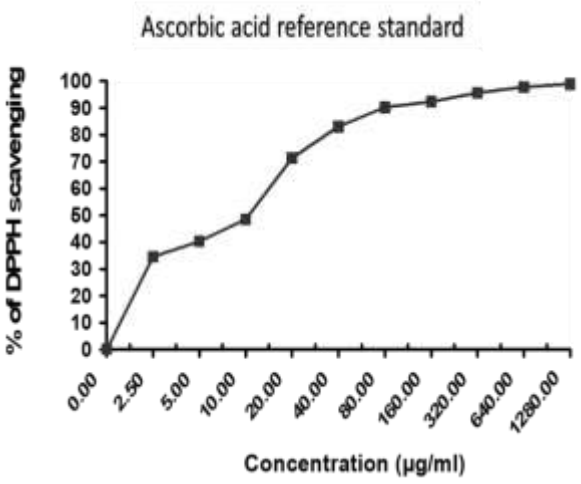

Fig.5: Ascorbic acid reference standard for the activity antioxidant

After exposure of spores of endophytic fungal extract to gamma radiation of 1.5 KGy dose, it was observed that an increase in the antioxidant activity occurred of IC_{50} 37.5 ±1.48 µg/ml, **Figure 6.**

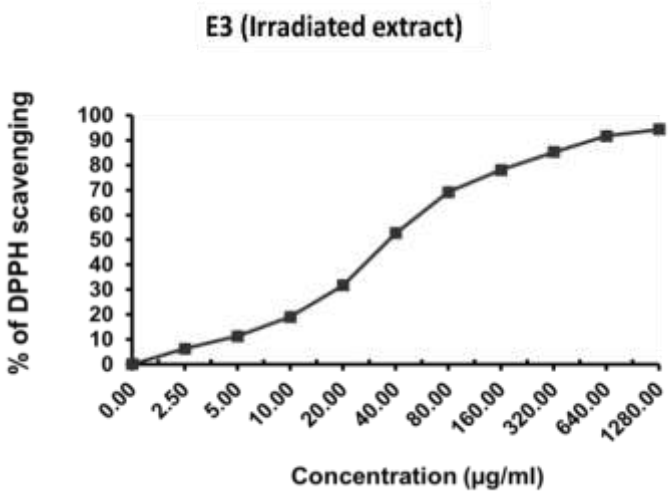

Fig.6: Antioxidant activity from irradiated ethyl acetate extract of endophytic fungi E3.

3.8. Cytotoxicity evaluation using viability assay.

The cytotoxicity activities of the endophytic fungus against two cancer cell lines; Hepatocellular carcinoma cells and Lung carcinoma cells (HepG-2 and A-549, respectively) were investigated before and after exposure to gamma radiation. From the viability test, the ethyl acetate extract of *A. terreus* had IC_{50} values of 20.74± 1.08 µg /ml and 26.89 ± 1.63 µg/ ml towards HepG-2 and A-549, respectively **(Figure 7).** The IC_{50} values of the extract became 48.91 ± 3.85µg/ml and 30.95 ± 1.97 µg/ ml for HepG-2 and A-549 after irradiation, respectively **(Figure 8).** Doxorubicin was used as standard reference of IC_{50} = 0.36 ± 0.02 µg/ml.

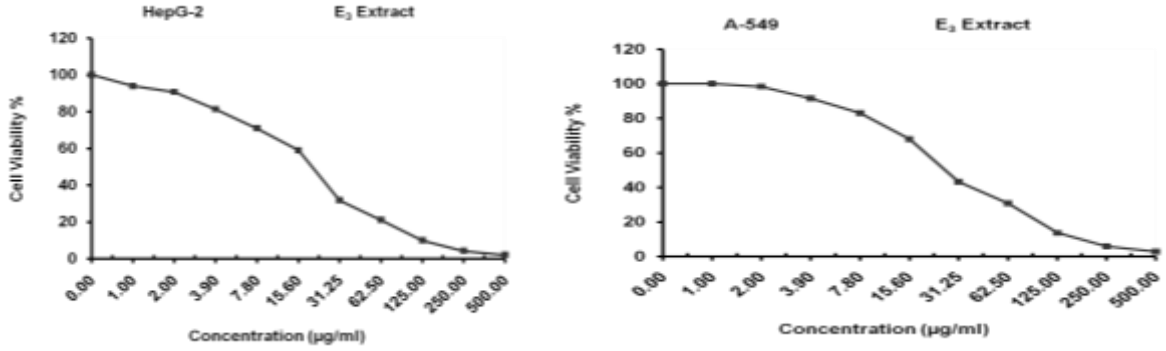

Fig.7: Cytotoxic activity of the ethyl acetate extract of *A. terreus* towards HepG-2 and A-549 cancer cell lines

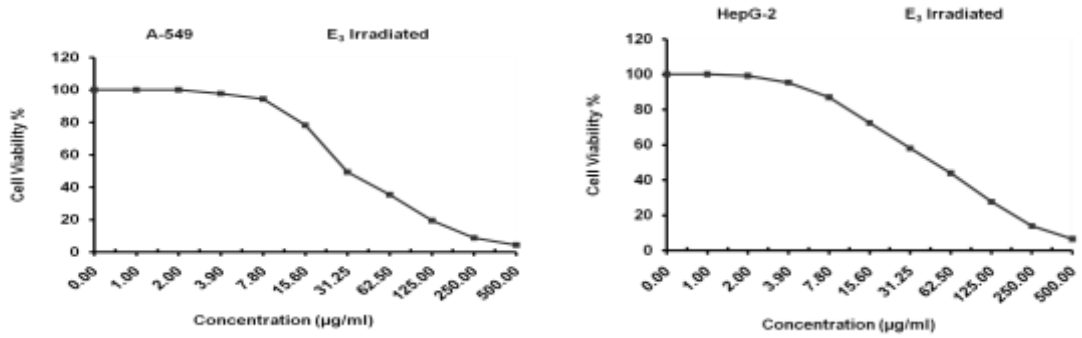

Fig. 8: Cytotoxic activity of the irradiated ethyl acetate extract of *A. terreus* towards HepG-2 and A-549 cancer cell lines

4. Discussion

Due to their tremendous potential to aid in the discovery of novel bioactive chemicals, endophytes have recently attracted a lot of attention in the field of microbial chemistry. Contrary to epiphytes or soil-associated microorganisms, it has been proposed that endophytes and their plant hosts produce a greater variety and number of biologically active chemicals because of their tight biological relationship [11]. Furthermore, because of this relationship's symbiotic nature, endophytic bioactive substances are likely to be less hazardous to cells because they do not harm the eukaryotic host system. This is crucial for the medical community since possible medications might not harm human cells [30]. New bioactive compounds as well as new endophytic fungi from *Azadirachta indica* have been described. Neem is a plant that is commonly employed for the exploration of endophytes and their secondary metabolites. To isolate endophytic fungi from its many components, the neem medicinal plant was used (park, leaves, and stem). Nine endophytic isolates which displayed antibiotic activity against the pathogenic test organisms were found in this study [31]. The screening of these endophytes were showed that the fungal isolate E3 had the most antimicrobial efficacy in terms of inhibition zone and it was identified morphologically and on the molecular level as *Aspergillus terreus* with 99.49% conformation to *A. terreus* accession number (MN429223.1) in the phylogenetic tree. *A. terreus* endophyte had been reported to produce a large number of bioactive metabolites like terrien which was found to have antioxidant, antimicrobial, and anticancer activity [32]. The isolated fungus was also deposited in the gene bank with accession number #ON532722. Optimization of different components of the medium (modified potato dextrose broth medium) was done using response surface methodology (RSM). Due to its improved efficiency, suitable design for integrated analysis of variants, and mathematical modeling with recommended values of the majority probable optimum conditions, together with matching product yield, RSM-based optimization is generally recognized. Compared to univariate solutions, the strategy is more accurate, more economically feasible, and more industrially acceptable [33]. Additionally, RSM was employed in various studies to enhance and increase the bioactive metabolites of isolated endophytic fungi [34].The

Placket- Burman model when was applied achieved an increase in the antimicrobial activity of the A.terreus ethyl acetate extract (17 mm) finds an agreement with the data of [35].

Butyrolactone V, a molecule derived from the endophytic fungus *A. terreus*, was shown to have high cytotoxicity against the breast cancer cell line MDA-MB-231 with an IC_{50} value of 22.2 M, significant antioxidant activity, and anti-schistosomal action [36]. The volatile nature of the bioactive compounds present in the ethyl acetate extract of neem endophytic fungi has been examined by GC–MS analysis. Thirty two compounds were found through GC-MS spectra and some of these compounds were found to be active and with great benefit ; Orcinol (Antifungal and antioxidant, [37]) , Z-10-Pentadecen-1-ol, n-Tetracosanol-1, Cyclotetracosane, Methyl 4,7,10,13-hexadecatetraen oate, 1H-2-Benzopyran-1-one, 3,4 dihydro-3,8-dihydroxy-3-methyl-, (-)-,Octadecane, Hexadecane, 5-Heptyl-3-deuteriotetrahy Drofuran-2-one, 1-Hydroxycyclododecane carbonitrile and 3-Tetradecene,(Z) were the major compounds. After irradiation, important new active compounds were found; 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (antimicrobial, anticancer, anti-inflammatory, anti-diabetic and immunostimulatory,[38]) , á-LAVANDULOL, 3-Methoxy-5-methylphenol,Terrein,1-Methylcyclohex-1-en-4- carboxylic acid, n-Octylidencyclohexane, 1,4-Bis{spiro[2,2,dicyano -4-phenylcyclobutane-1,1 '-bis [1,2,5] thiadiazolodicy anocyclohexanemethane} benzene, 1,2-Propadiene and 2-Cyclohexen-1-one, 3-methyl-6-(1-methylethy l). The predominant compounds which have a significant role as antimicrobial, cytotoxicity and antioxidant agents [39].

Irradiation with 1.5 KGy dose was found to be more efficient in improving the antimicrobial activity when various gamma irradiation doses (0.25–4KGy) were first examined. Antioxidant activity after irradiation was found to be (IC50 value) 85.5 ± 3.62 µg/ ml; this could be attributed to the hydroxyl groups found in lactone and aromatic rings. Free radicals could be stabilized by resonance, which allows hydrogen atoms to be easily released from the hydroxyl groups [40]. This efficacy was enhanced by irradiating the spores of the isolated *A.terreus* ON532722 where the IC_{50} value was become 37.5 ± 1.48 µg/ml. This is maybe attributed to the induction of gamma irradiation to the different bioactive compounds and increasing their yield and activity.

5. Conclusion

Endophytic fungi have great attention nowadays as a promising source for bioactive compounds production. In this study, the endophytic fungi of neem medicinal plant metabolites had been proven to have antimicrobial activity. Optimization of factors affecting the activity of endophytic fungi using experimental design showed an effective method as it not only save time but also showed the effect of the interaction between these factors on their antimicrobial activity. Also, in this study the effect of gamma irradiation on the extract of the fungal isolate was obvious. GC-MS showed a variety of important and effective compounds that had antimicrobial, antioxidant, and cytotoxicity activities. After irradiation, new compounds appeared with great biological activity. So, the production of new active compounds from endophytic fungi gained great attention.

6. Conflicts of interest

The authors affirm that they have no known financial or interpersonal conflicts that would have appeared to have an impact on the research presented in this study.

7. References

1. **Semwal, P., Painuli, S., Anand, J., Martins, N. C., Machado, M., Sharma, R., Batiha, G. E., Yaro, C. A., Lorenzo, J. M., & Rahman, M. (2022).** The Neuroprotective Potential of Endophytic Fungi and Proposed Molecular Mechanism: A Current Update. *Evidence-Based Complementary and Alternative Medicine*, *2022*. 6214264. https://doi.org/10.1155/2022/6214264
2. **Shalabi, A., Eskander, D., & badawe, mona. (2021).** Isolation of Secondary Metabolites from Marine Streptomyces sparsus ASD203 and Evaluation its Bioactivity. *Egyptian Journal of Chemistry*, *0*(0), 0–0. https://doi.org/10.21608/ejchem.2021.95284.4489
3. **Kalachaveedu, M., Senthil, R., Azhagiyamanavalan, S., Ravi, R., Meenakshisundaram, H., & Dharmarajan, A. (2023).** Traditional medicine herbs as natural product matrices in cancer chemoprevention: A trans pharmacological perspective (scoping review). *Phytotherapy Research*, 37, 1539-1573. https://doi.org/10.1002/ptr.7747
4. **Reddy, I. V., & Neelima, P. (2022).** Neem (*Azadirachta indica*): A review on medicinal Kalpavriksha. *International Journal of Economic Plants*, 9, 59-63. https://doi.org/10.23910/2/2021.0437d
5. **Khaled, F. M., Haitham, Q., Dmour Saif, M., Moath, A., & Yassin, A. K. A. (2023).**

Azadirachta indica (Neem) Leaf: chemical composition and antibacterial activity as determined by different polarity solvents. *Research Journal of Biotechnology*, 18, 2. https://doi.org/10.25303/1802rjbt55062

6. **Ahire, M. L., & Mundada, P. S. (2022).** 18 *Azadirachta indica* (Neem). Herbs, Shrubs, and Trees of Potential Medicinal Benefits, 365. https://doi.org/10.1201/9781003205067-18

7. **Yan, L., Zhu, J., Zhao, X., Shi, J., Jiang, C., & Shao, D. (2019).** Beneficial effects of endophytic fungi colonization on plants. *Applied microbiology and biotechnology*, 103,3327-3340. https://doi.org/10.1007/s00253-019-09713-2
8. **Hassan, S. E., Fouda, A., Radwan, A. A., Salem, S. S., Barghoth, M. G., Awad, M. A., Abdo, A. M., & El-Gamal, M. S. (2019).** Endophytic actinomycetes Streptomyces spp mediated biosynthesis of copper oxide nanoparticles as a promising tool for biotechnological applications. *JBIC Journal of Biological Inorganic Chemistry*, *24*(3), 377-393. https://doi.org/10.1007/s00775-019-01654-5
9. **Omar, S., Fahmi ,, A. E., Abdur-Rahman, M., Ghareeb, M., & Abdelaziz, M. (2022).** Biological and chemical evaluation of secondary metabolites from endophytic fungi isolated from Egyptian ornamental plants. *Egyptian Journal of Chemistry*, *0*(0), 0 0. https://doi.org/10.21608/ejchem.2022.173108.7160

10. **Verma, V.C., Gond, S.K., Kumar, A., Kharwar, R.N., Strobel, G. (2007).** The endophytic mycoflora of bark, leaf, and stem tissues of *Azadirachta indica* A. Juss (Neem) from Varanasi (India). *Microbial Ecology*, 54, 119–125. https://doi.org/10.1007/s00248-006-9179-9
11. **Chutulo, E. C., & Chalannavar, R. K. (2018).** Endophytic Mycoflora and Their Bioactive Compounds from *Azadirachta Indica*: A Comprehensive Review. *Journal of fungi (Basel, Switzerland)*, 4, 42. https://doi.org/10.3390/jof4020042
12. **Kumar, V., Soni, R., Jain, L., Dash, B., & Goel, R. (2019).** Endophytic fungi: recent advances in identification and explorations. *Advances in Endophytic Fungal Research*: *Present Status and Future Challenges*, 267-281.https://doi.org/10.1007/978-3-030-03589-1_13

13. **Hashem, A. H., Shehabeldine, A. M., Abdelaziz, A. M., Amin, B. H., & Sharaf, M. H. (2022).** Antifungal activity of endophytic aspergillus terreus extract against some fungi causing Mucormycosis: Ultrastructural study. *Applied Biochemistry and Biotechnology*, 194, 3468-3482. https://doi.org/10.1007/s12010-022-03876-x

14. **Zaki, A. G., El-Shatoury, E. H., Ahmed, A. S., & Al-Hagar, O. E. (2019).** Production and enhancement of the acetylcholinesterase inhibitor, huperzine a, from an endophytic *Alternaria brassicae* AGF041. *Applied Microbiology and Biotechnology*, 103, 5867-5878. https://doi.org/10.1007/s00253-019-09897-7
15. **El-Sayed, E. R., Hazaa, M. A., Shebl, M. M., Amer, M. M., Mahmoud, S. R., & Khattab, A. A. (2022).** Bioprospecting endophytic fungi for bioactive metabolites and use of irradiation to improve their bioactivities. *AMB Express*, 12, 46. https://doi.org/10.1186/s13568-022-01386-x

16. **Kortei, N. K., Odamtten, G. T., Obodai, M., Appiah, V., Akuamoa, F., Adu-Bobi, A. K., Annan, S. N., Armah, J. N., & Acquah, S. A. (2014).** Evaluating the effect of gamma radiation on the total phenolic content, flavonoids, and antioxidant activity of dried *Pleurotus ostreatus* ((Jacq. ex. Fr) Kummer) stored in packaging materials. *Advances in Pharmaceutics*, 2014, 1-8. https://doi.org/10.1155/2014/262807

17. **Spiteller, P., & Reck, B. (2015). Synthesis of secondary metabolites from higher fungi.** *Synthesis*, 47, 2885-2911. https://doi.org/10.1055/s-0034-1378774
18. **Qureshi, A.H., Ali, S., & Pandey, A.K. (2019).** Isolation and identification of endophytic fungi inhabiting *Aazdirachta indica* A. Juss. From different regions of Jabalpur (M.P) India, RJLBPCS 5, 274-281. https://doi.org/10.26479/2019.0503.22
19. **Barnett, H.L., Hunter, B.B. (1998).** Illustrated Genera of Imperfect Fungi. 4th edition. *American Phytopathology Society.*
20. **Moubasher, A.H. (1993).** Soil fungi in Qatar and other Arab countries. The Centre for

Scientific and Applied Research, P.O. Box 2713, Doha, Qatar.

21. **Kjer, J., Debbab, A., Aly, A.H. & Proksch, P. (2010).** Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. *Nature protocols*, 5, 479-490. https://doi.org/10.1038/nprot.2009.233
22. **Saitou, N. and Nei, M. (1987).** The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4(4), 406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454
23. **Tayung K, Barik BP, Jha DK and Deka DC (2010)**. Identification and characterization of antimicrobial metabolite from an endophytic fungus, *Fusarium solani* isolated from the bark of Himalayan yew. *Mycosphere*, 2(3), 203–213.
24. **Radji M, Sumiati A, Rachmayani R, Elya B (2011).** Isolation of fungal endophytes from Garcinia mangostana and their antibacterial activity. *African Journal of Biotechnology*, 1, 103-107. https://doi.org/10.5897/AJB10.1531
25. **Bauer, A. W., Kirby, W. M. M., Sherriss, J. C., and Turck, M. (1966).** Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology.*, **45**(4),493-https://doi.org/10.1093/ajcp/45.4_ts.493
26. **Abhini, K. N., Rajan, A. B., Fathimathu Zuhara, K., & Sebastian, D. (2022).** Response surface methodological optimization of L-asparaginase production from the medicinal plant endophyte *Acinetobacter baumannii ZAS1*. *Journal of Genetic Engineering and Biotechnology*, 20(1), 1-13. https://doi.org/10.1186/s43141-022-00309-4
27. **Box GEP, Wilson KB (1951).** On the experimental attainment of optimum conditions. *Journal of the Royal Statistical Society*, 13, 1-45.
28. **Mosmann, T. (1983):** Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *The Journal of Immunological Methods*, 65, 55-63. https://doi.org/10.1016/0022-1759(83)90303-4
29. **Gomha, S.M.; Riyadh, S.M.; Mahmoud, E.A., & Elaasser, M.M. (2015).** Synthesis and Anticancer Activities of Thiazoles, 1,3-Thiazines, and Thiazolidine Using Chitosan-Grafted-Poly (vinyl pyridine) as Basic Catalyst. *Heterocycles*; 91(6), 1227-1243. https://doi.org/10.3987/com-15-13210
30. **Alvin, A., Miller, K. I., & Neilan, B. A. (2014).** Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. *Microbiological Research*, 169(7-8), 483-495. https://doi.org/10.1016/j.micres.2013.12.009
31. **Kumari, P., Singh, A., Singh, D. K., Sharma, V. K., Kumar, J., Gupta, V. K., Bhattacharya, S., & Kharwar, R. (2021).** Isolation and purification of bioactive metabolites from an endophytic fungus *penicillium citrinum* of *Azadirachta indica. South African Journal of Botany*, 139, 449-457.
32. **da Silva IP, Brissow E, Kellner Filho LC, Senabio J, de Siqueira KA, Vandresen Filho S, Damasceno JL, Mendes SA, Tavares DC, Magalhães LG, Junior PAS, Januário AH, Soares MA (2017).** Bioactive compounds of *Aspergillus terreus*—F7, an endophytic fungus from *Hyptis suaveolens (L.) Poit. World Journal of Microbiology and Biotechnology*, 33, 62. https://doi.org/10.1007/s11274-017-2228-3
33. **Mahapatra S and Banerjee D (2013).** Optimization of a bioactive exopolysaccharide production from endophytic *Fusarium solani* SD5. *Carbohydr Polym*. 97, 627- 634. https://doi.org/10.1016/j.carbpol.2013.05.039
34. **Sahani, Kamana & K.P.J. Hemalatha. (2018).** Optimization of endophytic fungi *curvularia aeria* MTCC 12847 isolated from Tribulus terrestrial plant by RSM technology. *International Journal of Pharmaceutical Sciences Review and Research*, *52*, 154-159.
35. **Widjajanti, H., Nurnawati, E., Muharni, M., & Zahwa, E. D. (2022).** Optimization of antibacterial production of endophytic fungi with various sources of C, N, and pH using the response surface methodology. *Science and Technology Indonesia*, 7, 149-157. https://doi.org/10.26554/sti.2022.7.2.149-157**El-Hawary, S.S., Moawad, A.S., Bahr, H.S., Abdelmohsen, U.R., Mohammed, R. (2020).** Natural product diversity from the endophytic fungi of the genus *Aspergillus*. RSC Adv, 10,

22058–22079.
https://doi.org/10.1039/d0ra04290k

36. **Cruz, J. S., Da Silva, C. A., & Hamerski, L. (2020).** Natural products from endophytic fungi associated with rubiaceae species. Journal of Fungi, 6, 128. https://doi.org/10.3390/jof6030128
37. **Chandrasekaran, M., Raj, G. A., Jegan, S., & Venkatesalu, V. G. (2017).** Phytochemical analysis, antibacterial and Antifungal activities of different crude extracts of marine red alga *Gracilaria corticata* from the Gulf of Mannar south coast, Navaladi, South India. *International Journal of Pharmaceutical Sciences and Drug Research*, 9, 2017. https://doi.org/10.25004/ijpsdr.2017.090203
38. **Banakar, P., & Jayaraj, M. (2017).** Pharmacognosy, Phytochemistry and GC-MS analysis of Ethanolic stem extract of *Waltheria indica L*. - A potent medicinal plant. *Journal of Biologically Active Products from Nature*, 7, 369-378. https://doi.org/10.1080/22311866.2017.1400923
39. **Brewer, M. (2011).** Natural antioxidants: Sources, compounds, mechanisms of action, and potential applications. *Comprehensive Reviews in Food Science and Food Safety*, 10, 221-247. https://doi.org/10.1111/j.1541-4337.2011.00156.x.