Egypt. J. Bot. **Vol. 60**, No. 2, pp. 537-548 (2020)

Egyptian Journal of Botany
http://ejbo.journals.ekb.eg/

Efficacy of *Alternaria alternata* NMK1 Secondary Metabolites against Some Seed-Borne Fungi

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AN AGRICULTURAL soil was used to isolate *Alternaria alternata*. It was molecularly identified and was given an accession number MN645469 at the National Center for Biotechnology Information (NCBI) and a strain identifier to be *Alternaria alternata* strain NMK1. Four fractions were collected from the acetic acid extract of its filtrate. Fraction no. 2 showed highest antioxidant activity (71 and 64%, at 200 and 150µg/mL) followed by the crude extract (60 % at 200µg/mL). Safety of using the extracted fractions was tested against the normal human amnion (WISH) cell line. Lowest cytotoxicity values were recorded for fraction no. 2 (19%) followed by the crude extract (28%) at 50µg/mL. *Aspergillus flavus*, *Aspergillus niger* and *Penicillium griseofulvum* exhibited highest relative density percentages (28.4, 30.8 and 24.3%) on their respective isolation seeds; wheat, broad bean and kidney bean. Interestingly, fraction no. 2 caused 100% inhibition of these isolated fungal species at 100µg/mL. The minimum fungicidal concentration (MFC) was estimated to be 50µg/mL against *A. flavus* and 40µg/mL for *A. niger* and *P. griseofulvum*. Generally, subjecting each of the tested isolates to fraction no. 2 led to damage in their DNA as shown by the comet assay. Gas chromatography- mass spectroscopy (GC-MS) profiling of fraction no. 2 showed 12 major compounds with diverse possible biological activities being antiinflammatory, insecticide, anticancer, antineoplastic antifungal, and antimicrobial.

Keywords: *Alternaria alternata* NMK1, Antifungal activity, Comet assay, Secondary metabolites, Seed-borne fungi.

Introduction

Fungal contamination of various types of stored products such as cereals, legumes, fruits, vegetables, spices, etc. is considered a serious problem in countries where the climatic conditions as well as the agricultural and storage practices, are favorable for fungal growth (Aziz & Moussa, 2004). These seed-borne fungi that are growing on stored seeds are considered of crucial significance in the deterioration of seed quality. To consume fungal-contaminated seeds would be a major health hazard in such countries. The genera *Aspergillus*, *Fusarium* or *Penicillium* can be commonly detected (Sweeney & Dobson, 1998; Marin et al., 2013) on stored seeds. Species belonging to *Aspergillus* and *Penicillium* genera are the most dominant. They include *A. flavus*, *A. ochraceus*, *A. parasiticus*, *A. versicolor*, *P. verrucosum*, *P. chrysogenum*, *P. griseofulvum*, *P. cyclopium* and *P. citrinum* (Aziz & Mahrous, 2004). These fungi produce mycotoxins which can be nephrotoxic, teratogenic, mutagenic, immunosuppressive and carcinogenic to a variety of organisms including humans (Groopman et al., 1988; Massey et al., 1995). Furthermore, they are responsible for feed refusal (Alshannaq & Yu, 2017).

A number of strategies, involving chemical and physical methods, have been proposed for the prevention of fungal contamination of

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Received 1/1/ 2020; Accepted 22/3/ 2020
DOI: 10.21608/ejbo.2020.21766.1425
Edited by: Prof. Dr. Ahmed Ismaiel, Faculty of Science, Zagazig University, Zagazig, Egypt.
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susceptible crops (Cleveland & Bhatnagar, 1992). However, the impact of applying such methods was serious, leading to environmental hazards as well as health problems. This is owed to the existing toxic residues which are causal agents of carcinogenesis, imbalance of hormones, beside spermatotoxicity (Pandey, 2003; Kumar et al., 2007). Accordingly, the demand to present more ecofriendly methods to control fungal infection has recently arisen (Tolouee et al., 2010).

Alternaria is a worldwide distributed genus. *Alternaria* fungi can be saprophytes, weak facultative parasites, plant pathogens and endophytes (Thomma, 2003). Metabolites produced by species belonging to genus *Alternaria* are categorized into several groups including terpenoids, nitrogen-containing compounds, steroids, pyranones (pyrones), phenolics, quinones, etc. Some metabolites can be unique to one species of *Alternaria*; however more than one *Alternaria* species can produce most of these metabolites (Bottini & Gilchrist, 1981). Metabolites from *Alternaria* show biological activities in different areas. They could be cytotoxic, phytotoxic, and antimicrobial agents. Some of the metabolites produced by species of *Alternaria*, can be denoted as mycotoxins and phytotoxins when they are toxic to animals and plants, respectively (Strange, 2007; Mobius & Hertweck, 2009; Duke & Dayan, 2011). This has led to considerable interest within the communities of pharmacologists, plant pathologists as well as chemists which enhanced them to include these metabolites in their research programs involving application studies (Brase et al., 2009; Tsuge et al., 2013).

In view of the aforementioned, the present investigation was aimed to assess *Alternaria alternata* extracts as potential antifungal agents against some fungi isolated from some stored seeds. In addition, the safety of the tested extracts against the normal cell line human amnion (WISH) was assessed.

Materials and Methods

Isolation and identification of fungal species

A **s**oil sample was obtained from an agricultural soil inside Cairo University (Giza, Egypt). Local markets in Egypt were sources to purchase seeds of wheat (*Triticum vulgare*), kidney bean *(Phaseolus vulgaris*), and broad bean *(Vicia faba*). Seeds were disinfected by 2.5% sodium hypochlorite for one minute, rinsed three times in 10mL sterile distilled water, dried and homogenized with 90mL sterilized distilled water for two minutes. For fungal isolation, serial dilutions (ten-fold) were set from the soil sample or seeds suspensions. Samples were each plated on Petri dishes containing Dox′s agar medium supplemented with streptomycin (30µg/mL) and incubated at 30°C for 7 days. Colonies which resulted were isolated then identified by the help of Raper & Fennell (1965), Moubasher (1993) and Samson et al. (2010). The developed fungal colonies from seeds were counted. The obtained fungal cultures were maintained on PDA medium.

Identification, on molecular basis, of the selected soil isolate was performed by sequencing the nuclear ribosomal DNA internal transcribed spacer (ITS). Genomic DNA was collected *via* protocol of purification kit of GeneJet Plant genomic DNA. The region of 5.8S ribosomal RNA of ITS, was amplified with the use of ITS1 (5´TCCGTAGGTGAACCTTGCGG 3´) and ITS4 (5/TCCTCCGCTTATTGATATGC 3/) primers. Sequencing was conducted at GATC Company (Germany) for the PCR amplified product. The sequence was subjected to BLAST algorithm analysis of the NCBI database to acquire closely related phylogenetic sequences. It was then submitted to the GenBank of NCBI. A strain identifier and an accession number were given.

Production of secondary metabolites by Alternaria alternata

A two-step culture was performed for production of secondary metabolites from *Alternaria alternata* (isolated from soil sample). A sterilized solution of NaCl (0.9%, w/v) and Tween-80 (1%, v/v) was used to prepare spore suspension from 10-day-old PDA slants of *A. alternata*. Cultivation was carried out in two steps; first, 1-mL spore suspension (2×10^6 spores/mL) was used to inoculate 100mL modified Dox's medium (supplemented with 5g/L yeast extract) in 250mL-Erlenmeyer flasks. Flasks were incubated at 28°C for 24hrs under shaking (180rpm). For the second step of cultivation, the developed culture was used as 10% inoculum for fermentation medium (250mL modified Dox's medium in 500mL-Erlenmeyer flasks). Incubation was at 28°C for 10 days under shaking (180rpm). The produced culture broth was filtered using cheese cloth to obtain mycelia and filtrate. The latter was utilized in the following experiment.

Preparation of Alternaria alternata extracts

Fungal culture filtrate (15L) was three times extracted with ethyl acetate. The collected solvent extract was evaporated (40°C) under vacuum till dryness using rotary evaporator and then weighed. This is the crude extract.

Separation of active metabolites from fungal extract

Fungal crude extract was purified by preparative paper chromatography Whatman 3MM and the solvent system was acetic acid: water (15:85, v/v). The paper chromatography detection was carried out by viewing at 366nm UV light. Four bands were cut and methanol was used to elute them (Harborne, 1984).

Antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was carried out according to Sharma & Bhat (2009). A solution containing DPPH radical in 0.004% methanol was prepared. It was kept in dark conditions till use. Methanol was used to prepare different concentrations of extract. Twenty µL of each concentration was added to 180µL DPPH solution in a 96-well plate. Reaction mixtures were vortexed then incubated for half an hour at the room temperature. Absorbance was then read at 520nm. Methanol was the blank while the DPPH solution without extract was the –ve control. α-tocopherol was employed as +ve control. The scavenging activity was calculated according to the formula: Scavenging activity (%)= $(A_1 – A_0/A_0) \times 100$, where A_0 is absorbance of –ve control and A_1 is absorbance of used treatment.

Cytotoxic activity

Cell culture preparation for viability test

The effects of different samples on viability of the human normal cell line, human amnion (WISH) were assessed (Moldeus et al., 1978). Dulbecco's modified Eagle's medium (DMEM) with 4.5g/L glucose, and with supplements of 10% (v/v) inactivated fetal calf serum, 1% non-essential amino acids, 1% glutamine, 100U/mL penicillin, and 10mg/mL streptomycin at pH value of 7.4, was utilized to grow cells. Cells are incubated in a humidified atmosphere of O_2 (95%) and CO_2 (5%) at 37°C. At 70–80% confluence, the cells were trypsinized, then centrifuged (250 × g for 5min at 48°C), re-suspended in fresh medium and transferred to micro titer wells (2×10^4 cells/well). After that, they were incubated in a serum-free medium which contained the sample after attachment.

Methylthiazolyl tetrazolium (MTT) assay

Measuring viability of cells is dependable on conversion of MTT yellow color to the purple color of formazan salt *via* succinate dehydrogenase of the mitochondria (De Las Heras et al., 2001). Cells (2×10^4 cells/well) were incubated with sample for 24h at 37°C, control wells contained no treatment. A phosphate buffered saline solution was used to wash cells. They were kept for 4hrs in a serum-free medium containing MTT (0.5mg/mL, 100mL). Reading absorbance was at 570nm. Percentage of resulting cytotoxicity was calculated as percentage of control.

Antifungal assays

Spore suspensions of the postharvest fungi *Aspergillus flavus, A. niger* and *Penicillium griseofulvum* were prepared as previously mentioned. The spore germination assay was performed. Fifty µL of spore suspension were transferred to each well of a microtiter plate containing 100µL liquid Dox's medium with each tested fraction to yield a final concentration of 100µg/mL. DMSO (1%) and Itraconazole replaced tested fractions and were used as negative and positive controls, respectively. The plate was kept in an incubator at 30°C for 16 hrs. All tests were conducted in three replicates. Spores germination was considered when the germ tube length was at least twice that of the spore (Griffin, 1994). A hemocytometer was used for counting germinated spores. For detecting spore germination, about 100 spores were observed per replicate. The percentage of inhibition of the germination of spores was counted as follows:

Inhibition (%)= [(negative control – treatment)/ negative control] × 100.

Different concentrations of 10-100 and 50-150µg/mL of fraction no. 2 and itraconazole, respectively, were assayed in order to determine its minimum fungicidal concentration (MFC) against each tested fungal species.

Alkaline single-cell gel electrophoresis (SCGE) or alkaline comet assay was performed to elucidate the action of fraction no. 2 extracted from *A. alternata* strain NMK1 on DNA of the isolated postharvest fungal spores. The method followed was adopted from Miloshev et al. (2002)

and Nemavarkar et al. (2004). On slides which are clean, dry and frosted, agarose (0.5%) was spread evenly. Slides were dried in air. Spores of *Aspergillus flavus, A. niger* and *Penicillium griseofulvum* or those treated with fraction no. 2 were washed in sterilized deionized water and resuspended in a suspending buffer (1M sorbitol, 25mM KH_2PO_4, pH 6.5). Approximately, 5 × 10^6 spores were mixed with low melting agarose (0.7%) which contained 2mg/mL of diluted Macerozyme R-10 (3mL/100mL buffer). Cells were evenly spread on a slide coated with agarose then covered with cover slips. They were incubated at 30°C for 30min so that the spore cell walls would be digested to obtain the spheroplasts. The following procedures were accomplished in cold conditions (10°C) for minimizing endogenous enzymes activity. Covers were removed and then slides were immersed in EDTA (30 mM, pH 12.4) for the purpose of unwinding the DNA. Gel-slides were then electrophoresed in the same EDTA buffer at 30mA, 0.7V/ cm. After that, gels-slides were submerged in Tris HCl (10mM, pH 7.4) for 2-3 min to be neutralized. This was consecutively followed by incubation in 76% then 96% ethanol. Slides were air-dried then stained in 2mg/mL ethidium bromide. A fluorescence microscope (Carl Zeiss) at 400 x, with an exciting filter of 515-560nm and a barrier filter of 590nm, was used to obtain images and quantify strand breaks of the DNA in the samples tested. Data are presented as tail length (px), percentage of DNA in the tail and the tail moment. Standard errors were determined on 50 comets basis for each treatment. The tail moment was evaluated as follows:

Tail moment= (Tail length × % DNA in Tail)/ 100.

GC-MS analysis

Fraction no. 2 obtained from crude fungal extract of *Alternaria alternata* strain NMK1 gave highest antifungal activities and exhibited good antioxidant activities. Therefore, it was considered to identify its active principles through GC-MS analysis. A Thermoquest-Finnigan Trace GC-MS column (60m\ 0.25mm i.d. and 0.25μm film thickness) supplied with a DB-5 (5% (w/v) phenyl) methylpolysiloxane was used. Temperature of injection was 220ºC while temperature of oven was elevated from 40ºC (3 min hold) to 250ºC at a 5ºC/min rate. It was held for two minutes at 250ºC; the temperature of transfer line was 250ºC. One μL of the sample was used in injection and at 1.0mL/min flow rate with helium as the carrier gas. The scanning rage of mass spectrometer was from 40 to 500m/z with 70eV ionizing voltage. Compounds identification was on the basis of the standard mass library of the National Institute of Standards and Technology (NIST Version 2.0).

Statistical analysis

Data were presented as mean. The standard error (SE) was determined. The least significant difference (LSD) was calculated using One-Way ANOVA: Post Hoc Multiple comparisons at $p<$ 0.01. Statistics were carried out using SPSS 20.0 software.

Results and Discussion

Molecular identification of Alternaria alternata

Alternaria alternata was isolated from an agricultural soil. It was purified and morphologically identified. This identification was further confirmed by ITS sequencing. The new 454 and the traditional Sanger technologies were combined in order to sequence the PCR product. The obtained sequence of the nucleotide was deposited in the GenBank of the NCBI. The isolate was given a strain identifier to be *Alternaria alternata* strain NMK1 with accession number: MN645469.

GC-MS analysis of fraction no. 2

The acetic acid extract of *Alternaria alternata* strain NMK1 was fractionated using paper chromatography. Four fractions were obtained. All fractions were tested for their antioxidant, cytotoxic and antifungal activities.

Twelve compounds were identified in fraction no. 2 of *Alternaria alternata* strain NMK1 by GC-MS analysis. The compounds with their respective retention time (RT), relative concentration and previously reported activities are given in Table 1. The unknown compounds spectrum of this fraction was compared with spectra of the stored known compounds in NIST library. Interestingly, various applicable activities can be noticed. The compounds 2,4(1H,3H) quinolinedione (2.65%), 5-chloro-2-(4chlorophenyl) benzoxazole (3.15 %) and cyclopropanecarboxylic acid (4.22%) were previously tested (Loh et al., 1980; Lalouti et al., 2014; Boersen et al., 2015) and proved their antiinflammatory activity. Another compound, Propane, 1,2-dichloro- (1.89%), was shown to be an insecticide (IARC, 1986). Other biological activities for the other compounds in the current fraction will be discussed in the following sections.

TABLE 1. List of components and their biological activity of fraction no. 2 obtained from *Alternaria alternata* strain NMK1 through GC-MS analysis.

RT	Compound	Relative concentration	Reported activities
10.782	2,4(1H,3H) Quinolinedione	2.65	Antiinflammatory (Lalouti et al., 2014)
11.989	Propane, 1,2-dichloro	1.89	Insecticide (IARC, 1986).
12.172	2Pyridinecarboxaldehyde, N-oxide	4.39	Anticancer activity (Shiba et al., 1983)
12.561	Tricyclo[4.2.1.1(2,5)]dec-3-ene-9,10-dione	6.15	Antifungal activity (Hameed et al., 2016; Ilkay et al., 1999)
12.641	Clomesone	3.30	Antineoplastic (Shealy & Krauth, 1993)
12.733	1H-Benzotriazole, 4-nitro	4.13	Antimicrobial (Singh et al., 2017)
12.767	Butenedinitrile	2.11	Antimicrobial (Sheikhshoaie et al., 2018)
12.899	Dioxo-4-azatricyclo	2.62	Antimicrobial, anticancer (Struga et al., 2008)
12.933	6-Methoxybenzofuroxan	2.0	Antibacterial activity (Šarlauskas et al., 2014)
13.225	5-Chloro-2-(4chlorophenyl) benzoxazole	3.15	Antimicrobial, Antiinflammatory (Loh et al., 1980)
14.650	2,4,6-Cycloheptatrien-1-one oxime	2.89	Antimicrobial (Saniewski et al., 2014)
15.165	Cyclopropanecarboxylic acid	4.22	Antiinflammatory (Boersen et al., 2015)

Antioxidant activity of extracted fractions

To test the antioxidant activity of extracted fractions of *Alternaria alternata* strain NMK1, the DPPH scavenging assay was performed (Fig. 1). Fraction no. 2 showed the highest antioxidant activity (71 and 64% at 200 and 150μg/mL, respectively) followed by the crude extract (60 %) at the concentration 200μg/mL when compared with α-tocopherol as a standard antioxidant (89% at 200μg/mL). The good activity exhibited by fraction no. 2 may be attributed to that in the GC-MS profiling of this fraction (Table 1), the benzoxazole derivative, 5-chloro-2-(4chlorophenyl)benzoxazole, was encountered (3.15%). In this relation, Kashid et al. (2019) synthesized new benzoxazole derivatives which were evaluated for their antioxidant activity. They achieved good activities when compared to the used standards.

Cytotoxic activity of extracted fractions

The safety of using the extracted fractions was taken into consideration. Accordingly, the cytotoxicity assay was conducted against the normal human cell line, human amnion (WISH). Generally, raising the concentration of each of the test treatments caused an increase in cytotoxicity of normal cells (Fig. 2). Fraction no. 2 was the least cytotoxic (19-51%) followed by the crude extract (28-58%) at 50-200μg/mL, while highest cytotoxicity effects were recorded for fraction no. 4 (69-84%) then fraction no. 3 (63-77%) at concentrations from 50 to 200μg/mL. The promising fraction no. 2 could be considered of low cytotoxicity to the tested human normal cell line at the concentration 50μg/mL exhibiting 81 % cell survival. On the other hand, among the compounds encountered in the promising fraction no. 2 (Table 1) is clomesone (3.30%) which was previously reported to be antineoplastic (Shealy & Krauth, 1993). In addition, 2-pyridinecarboxaldehyde, N-oxide (4.39%) and -dioxo-4-azatricyclo- (2.62%) were shown to have anticancer activities (Shiba et al., 1983 and Struga et al., 2008, respectively).

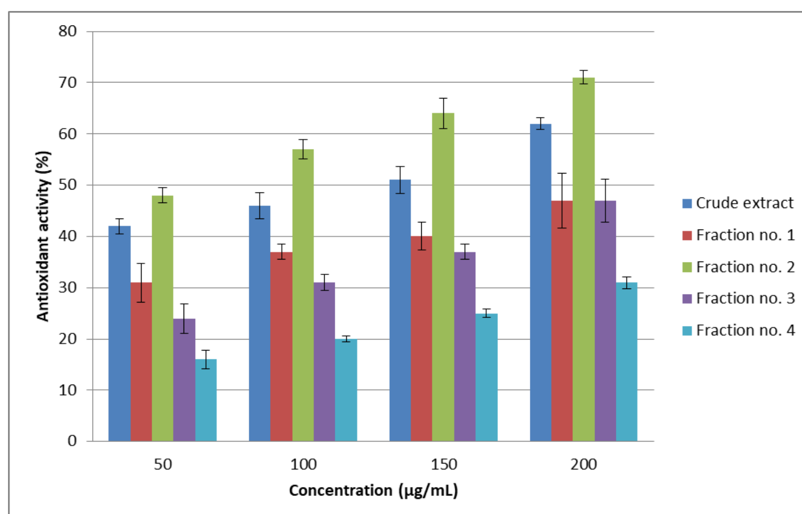

Fig. 1. Antioxidant activity of different fractions from *Alternaria alternata* strain NMK1 at different concentration levels [Bars show means. Error bars show mean ± SE.].

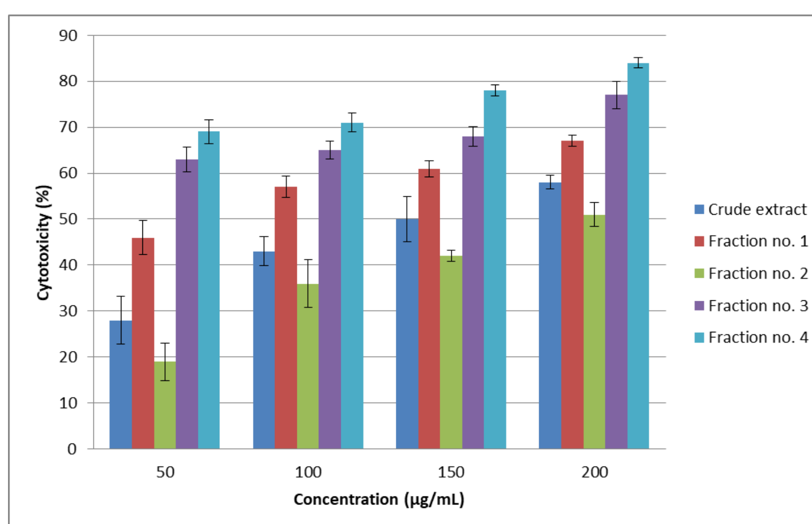

Fig. 2. Cytotoxic activity of different fractions from *Alternaria alternata* strain NMK1 at different concentration levels [Bars show means. Error bars show mean ± SE].

Isolation of seed-borne fungi

Fungal occurrence on different seeds is shown in Table 2. All seeds samples studied in this work were contaminated with molds. It was reported by Domsch et al. (1980) that fungal contamination of food materials is due to dust natural contamination followed by the storage in humid conditions. In each of the studied cases in the current work, genus *Aspergillus* was the most dominant achieving 61.6, 64.1 and 48.7% of the total count for seeds of wheat, broad bean and kidney bean, respectively. It was followed by genus *Penicillium* with relative densities of 29.2, 28.3 and 39.7% on the respective seeds. Concerning wheat grains, a total of 12.4 × 10^3 colonies/g of grain were isolated. *Aspergillus flavus* was the highest in count achieving a relative density of 28.4 % and was followed by *A. niger* (R.D. 24.3 %). With regard to broad been, the total fungal count was 8.1 × 10^3 colonies/g of seed. *Aspergillus niger* was of highest density (30.8%). *A. flavus* was next with 27.1% R.D. For kidney bean, a total count of 7.8 × 10^3 colonies/g was obtained. *Penicillium griseofulvum* accounted for the highest fungal density (24.3%) while *Aspergillus flavus* came next (21.8%). In this context, Aziz et al. (2006) isolated fungi from different seeds e.g. soybean, kidney bean, yellow corn, wheat, and barley. They noticed that the most frequently encountered fungal genera were *Aspergillus* and *Penicillium*. Also, Youssef & Mohamed (2020) found that *Penicillium chrysogenum*, *Aspergillus niger* and *Aspergillus flavus* were the markedly detected fungal strains isolated from stored seeds of rice, popcorn and bean.

TABLE 2. Occurrence of fungal species on stored seeds.

Fungal species	Wheat		Broad bean		Kidney bean	
	T. C./g (× 10^3)	R.D. (%)	T. C./g (× 10^3)	R.D. (%)	T. C./g (× 10^3)	R.D. (%)
Aspergillus flavus	3.5	28.4	2.2	27.1	1.7	21.8
A. niger	3.0	24.3	2.5	30.8	1.5	19.2
A. ochraceus	1.1	8.9	0.5	6.2	0.6	7.7
Cladosporium herbarum	0.2	1.6	---	---	0.1	1.3
Fusarium moniliforme	0.4	3.2	0.1	1.2	0.4	5.1
F. oxysporum	0.1	0.8	0.5	6.2	0.3	3.8
Penicillium citrinum	1.5	12.2	1.0	12.3	1.2	15.4
P. griseofulvum	2.1	17.0	1.3	16.0	1.9	24.3
Rhizopus stolonifer	0.5	4.1	---	---	0.1	1.3
Total	12.4 × 10^3		8.1 × 10^3		7.8 × 10^3	

T. C./g: Total count / gram of grain. R.D.: Relative density as percentage of total count.

From the results of the previous experiment, the most dominant fungal species isolated from wheat, broad bean and kidney bean were *Aspergillus flavus*, *A. niger* and *Penicillium griseofulvum*, respectively. Accordingly, they were chosen for further tests in this study.

Antifungal assays

The antifungal activity of the fractions extracted from *Alternaria alternata* strain NMK1 is shown in Table 3. Different degrees of growth (spore germination) inhibition of test fungi were noticed for the isolated fractions. *Aspergillus flavus* was apparently the most resistant followed by *A. niger* then *Penicilliun griseofulvum*. Obviously, fraction no. 2 was the most potent fungal inhibitor causing 100 % inhibition of germination of spores of the tested fungi. The results obtained for values of MFC for fraction no. 2 were very promising (Table 4). The values were estimated to be 50, 40 and 40 as compared to 120, 110 and 110µg/mL by itraconazole against *Aspergillus flavus*, *A. niger* and *Penicillium griseofulvum*, respectively. It is worthy to elucidate that fraction no. 2 exhibited low cytotoxicity against the tested WISH cell line at the concentration 50µg/mL (81% cell survival). On the other hand, it can be deduced that one or more of the active compounds found in fraction no. 2 could be responsible for mediating the inhibitory action. The results of GC-MS analysis of the promising fraction no. 2 (Table 1) show the presence of the compound tricyclo[4.2.1.1(2,5)]dec-3-ene-9,10-dione (6.15%), which previously revealed antifungal activities (Ilkay et al., 1999; Hameed et al., 2016;). Furthermore, the compounds 1H-benzotriazole, 4-nitro- (4.13%), -butenedinitrile (2.11%), -dioxo-4-azatricyclo- (2.62%), 5-chloro-2-(4chlorophenyl) benzoxazole (3.15%) and 2,4,6-cycloheptatrien-1-one oxime (2.89 %) proved their antimicrobial activities by Singh et al. (2017), Sheikhshoaie et al. (2018), Struga et al. (2008), Loh et al. (1980) and Saniewski et al. (2014), respectively. While the compound 6--methoxybenzofuroxan (2.0%), was reported by Sarlauskas et al. (2014) to have antibacterial activity.

The mechanism of antifungal activity could be attributed to a change that occurred in cell membrane permeability which is ascribed to interaction between active secondary metabolites in these fractions and electronegative charges of cell membrane of tested fungi. This would lead to the leakage of proteinaceous constituents as well as intracellular electrolytes (Khalil et al., 2014). It was mentioned that in the mechanisms of antifungal activity, polyene compounds attach to ergosterol in cell membrane forming large pores which disrupt functions of the cell while azole compounds inhibit biosynthesis of ergosterol (Cowen et al., 2014). Ouf et al. (2018) synthesized novel azole derivatives and tested their antifungal activities. The MFC for the novel compounds was 4µg/mL in case of *Candida albicans* while for *Microsporum gypseum* and *Trichophyton mentagrophytes*, the MFC ranged from 8 to 32µg/mL. The tested compounds revealed their antifungal properties regarding ergosterol biosynthesis. Also, the alkaloid Quinazoline was isolated from *Aspergillus nomius* (Ali et al., 2017). The compound was inhibitory to *Bacillus subtilis*, *Staphylococcus aureus*, *Alcaligenes faecalis*, *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Candida albicans* at 31.25, 62.5, 62.5, 62.5, 250, 31.25 and 125µg/mL, respectively.

TABLE 3. Antifungal activity of different fractions (100 µg/mL) extracted from *Alternaria alternata* strain NMK1.

Fraction no.	Test organism (% inhibition)			
	Aspergillus flavus	***Aspergillus niger***	***Penicillium griseofulvum***	**LSD**
-ve control	^{f} 0 ± 0	0 ± 0	0 ± 0	0.00
+ve control	^{b} 89 ± 1.77 ^{b}	^{b} 92 ± 3.01 ^{ab}	^{a} 98 ± 1.53 ^{a}	7.06
Crude extract	^{c} 75 ± 1.43 ^{c}	^{c} 83 ± 2.32 ^{b}	^{b} 90 ± 2.11 ^{a}	6.54
1	^{b} 90 ± 1.94 ^{b}	^{b} 92 ± 1.05 ^{b}	^{a} 97 ± 1.22 ^{a}	4.71
2	^{a} 100 ± 0	^{a} 100 ± 0	^{a} 100 ± 0	0.00
3	^{d} 68 ± 1.39 ^{c}	^{d} 77 ± 2.18 ^{b}	^{c} 86 ± 1.14 ^{a}	5.27
4	^{e} 51 ± 1.68 ^{a}	^{e} 54 ± 1.99 ^{a}	^{d} 43 ± 1.73 ^{b}	6.04
LSD	3.33	4.30	3.14	

Data presented are mean ± SE. -ve control: 1% DMSO. +ve control: itraconazole. The Least Significant Difference (LSD) was calculated at 99% confidence interval (probability of error p= 0.01). Means followed by the same letters are statistically non-significant. Right and left superscript letters are for LSD in column and row, respectively.

TABLE 4. MIC values (µg/mL) of fraction no. 2 extracted from *Alternaria alternata* strain NMK1.

	Aspergillus flavus	*Aspergillus niger*	*Penicillium griseofulvum*
+ve control	120	110	110
Fraction no. 2	50	40	40

+ve control: itraconazole.

Another presumed mechanism for the antifungal activity is an interaction between secondary metabolites and fungal DNA which would cause inhibition of mRNA and consequently protein synthesis (Khalil et al., 2014). To study the possible effect of constituents of fraction no. 2 on DNA of tested fungi, the comet assay was performed. The comet assay is considered a sensitive, direct and inexpensive method for studies in genetic toxicology (Azqueta & Collins, 2013). Figure 3 shows a micrograph which is representative of the undamaged and the damaged DNA in the groups tested. In Fig. 3A, the undamaged DNA seems clear-cut and nearly spherical which was still contained inside cells. On the other hand, damaged DNA has a characteristic appearance of comets (Fig. 3B). Various degrees of DNA damage are represented in Table 5. In general, subjecting spore cells of each of tested fungi to fraction no. 2 led to an increase in the monitored DNA damage parameters. The parameters used were tail length, % of DNA in tail, and tail moment. Tail length is only used at low DNA damage levels, since as soon as the tail is formed it does not change (Collins, 2004). Eventually, as the damage increases, the intensity of tail is enhanced. The DNA percentage in comet tail is considered as another useful parameter, since it is in linear relation with frequency of breaking. On the other hand, the tail moment combines the tail length with the tail intensity in one value. Therefore, tail moment is the most convenient parameter to use (Lu et al., 2017). Accordingly, in the present study, the tail moment was calculated to be used to compare between tested fungi (Table 5). It seems that cells of *Aspergillus flavus* were highest in resistance with least percentage of change in tail moment value (418.2%) and it was followed by *Aspergillus niger* (425.8%) then *Penicillium griseofulvum* (427.1%) which happens to be in accordance with findings from the preceding experiment of antifungal activity measured *via* spore germination. It was revealed through the analysis conducted by Shishodia et al. (2019) that in *Aspergillus* species, the enzymes produced from cell wall remodeling and energy metabolism or in response to oxidative stress are the factors accountable for allowing resistance against the antifungal drugs.

Conclusion

From the foregoing discussed results, it can be concluded that valuable secondary metabolites could be extracted from the soil fungus *Alternaria alternata* strain NMK1. The extracted fraction no. 2 showed a good antioxidant activity. Furthermore, it demonstrated potent antifungal activities at low cytotoxic levels against some seed-borne fungi which could make it of high applicable importance. Using this fraction as a natural preservative for stored seeds can be recommended, however, further *in vivo* tests using laboratory animals should be considered.

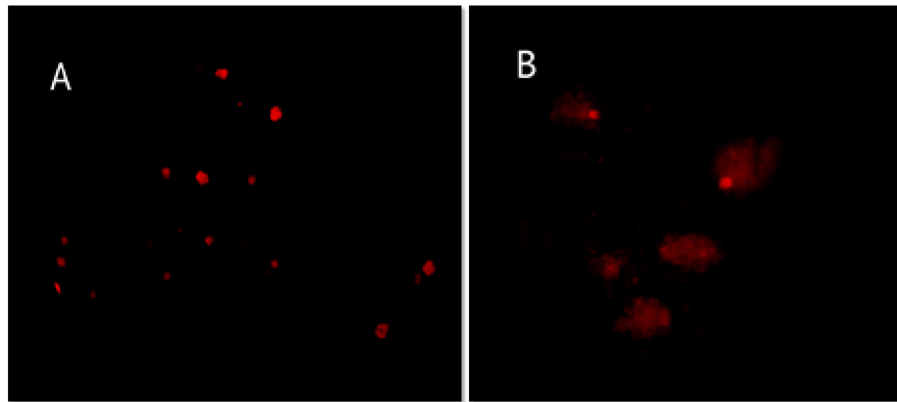

Fig. 3. Representative micrograph for undamaged (A) and damaged (B) DNA in different groups.

TABLE 5. DNA damage parameters of comet assay of fungal spore cells exposed to fraction no. 2 from *Alternaria alternata* strain NMK1.

Group		Tail length (px)	% DNA in tail	Tail moment
Aspergillus flavus	C	[c] 2.60 ± 0.46	[d] 20.45 ± 2.53	[c] 0.55 ± 0.08
	T	[b] 8.67 ± 1.23	[b] 31.38 ± 2.55	[b] 2.85 ± 0.48
	% change	**233.4**	**53.4**	**418.2**
Aspergillus niger	C	[c] 3.33 ± 0.50	[cd] 21.94 ± 2.41	[c] 0.66 ± 0.08
	T	[ab] 10.20 ± 1.27	[ab] 32.89 ± 2.77	[b] 3.47 ± 0.52
	% change	**266.3**	**49.9**	**425.8**
Penicillium griseofulvum	C	[c] 3.56 ± 0.47	[c] 26.56 ± 2.36	[c] 0.81 ± 0.07
	T	[a] 12.03 ± 1.38	[a] 37.50 ± 2.57	[a] 4.27 ± 0.55
	% change	**237.9**	**41.1**	**427.1**
LSD		2.21	5.56	0.65

Data presented are mean ± SE. 1 px = 0.24µm. % change = [(T – C) / C] × 100. The Least Significant Difference (LSD) was calculated at 99% confidence interval (probability of error p= 0.01). Means followed by the same letters are statistically non-significant.

Conflict of Interest

The authors declare no conflicts of interest.

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فعالية المركبات الثانوية لفطرة *الترناريا الترناتا* NMK1 ضد بعض الفطريات المحمولة على البذور

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تم عزل فطرة *الترناريا الترناتا* NMK1 من التربة وتعريفها على المستوى الجزيئى و اعطائها رقم انضمام MN645469 فى بنك الجينات. تم الحصول على أربعة اجزاء من مستخلص حمض الخليك للراشح الفطرى. اظهر الجزء رقم 2 أعلى نشاط مضاد للأكسدة بنسبة 71 و 64% عند تركيزات 200 و 150 ميكروجرام/مل يليه المستخلص الخام (60% عند تركيز 200 ميكروجرام/ مل). تم اختبار مدى سلامة استخدام هذه الاجزاء ضد خلايا WISH الطبيعية. اظهر الجزء رقم 2 اقل نسبة سمية (19%) يليه المستخلص الخام (28%) عند تركيز50 ميكروجرام/مل. اظهرت فطريات *اسبرجللس فلافس*، *اسبرجللس نيجر* و *بينيسيلليوم جريزيوفولفم* اعلى كثافة نسبية على بذور القمح و الفول و الفاصوليا (28.4، 30.8، 24.3%). حقق الجزء رقم 2 تثبيطا بنسبة 100% لنمو هذه الفطريات عند تركيز 100 ميكروجرام/مل. و كان الحد الادنى للتركيز المثبط هو 50 ميكروجرام/مل لفطرة *اسبرجللس فلافس* و 40 ميكروجرام/مل لفطرتى *اسبرجللس نيجر* و*بينيسيلليوم جريزيوفولفم*. و قد تم اثبات الضرر من خلال فحص المذنب للحمض النووى. اظهرت صورة ال GC-MS وجود اثنى عشر مركبا و لهم تطبيقات بيولوجية واسعة منها التضاد للالتهاب، مبيد للحشرات، تضاد للسرطان، تضاد للأورام، تضاد للفطريات و تضاد للميكروبات.