Ministry of Higher Education and Scientific Research
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Investigation of The Biological Activities of *Alternaria alternata* Extracts

Graduation Research Submitted to the Department of Biotechnology as Part of Requirements for the Degree of Bachelor in Biotechnology Sciences

By

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بِسمِ اللهِ الرَّحمن الرَّحيم

قَالَتْ يَا أَيُّهَا الْمَلَأُ إِنِّي أُلْقِيَ إِلَيَّ كِتَابٌ كَرِيمٌ ﴿٢٩﴾ إِنَّهُ مِنْ سُلَيْمَانَ وَإِنَّهُ بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ ﴿٣٠﴾

صدق الله العظيم

سورة النمل / الآية: ﴿٢٩-٣٠﴾

Dedication

We dedicate the fruit of our effort to ...

Our god and our prophet Mohammed

Our dear mothers and fathers

Our loving sisters and brothers

And

The spirits of the martyrs in our aggrieved country

Tabarak

Dawood

Zahraa

Mohammed

Yousif

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Researchers

إقرار المشرف وترشيح رئاسة قسم التقانة الأحيائية

اشهد بأن إعداد هذا البحث الموسوم بـ:

Investigation of The Biological Activities of *Alternaria alternata* Extracts

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نشهد باننا اعضاء لجنة المناقشة، اطلعنا على هذا البحث الموسوم بـ

Investigation of The Biological Activates of *Alternaria alternata* Extracts

الذي قدمه الطلبة (تبارك و داود و زهراء ومحمد ويوسف) في محتوياته وفيما لها علاقة به، ونعتقد بانهم جديرون بالقبول لنيل درجة البكالوريوس في علوم التقانة الاحيائية بتقدير ( ).

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مصادقة رئاسة قسم التقانة الاحيائية

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Abstract

The current study included investigating the vital activities of the fungus extract of *Alternaria alternata*, in which the characteristics of the fungus, its environment and the ideal conditions for its growth were identified as well as "the phenotypic and taxonomic characteristics of it. It was also investigated for its cytotoxic activity against pathogenic bacterial cells and cancerous cells, and pathogenic antifungal activity. The study also investigated the immune effects of this fungus through previous studies in this field.

It was reached through this study that this fungus and its extracts have many studies in which the researchers found the positive results of the experiments and the extreme importance of this, which is widely spread in various environments. Therefore, we recommend through this study to future experiments to take advantage of the extracts of this fungus to treat incurable diseases.

Keywords: ***Endophyte fungus, Alternaria alternata,*** **Bioactivity**

Contents

1. Literature Review

1.1: Habitat, Characteristics and Growth of *Alternaria alternata*

Alternaria is a ubiquitous genus containing common saprophytes in soil, air, and plant pathogens, *Alternaria alternata* has been recorded causing leaf spot and other diseases on over 380 host species of plant. It is an opportunistic pathogen on numerous hosts causing leaf spots, rots and blights on many plant parts (Webster and Weber, 2007). It can also cause upper respiratory tract infections and asthma in humans with compromised immunity (Wiest, 1987). This species is a causative agent of subcutaneous phaeohyphomycosis and mycotic keratitis (Ellis *et al.*, 2016).

Alternaria alternata is often found in areas with humid climates, or where there has been significant rainfall, the fungus lives in seeds and seedlings, and is also spread by spores. This disease flourishes in dead plants that have been left in gardens over winter. Additionally, when dead infected debris is added to compost pile it can spread to other vegetables throughout the garden specially tomato and apple plants (figure.1), in addition to spoiling fruits and vegetables, many *Alternaria* species are also capable of producing a wide range of secondary metabolites. Most of these metabolites are (such as Alternariole ALT) that play an important role in the pathogenesis of plants. Others can be considered mycotoxins and are harmful to humans and animals that consume the contaminated vegetable foods. Relatively little is known about the toxicity of *Alternaria* toxins in comparison with mycotoxins produced by other fungi such as *Aspergillus, Penicillium* and *Fusarium* (Patriarca, and Fernández, 2018).

Colonies are fast growing, black to olivaceous-black or greyish, and are suede-like to floccose. Microscopically, branched acropetal chains (blastocatenate) of multicellular conidia (dictyoconidia) are produced sympodially from simple, sometimes branched, short or elongate conidiophores.

Conidia are clavate, pyriform, sometimes ovoid or ellipsoidal, often with a short conical or cylindrical beak, pale brown, smooth-walled or verrucose. Temperature: optimum 25-28^{O}C; maximum 31-32^{O}C (figure.2) (Ellis *et al.*, 2016).

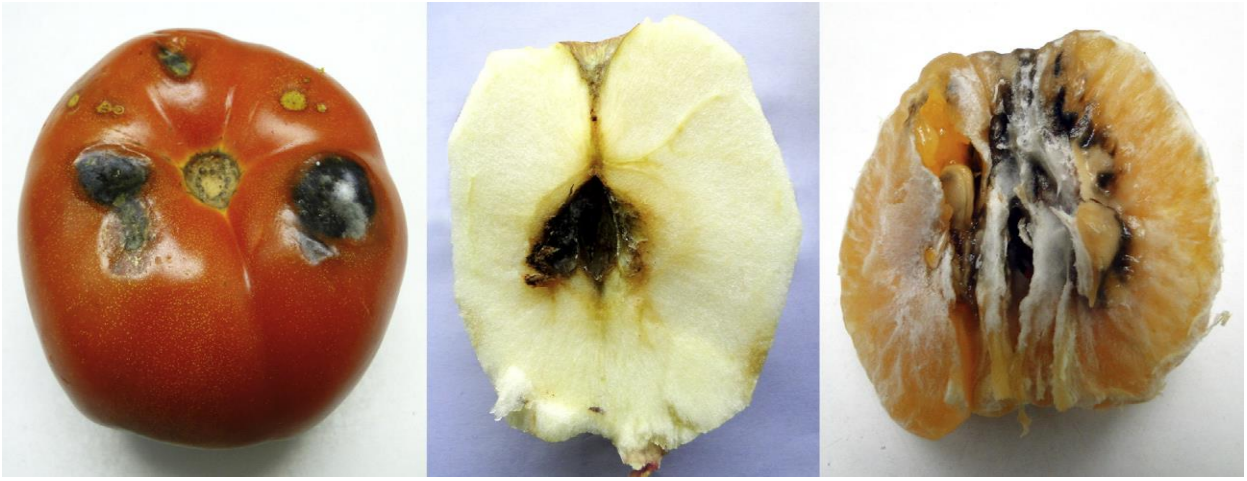

Fig.1: Fruits infected by *Alternaria* (A) Black mould of tomato fruit, (B) moldy core-rot of apple, (C) black heart rot of mandarin (Patriarca, and Fernández, 2018)

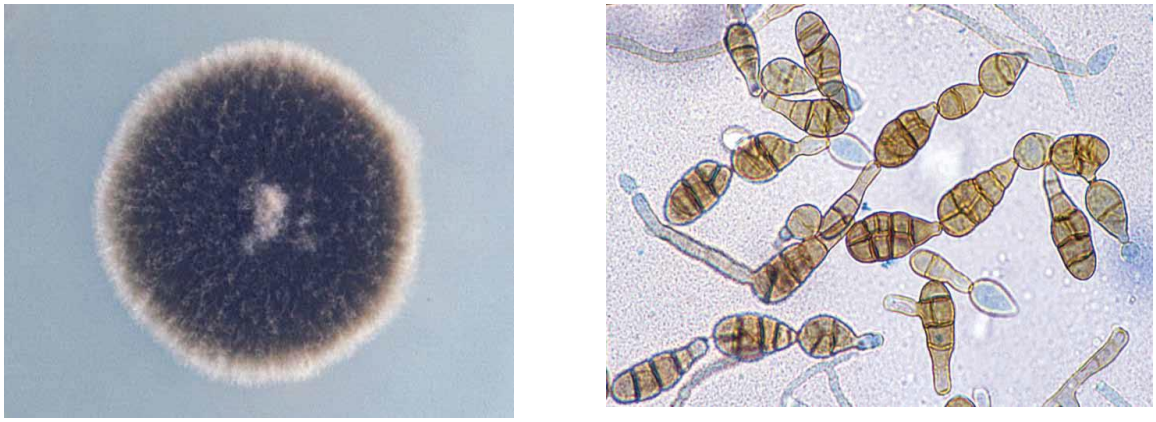

Fig.2: *Alternaria alternata* showing: (A) colonies are black to olivaceous-black or Greyish, and are suede-like to floccose, (B) branched acropetal chains and multicelled, clavate to pyriform conidia with short conical beaks (Pastor and Guarro, 2008).

Classification of *Alternaria alternata* according to Webster and Weber, (2007)

Kingdom: Fungi

Division: Ascomycota

Class: Euascomycota

Order: Pleosporales

Family: Pleosporaceae

Genus: *Alternaria*

Species: *A. alternata*

1.2: Biological Activities of *Alternaria alternata* Extracts

In spite of more investigations on the toxic potential of *Alternaria alternata* mycotoxins for human consumption and hazard effects of its pathogenic isolates, there are many of studies for biological applications of its extracts including:

1:2:1: Antibacterial and Anticancer activity

Studies on Tentoxin (bioactive metabolites of this fungal endophyte *Alternaria alternata*) showed its cytotoxic activity against both lung cancer cell line A549 and breast cancer cell line MDA-MB-231(KI-Bong *et al*., 2002). A crude extract of this fungus isolated from *Coffea arabica* leaves showed high antimicrobial activity with significant inhibition zones (Fernandes *et al*., 2009).

In a study performed by Arivudainambi *et al.*, (2014), they showed that the entophytic microorganism *A. alternata* seems to produce bioactive and probably chemically novel compounds bearing pharmaceutical potential. The ethyl acetate extract of *A. alternata* VN3 had the maximum inhibition zone of 21.4±0.07 mm and 21.5±0.25 mm for drug-resistant bacteria *Staphylococcus aureus* strain 10 and *Pseudomonas aeruginosa* strain 2, respectively. Also, *A.*

alternata VN3 showed moderate anticancer activity against MCF-7 and MDA MB-231 cell lines. At 30 µg/ml, cell viability was decreased to 75.5% and 71.8% for MCF-7 and MDA MB-231 cells, respectively.

Methanolic extract of this fungus showed antibacterial activity against four selected clinical pathogens namely, *Pseudomonas aeruginosa* (3.98±0.41) mm, *Escherichia coli* (4.99±0.68) mm, *Staphylococcus aeureus* (5.00±0.71) mm and the maximum zone formation against *Klebsiella pneumonia* was found (5.04 ± 0.29) mm (Kamal *et al*., 2015). *A. alternata* acts as a good source for the production of silver nanoparticles AgNPS, these nanoparticles showed antibacterial activity against *S. aureus* and *E. coli* at 30µg/ml with zone of inhibition 13 mm against *S. aureus* and 17 mm against E. coli (Majeed, 2017)

Ethyl acetate (EA) fraction of *A. alternata* isolated from *Azadirachta indica* showed antimicrobial activity against Gram positive bacteria, *Bacillus subtilis, Listeria monocytogenes and S. aureus and Gram negative, E. coli and Salmonella typhimurium* at inhibition zones (14 ± 1.5, 14 ±1.5, 12±1.0, 11±1.0 and 12± 1.5*)* mm respectively with MIC values of 300–400 µg/ml against both Gram positive and Gram negative bacteria tested (Chatterjee *et al.*, 2019).

Cytotoxic assay of Tentoxin against HeLa cancer cell line was carried out and results revealed strong cytotoxicity for tentoxin with a CC_{50} of 22.5 µg/ml (Abdou and Dawoud, 2020).

1.2.2: Antifungal Activity

The silver nanoparticles AgNPS nanoparticles of this fungus showed excellent antifungal activity against *Aspergillus niger* and *Candida albicans* by disc diffusion method at 30 µg/ml which showed the 19 mm zone of inhibition for *A. niger* followed by *C. albicans* showed 16 mm, these AgNPs works but some studies suggest that these nanoparticles being a small in size easily enters into the cell and interfere with DNA by the production of reactive oxygen

species (ROS) which damages the cell and hence cause the cell death (Majeed, 2017).

The ethyle acetate fraction *A. alternata* of and bioactive compound, p-Coumaric acid (PC), showed concentration- dependent broad-spectrum anti-microbial activity against yeasts and mycotoxigenic molds with MICs ranging from 7.8 to 250 μg/mL. The *in vitro* production of aflatoxin B1 (AFB1) from *Aspergillus flavus* and fumonisin B1 (FB1) from *Fusarium verticillioides* was completely inhibited by EA and PC at 400 μg/mL. The synthesis of the membrane-bound ergosterol from *A. flavus* and *F. verticillioides* was strongly inhibited by PC at 200 μg/mL (Sudharshana *et al.*, 2019).

The endophyte *A. alternata* extract that isolated from the leaves of the medicinal plant *Bidens bipinnata* was found to exert antifungal activity against several fungal species: *Sporoidiobolus salmonicolor, Kluyveromyces marxianus, C. albicans, A. niger, A. fumigatus, A. terrus* and *Phanerochaete avellanea* with different inhibition zones according to different extraction methods of this fungus (Abdou and Dawoud, 2020).

1.2.3: Physiological and Immunological Effects

A study performed by Zhu *et al.*, (2014), they tested the effect of spores exposure of pathogenic isolate of *Alternaria alternata* on virus-induced airway epithelial immunity and they indicated that *Alternaria* treatment was found to significantly enhance the production of proinflammatory cytokines (e.g., IL-6 and IL-8) induced by virus infection or dsRNA treatment.

A crude extract of this fungus isolated from *Coffea arabica* leaves displayed a cytotoxic activity of approximately 50% towards HeLa cells *in vitro* high antimicrobial activity with significant inhibition zones (Fernandes *et al.*, 2009). Endophytic fungi have also been reported as a good source of antioxidant metabolites, Ethyle acetate extract of this fungus exhibited very strong free radical scavenging activity during 2, 2-Diphenyl-1-picrylhydrazyl

DPPH free radical scavenging assay in the presence of different concentration of Ethyle acetate fraction of this fungus, Inhibition Concentration (IC_{50}) value of ethyle acetate fraction of *A. alternata* was calculated as 38± 1.7 µg/ml from the standard curve of percentages of inhibition (Chatterjee *et al.*, 2019).

1.3: Secondary Metabolites from the Crude Extract of *A. alternata* by Gas Chromatography and Mass Spectrometry

Twenty six bioactive compounds were identified in methanolic extract of *Alternaria alternata*. The identification of bioactive chemical compounds is based on the peak area, retention time molecular weight and molecular formula. GC-MS analysis of *A. alternata* revealed the existence of the α-acetyl-L-serine, 2(5H)-furanone, 6-oxa-bicyclo[3.1.0]hexan-3-one, D-glucose,6-O-α-D-GALACTOPYRANOSYL, DL-arabinose, ε-N-fommyl-L-lysine, 2-[4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), thrietol, 2-O-heptyl, 2-deoxy-2- fluoro-1,6-anhydro-ß-d-glucopyranose, d-ribo-hexos-3-ulose, A-D-glucopyranoside, O-α-D-glucopyranosyl-(1.fwdarw.3)-ß-D-fru, maltose, 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl, desulphosinigrin, uric acid, midazole-4-carboxylic acid, 2-fluoro-1-methoxymethyl-ethyl ester, geranyl isovalerate, 1-nitro-ß-d-arabinofuranose, tetraacetate, glycyl-D-asparagine, α-D-xylofuranose, cyclic 1,2:3,5-bis(butylboronate), estra -1,3,5(10)-trien-17ß-ol, glucobrassicin, N-2,4-Dnp-L-arginine, dasycarpidan-1-methanol, acetate(ester) and 5alpha-androstane-3,17-monooxime (Kamal *et al*., 2015).

A bioactive compound (p-Coumaric acid) (C9H8O3) (PC) with a molecular weight of 164.04 Da obtained from and this fungus isolated from Catharanthus roseus leaves (Sudharshana *et al.*, 2019). In a study performed by Abdou and Dawoud (2020), they presented the isolation and identification of the bioactive metabolites Alterneriol, Sterigmatocystin and tentoxin from the

endophyte *A. alternata* that isolated from the leaves of the medicinal plant *Bidens bipinnata*.

2. The Aim of the Study

The study aims to investigate about the bioactive compounds in the endophyte fungus *Alternaria alternata* and the effects of cellular, anti-microbial and physiological activities of this fungus extracts.

3. Conclusions and Recommendations

3.1 Conclusions

The results of this study showed that *A. alternata* is dispersed and adaptable to different environments have high biological activities and produce many important secondary metabolites.

3.2 Recommendations

We recommend through this study to future experiments to take advantage of the extracts of this fungus to treat incurable diseases

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الخلاصة

أشتملت الدراسة الحالية للتحري عن الفعاليات الحيوية لمستخلصات الفطر *Alternaria alternata* ، حيث تم التعرف عن صفات الفطر وبيئته والظروف المثالية لنموه فضلا" عن الصفات المظهرية والتصنيفية له. كما تم التحري عن فعالياته الضد خلوية للخلايا البكتيرية الممرضة والخلايا السرطانية، والفعالية المضادة للفطريات المرضية . أشتملت الدراسة أيضا" على التحري عن التأثيرات المناعية لهذا الفطر من خلال الدراسات السابقة عن هذا المجال .

تم التوصل من خلال هذه الدراسة أن لهذا الفطر ومستخلصاته دراسات عديدة وجد فيها الباحثون النتائج الإيجابية للتجارب والأهمية القصوى لهذا الفطر الذي يعد منتشرا" بشكل واسع في مختلف البيئات . لذا نوصي من خلال هذه الدراسة الى تجارب مستقبلية للاستفادة من مستخلصات هذا الفطر لعلاج الامراض المستعصية

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جامعة ديالى/كلية العلوم
قسم التقانة الاحيائية
الدراسة الصباحية

التحري عن الفعاليات الحيوية لمستخلصات الفطر *Alternaria alternata*

بحث تخرج مقدم الى

مجلس قسم التقانة الاحيائية/كلية العلوم/جامعة ديالى

وهو جزء من متطلبات نيل درجة البكالوريوس في التقانة الاحيائية

من قبل

تبارك فائز جبار
داود غازي داود
زهراء مهدي محمد
محمد أمين سمين
يوسف خليل إسماعيل

باشراف

د. أنعام فؤاد حسين

٢٠٢٠م – ١٤٤١هـ