COMPARATIVE BIOLOGICAL EVALUATION OF FOUR ENDOPHYTIC FUNGI ISOLATED FROM *NIGELLA SATIVA* SEEDS

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Abstract:

In this study, a total of four endophytic fungi have been isolated from *Nigella sativa* (Ranunculaceae) seeds growing in Egypt. The isolated fungi were identified morphologically and microscopically up to species to be; *Alternaria pluriseptata*, *Aspergillus tubingensis*, *Aspergillus parasiticus* and *Eurotium pseudoglaucus*. The extracts of the all identified fungi were screened biologically for antileukemic, cytotoxic, antimalarial, antileishmanial, antimicrobial, antioxidant and anti-inflammatory activities as well as for cannabinoid and opioid receptor binding affinities. All the examined fungal extracts showed good antileukemic activities with Ic_{50} values ranging from 0.025 to 0.8 µg/ml, while two of them showed cytotoxic activities against Pig kidney epithelial (LLC-PK1) cells. Two fungal extracts exhibited antimalarial activities, three showed antioxidant activities and two exhibited anti-inflammatory activities

Keywords: endophytic fungi, antileukemic, cytotoxic, *Nigella sativa*, *Aspergillus tubingensis*.

1. Introduction

Endophytic fungi are defined as fungi that inhabit inside healthy plant tissues in their a part or whole life cycle without causing any apparent harm (Petrini, 1991; Schulz et al., 1999).The literal translation of the word endophyte is originated from the Greek words ,endon meaning inside and phyton meaning plant (Jalgaonwala et al., 2017). Researchers have shown that endophytic fungi can protect their host plants from several living dangers such as pathogens through secreting antimicrobial compounds and increasing plant resistance to infection (Gao et al., 2010). In addition, it was found that some endophytic fungi can give their host plant the ability to accommodate different types of stresses as drought (Elmi and West, 1995), excess salt (Waller et al., 2005) and heat (Márquez et al., 2007). Endophytic fungi can be considered as a source of chemically novel bioactive secondary metabolites belonging to different chemical classes (Tenguria et al., 2011). Several bioactive metabolites have been characterized from endophytic fungi over last years, and those isolated compounds found to be belong to diverse structural groups such as; alkaloids (Metwaly et al., 2015), isochromenes,(Metwaly, A.M. et al., 2014b) benzopyranones (Metwaly, A. et al., 2014), α-pyrones (Metwaly, A.M. et al., 2014a; Metwaly et al., 2017), steroids (Hussain et al., 2009), terpenoids (Bilal et al., 2018), peptides (Tawfike et al., 2018), polyketones (Zheng et al., 2018), quinones (Stierle and Stierle, 2015), phenolics(Das et al., 2018), xanthones (Li et al., 2016), isocoumarines (Orfali et al., 2015), perylene derivatives (Chagas et al., 2016), furandiones (Li et al., 2015) and butenolide derivatives (Guo et al., 2016) . Recently, there has been a noticeable interest of researches on endophytic fungi producing important metabolites with a great diversity of biological activities such as antileukemic (Metwaly, A.M. et al., 2014a), anti-cancer (Chandra, 2012), antimicrobial(Kumar and Kaushik, 2012), anti-inflammatory (Deshmukh et al., 2009), antimalarial (Wiyakrutta et al., 2004), antileishmanial (Metwaly et al., 2013) and antioxidant (Huang et al., 2007).

2. Materials and Methods

2.1. Fungal material

2.1.1. Isolation of endophytic fungi

The fungi were isolated from surface sterilized fresh seeds of an apparently healthy *Nigella sativa* collected from botanical garden of faculty of pharmacy, Al-Azhar University, Cairo, Egypt. The seeds were rinsed with water and followed by surface sterilization in 70% EtOH for 1 min, rinsed again with sterilized water, then cut into two halves and deposited in a petri dish containing PDA medium (200 g potato, 20 g glucose, and 15 g agar in 1 L distilled water, supplemented with 100 mg/ L chloramphenicol) and cultivated at 28°C for 3 days. The hypha tips were observed and transferred to new PDA plates and subcultured until pure culture was obtained.

2.1.2. Identification of endophytic fungi

The fungi were identified by the Regional Center for Mycology and Biotechnology, Cairo, Egypt. Pure colony of each fungal isolate was inoculated on different medium (potato-dextrose agar, Czapek's agar, Czapek's yeast extract agar

(CYA) and malt extract agar) and incubated for 1-7 days. Cultural features for each fungal isolate in addition to microscopic examination of reproductive structure were recorded. identification was based on current universal keys (Fisher and Cook, 1998; Hoog et al., 2000) and on the data base identification program of the Regional Center for Mycology and Biotechnology (RCMB) for fungi using an Image Analysis System.

2.1.3. Mass cultivation

Each endophytic fungus was grown on PDA at 28 °C for 5 days. Ten pieces (0.5×0.5 cm^2) of mycelial agar plugs were inoculated into ten 1000 mL Erlenmeyer flasks containing sterilized (100 g Asian rice and 100 ml distilled water). The flasks were incubated under static conditions at room temperature for 40 days.

2.3. Extraction

Each fungus was extracted by adding 1 L EtOAc (twice) to each flask and homogenized. The homogenized suspensions were collected, filtrated, concentrated under vacuum and prepared for biological assay.

2.4. Antileukemic assay

Human acute leukemia HL60 cells and human chronic leukemia K562 cells were purchased from American Type Culture Collection, Rockville MD, USA. Both cell lines were grown in suspension culture at 37 ˚C in RPMI-1640 medium supplemented with 10% non-dialysed fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL of penicillin and 10 µg/mL of streptomycin. For the cell growth inhibition assay, HL60 and K562 cells were seeded at 1 x 10^5 cells/well in Costar 24-well plates. Cells could grow undisturbed for 24 h before addition of test samples. After 48 h incubation with samples at 37 ˚C, viable cell counts were made by using the trypan blue exclusion method to assess cell viability (Roper and Drewinko, 1976).

2.5. Cytotoxicity Assay

Cytotoxicity was determined against HepG2 (human hepatocarcinoma), Vero (African green monkey kidney fibroblasts) and LLC-PK_1 (pig kidney epithelial) cells as described earlier (Mustafa et al., 2004). Doxorubicin was used as a positive control.

2.6. Antimalarial assay

Crude extracts were tested for activity against chloroquine-sensitive (D6, Sierraleon) and -resistant (W2, Indo-china) strains of *Plasmodium falciparum* using previously reported method (Bharate et al., 2007). The anti-malarial screen tests samples for their ability to inhibit the chloroquine-sensitive (D6) and/or chloroquine-resistant (W2) *Plasmodium falciparum* protozoan. Crude extracts are initially tested against the D6 *P. falciparum* strain.

Primary Screen;

at 15867 ng/mL in duplicate, and percent inhibitions (% inh.) are calculated relative to negative and positive controls. Extracts showing ≥50% inhibition proceed to the Secondary Assay.

Secondary Assay;

In the secondary antimalarial assay, samples dissolved to 20mg/mL (crude extracts and some column fractions) are tested at 47600, 15867, and 5289ng/mL and

IC_{50}s (test concentration in ng/mL that affords 50% inhibition of the protozoan relative to negative and positive controls) vs. both the D6 and W2 strains are reported. Samples dissolved to 2mg/mL are tested at 4760, 1587, and529ng/mL and IC_{50}s vs. both the D6 and W2 strains are reported. In addition to the *P. falciparum* strains, samples are tested in the VERO mammalian cell line as an indicator of general cytotoxicity. The selectivity indices (SI) – the ratio of VERO IC_{50} to D6 or W2 IC_{50} - are calculated. All IC_{50}s are calculated using the XLFit fit curve fitting software. The antimalarial drug controls chloroquine and artemisinin are used as positive controls

2.7. Antileishmanial assay

The anti-leishmanial screen (LEM) tests samples for their ability to inhibit *Leishmania donovani*, a fly-borne protozoan that causes visceral leishmaniasis. Crude extracts are initially tested in a Primary Screen**.**

Primary Screen; at 80µg/mL in duplicate and percent inhibitions (% inh.) are calculated relative to negative and positive controls. Extracts showing ≥50% inhibition proceed to the secondary assay (Abdel-Mageed et al., 2012).

2.8. Antimicrobial screen

Crude extracts tested for antimicrobial activity against 90906, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591, *Staphylococcus aureus* ATCC 2921, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Mycobacterium intracellulare* ATCC 23068 *Candida albicans* ATCC 90028, *Candida glabrata* ATCC90030, *Candida krusei* ATCC 6258, *Aspergillus fumigates* ATCC and *Cryptococcus neoformans* ATCC 90113, Ciprofloxacin and Amphotericin B were used as positive standards.

Primary Screen;

Crude extracts are initially tested at 50µg/mL in duplicate and percent inhibitions (% inh.) are calculated relative to negative and positive controls. Extracts showing ≥50% inhibition proceed to the Secondary Assay.

Secondary Assay;

In the Secondary Assay, samples dissolved to 20mg/mL (crude extracts and some column fractions) are tested at 200, 40, 8µg/mL and IC_{50}s vs. all 10 microbial strains are reported. Samples dissolved to 2mg/mL (pure compounds and some column fractions) are tested at 20, 4, 0.8µg/mL and IC_{50}s vs. all 10 microbial strains are reported. Pure compounds that have an IC_{50} of ≤7µg/mL in the Secondary OI assay proceed to the Tertiary Assay.

The MIC is the lowest test concentration (in µg/mL) that inhibits the organism 100%. The MFC or MBC is the lowest test concentration (in µg/mL) that kills the organism. While a pure compound may have an MIC, the cells may still be alive, just not growing. The MFC and MBC is a way to monitor the "cidality" or the killing ability of the test sample. All IC_{50}s are calculated using the XLFit fit curve fitting software.

2.9. Anti-inflammatory assays

Anti-inflammatory activity was determined in terms of the inhibition of NF-*κ*B - mediated transcription and inhibition of intracellular generation of reactive oxygen species (ROS) and nitric oxide (NO). Inhibition of NF-*κ*B mediated transcription was determined in human chondrosarcoma (SW1353) cells by a reporter gene assay as described earlier(Ma et al., 2007). Sp-1 was used as a control transcription factor to evaluate the toxicity of tested compounds in the same assay. Parthenolide was used as the positive control. Inhibition of intracellular NO production as a result of iNOS activity was assayed in mouse macrophages (RAW 264.7 cells) as described before (Quang et al., 2006). Parthenolide was included in each assay as the positive control. Inhibition of intracellular ROS generation (antioxidant activity) was assayed in human promyelocytic leukemia (HL-60) cells by using DCFH-DA as described previously (Reddy et al., 2007). Trolox was used as a positive control.

2.10. Antioxidant assay

Antioxidant activity was determined by the DCFH-DA (20,70-dichlorofluorescein diacetate) method in myelomonocytic HL-60 cells as described

earlier(Abbas et al., 2007). Briefly, for the assay, cells were plated at a density of 1.25105 cells per well in 96-well plates. After treatment with different concentrations of the test samples for 30 min, cells were stimulated with 100 ng mL1 phorbol 12-myristate-13-acetate (PMA, Sigma) for 30 min. DCFH-DA (molecular probes, 5mg mL1) was added and further Antimicrobial assay incubated for 15 min. Plates were read on a polar star at an excitation wavelength of 485 nm and emission at 530 nm to measure the level of DCF production.

2.11. Opioid and Cannabinoid Receptor Binding Assay

This assay is designed to use a series of controls to determine the binding affinity of the test extracts against Kappa (κ), Delta (δ) and Mu (μ) opioid receptors in addition to Cannabinoid Receptors CB 1 and CB 2. 10uM of a positive control [*nor*-Binaltorphimine dihydrochloride (κ), DPDPE (δ), or DAMGO (μ)] was used to ascertain non-specific binding (NSB) and 1% ethanol or DMSO in Tris-EDTA buffer was used to ascertain total binding. For Cannabinoid Receptor Binding Assay 10uM of a CP-55,940 was used to ascertain non-specific binding and 1% ethanol or DMSO in Tris-EDTA buffer was used to ascertain total binding. To eliminate the possibility of contamination in the test extracts, controls or the radioligand, wells with 1% ethanol or DMSO with no membrane were tested. The test have been done using a 96-well format as described in the scientific literature (Bradford, 1976; Kumarihamy et al., 2015).

3. Results

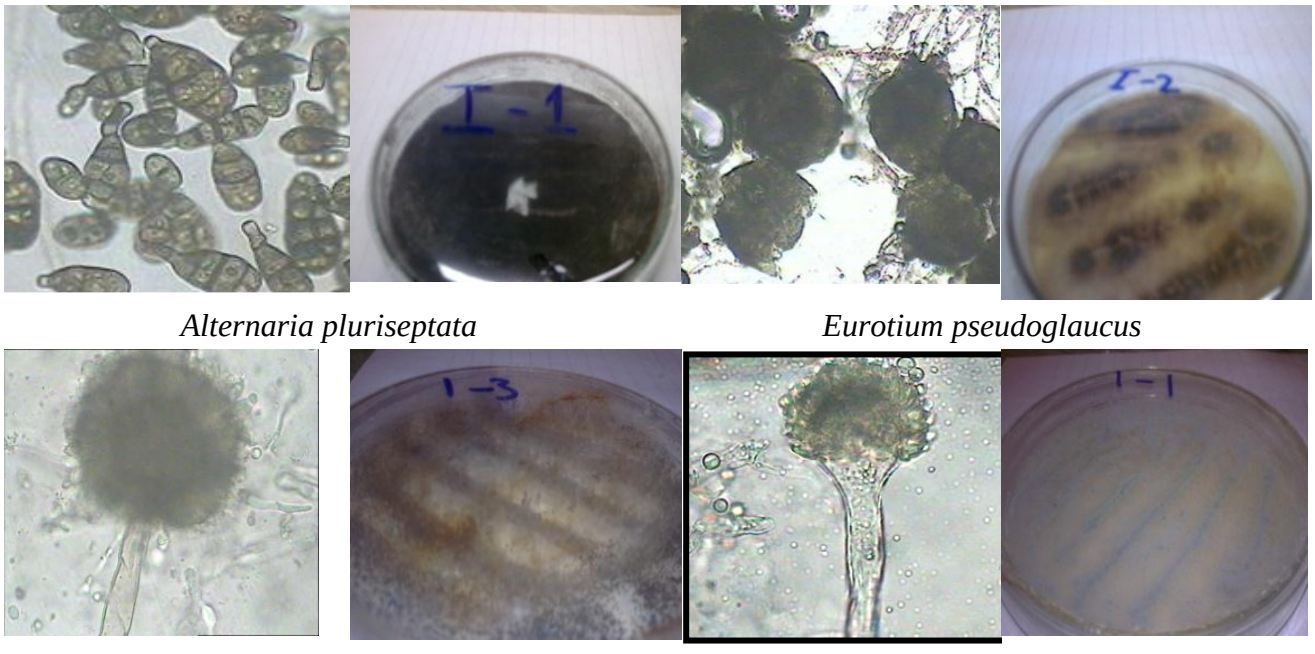
3.1. Isolation and identification of the endophytic fungi

In this study, a total of four endophytic fungal isolates were isolated on PDA from 20 seed segments of *Nigella sativa* (Ranunculaceae) seeds growing in Egypt and identified as *Aspergillus tubingensis*, *Aspergillus parasiticus*, *Alternaria pluriseptata and Eurotium pseudoglaucus* (Fig. 1). Morphological and microscopical characters of

the identified fungi have been summarized in Table 1. The fungal identification was carried out by the regional center for mycology and biotechnology, (Cairo, Egypt). The fungi were grown on Asian rice as a solid media and then extracted with ethyl acetate. All fungal extracts were biologically screened for antileukemic, cytotoxic, antimalarial, antileishmanial, antimicrobial, antioxidant and anti-inflammatory activities as well as for cannabinoid and opioid receptor binding affinity.

Table 1: Morphological and microscopical characters of the identified fungi

	Culture Examination	**Microscopic Examination**
Alternaria pluriseptata	Colonies on PDA grow rapidly, effuse, black, reaching 3-5 cm diameters in two days at 25˚C, Reverse is black.	Conidiophores 4.3µm thick. Conidia obclavate, golden brown in colour, smooth 32.0X15.5 µm, with a short, pale beak 4.0 µm thick, with 2-7 transverse septa.
Aspergillus parasiticus	Colonies fast-growing, reaching 5-7cm diameters in four days at 25˚C on malt media; usually consist of a dense felt of yellowish green mycelia, with pale yellow reverse.	Conidiophores are coarsely roughened, geenish yellow in colour, 10.4 µm. Conidia globose, 3.5µm. Chlamydospore was absent. Vesicles; subglobose, 24.5 µm. Sterigmata; one series
Aspergillus tubingensis	Colonies fast-growing, reaching 4-5 cm diameters in four days at 25˚C on malt media; usually consist of a compact white basal mycelium with black conidial heads. Reverse is colorless	Conidiophores; 15.8 µm. Conidial heads; Globose to radiate, mostly 200-300 µm. Conidia globose, 3.1µm. Chlamydospore was absent. Vesicles; globose, 35.7 µm. Sterigmata in two series
Eurotium pseudoglaucus	Colonies on PDA restricted in growth, yellow-orange to brown, reverse, orange in center and lighter toward in margin.	Conidiophores 5.3 µm. Conidia subglobose 6.0 µm Chlamydospore was absent. Vesicles; 14.0 µm. Sterigmata; one series. Cleistothecia; Abundant, spherical. Asci; 11 µm contain ascospores.



Alternaria pluriseptata *Eurotium pseudoglaucus*

Aspergillus tubingensis *Aspergillus parasiticus*

Figure 1. The isolated fungi under a microscope and as pure strains on malt agar plate

3.2. Antileukemic and cytotoxic assay results

Alternaria pluriseptata, Aspergellus parasiticus, Aspergillus tubingensis and *Eurotium pseudoglaucus* extracts showed good antileukemic activities against K562

cells with IC_{50} values of 3.5, 0.4, 0.8 and 0.05 µg/ml and against HL60 cells with IC_{50} values of 0.2, 0.16, 0.7 and 0.025 µg/ ml, respectively. These fungi have been chosen for cytotoxic activity examination against HepG2 (human hepatocarcinoma), Vero (African green monkey kidney fibroblasts) and LLC-PK1 (pig kidney epithelial). The endophytic fungal extracts of *Alternaria pluriseptata* and *Aspergillus tubingensis* showed moderate cytotoxic activity against LLC-PK_{11} with IC_{50} values of 66 and 20 µg/ml, respectively as shown in Table 2 and fig. 2.

Table 2: Antileukemic and cytotoxic activities result for the isolated endophytic fungi

	IC_{50} µg/ml				
	K562 cells	HL60 cells	HepG2 cells	Vero cells	LLC-PK1 cells
Alternaria pluriseptata	3.5	0.2	NA	NA	66
Aspergellus parasiticus	0.4	0.16	NA	NA	NC
Aspergillus tubingensis	0.8	0.7	NA	NA	20
Eurotium pseudoglaucus	0.05	0.025	NA	NA	NA

[a] NA: Extracts having IC_{50} value > 100µg/ml

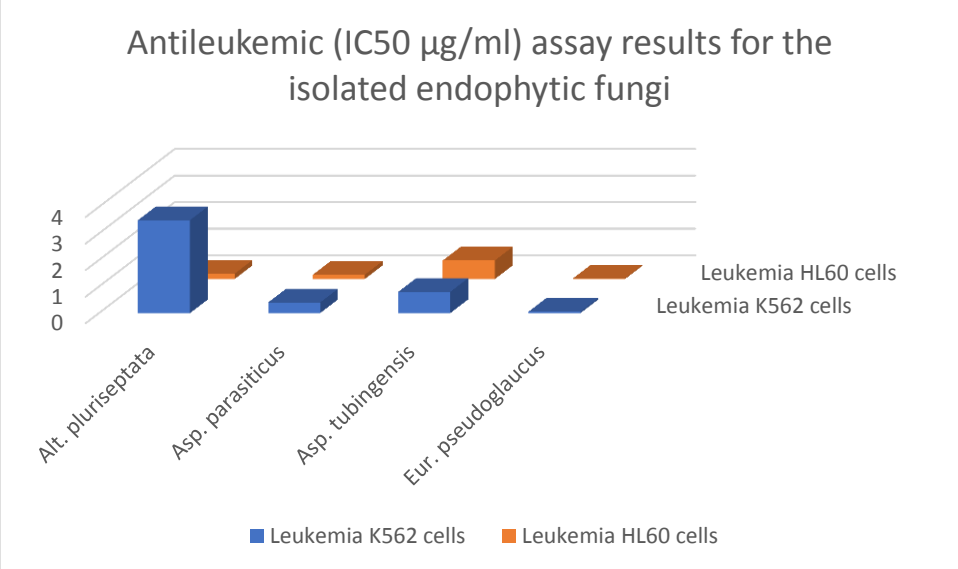


Fig. 2. Antileukemic result for the isolated endophytic fungi

3.3. Antimalarial assay results

The extract of *Alternaria pluriseptata* and *Eurotium pseudoglaucus* showed antimalarial activities against *Plasmodium falciparum* D6 clone with IC_{50} values of 12 and 8 µg/mL and against W2 clone with IC_{50} values of 14 and 8 µg/mL, respectively as shown in Table 3.

Table 3: Antimalarial activity results for the isolated endophytic fungi

	P. falciparum D6 IC_{50} µg/ml	*P. falciparum W2* IC_{50} µg/ml
Alternaria pluriseptata	12	14
Aspergellus parasiticus	NA	NA
Aspergillus tubingensis	NA	NA
Eurotium pseudoglaucus	8	8

[a] NA: Extracts having IC_{50} value > 25.8 µg/ml

3.4. Antileishmanial assay results

All fungal extracts of *Alternaria pluriseptata, Aspergillus parasiticus, Aspergillus tubingensis and Eurotium pseudoglaucus* inhibited the growth of *L. donovani* growth with % Inhibition of 48.8, 5.6, 11.9, 7.6 and 49.8 respectively.

Table 4: Antileishmanial assay results

	L. donovani	
	% Inh.	IC_{50} µg/ml
Alternaria pluriseptata	48.8	-
Aspergillus parasiticus	5.6	-
Aspergillus tubingensis	7.6	-
Eurotium pseudoglaucus	49.8	-

[a] NA: Extracts having IC_{50} value > 80µg/ml

3.5. Antimicrobial assay results

All endophytic fungal extracts have been examined for their ability to inhibit a panel of 5 bacteria and 5 fungi those are pathogenic to humans including; *Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli, Pseudomonas aeruginosa, Mycobacterium intracellulare, Candida albicans, Candida glabrata, Candida krusei, Aspergillus fumigates and Cryptococcus neoformans*. Some extracts exhibited inhibitory effects with different percentages against the examined organisms ranging from 1 to 34 (Table 5 and 6).

Table 5: Antibacterial assay results

	S. aureus		*MRS*		*E. coli*		*P. aeruginosa*		*M.Intracellulare*	
	% Inh.	IC_{50}	% Inh.	IC_{50}	% Inh.	IC_{50}	% Inh.	IC_{50}	% Inh.	IC_{50}
Alternaria pluriseptata	9	-	5	-	4	-	2	-	2	-
Aspergillus parasiticus	5	-		-	6	-	2	-	-	-
Aspergillus tubingensis	3	-	2	-	17	-	4	-	-	-
Eurotium pseudoglaucus	14	-	21	-	17	-	-	-	-	-

Table 6: Antifungal assay results

	C. albicans		*C. glabrata*		*C. krusei*		*A. fumigatus*		*C. neoformans*	
	% Inh.	IC_{50}	% Inh.	IC_{50}	% Inh.	IC_{50}	% Inh.	IC_{50}	% Inh.	IC_{50}
Alternaria pluriseptata	8	-	4	-	27	-	7	-	7	-
Aspergillus parasiticus	1	-	3	-	2	-	8	-		-
Aspergillus tubingensis	6	-	6	-	30	-	10	-	9	-
Eurotium pseudoglaucus	10	-	3	-	34	-	4	-	21	-

	Anti-inflammatory activity assay results IC_{50}µg/ml		
	iNOS	*NF-kB*	*SP-1*
Alternaria pluriseptata	*100*	*NA*	*NA*
Aspergellus parasiticus	*NA*	*NA*	*NA*
Aspergillus tubingensis	*92*	*NA*	*NA*
Eurotium pseudoglaucus	*NA*	*NA*	*NA*

3.6. Anti-inflammatory assay results

All the fungal extracts have been subjected to screen anti-inflammatory activities. The activity was determined in terms of the inhibition of nuclear factor (NF-*κ*B) mediated transcription and inhibition of intracellular generation of reactive oxygen species (ROS) and nitric oxide synthase (NOS). The endophytic fungal extracts of *Alternaria pluriseptata* and *Aspergillus tubingensis* showed nitric oxide synthase inhibitory (iNOS) activities with IC_{50} values of 100and 92 µg/ml, respectively, as shown in Table 7.

Table 7: Anti-inflammatory assay results

[a] NA: Extracts having IC_{50} value > 100µg/ml

3.7. Anti-oxidant assay results

Anti-oxidant activities were determined by the DCFH-DA (20,70-dichlorofluorescein diacetate) method in myelomonocytic HL-60 cells. The endophytic fungal extracts of *Alternaria pluriseptata*, *Aspergillus tubingensis* and *Eurotium pseudoglaucus* showed anti-oxidant activities with IC_{50} values of 19, 8 and 18 µg/ml, respectively.

Table 8: Anti-oxidant assay results

	Antioxidant activity IC_{50} µg/ml
Alternaria pluriseptata	*19*
Aspergellus parasiticus	*NA*
Aspergillus tubingensis	*8*
Eurotium pseudoglaucus	*18*

[a] NA: Extracts having IC_{50} value > 100µg/ml

3.8: Opioid receptors binding assay results

The extracts of *Alternaria pluriseptata, Aspergillus tubingensis* and *Eurotium pseudoglaucus* exhibited very weak opioid receptors binding affinities against Delta receptors with % inhibition values of 0.62, 4.89 and 9.17 and against Mu receptors % inhibition values of 1.27, 14.82 and 11.87, respectively *Aspergillus tubingensis* extract weakly inhibited Kappa receptors with a value of 11.01%.

Table. 9: Opioid receptors binding assay results

	Kappa receptors %Inh.	Delta receptors % Inh.	Mu receptors % Inh.
Alternaria pluriseptata	-	0.62	1.27
Aspergellus parasiticus	-	-	-
Aspergillus tubingensis	11.01	4.89	14.82
Eurotium pseudoglaucus	-	9.17	11.87

3.9. Cannabinoid receptors binding assay results

Alternaria pluriseptata, Aspergillus tubingensis and *Eurotium pseudoglaucus* extracts were found to have weak inhibitory effects against CB 2 receptors with % inhibition of 6.29, 8.69 and 14.88, respectively.

Table 10: Cannabinoid receptors binding assay results

	CB 1 receptors %Inh	CB 2 receptors %Inh
Alternaria pluriseptata	-	6.29
Aspergellus parasiticus	-	-
Aspergillus tubingensis	-	8.69
Eurotium pseudoglaucus	-	14.88

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التقييم البيولوجي المقارن لأربعة فطريات معزولة من بذور حبة البركة

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الخلاصة :في هذه الدراسة ، تم عزل ما مجموعه أربعة فطريات الفطرية من بذور نيجيلا ساتيفا التي تنمو في مصر . تم التعرف على الفطريات المعزولة شكلياً ومجهريًا حتى تكون الأنواع ؛

Alternaria pluriseptata و Aspergillus tubingensis و Aspergillus parasiticus و Eurotium pseudoglaucus.

تم فحص خلاصات من الفطريات التي تم تحديدها جميعها بيولوجيا بحثًا عن تأثير مضاد لسرطان الدم وتأثير مضاد للخلايا السرطانية وتأثير مضاد للشمانيا وتأثير مضاد للملاريا وتأثير مضاد الميكروبات و تأثير مضادات الأكسدة وتأثير مضاد للالتهابات