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ISOLATION, PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR MYCOTOXIN FROM *Alternaria alternata*

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ABSTRACT

The isolate of *Alternaria alternata* that isolated from soil produced extracellular secondary metabolites (Mycotoxin) of carbohydrate with reducing nature which had inhibitory effect against Gm positive & Gm negative standard and pathogenic bacteria in solid media. Three types of media Malt Extract Agar (MEA), Potato Dextrose Agar (PDA) and Potato Carrot Agar (PCA) can be using as solid or liquid media for production this mycotoxin. The mycotoxin extracted from free cell supernatant by simple extraction method using ethanol 90%. The cytotoxicity of extracted mycotoxin against human blood cells (RBCs) was (25) ppm. The acute toxicity of extracted toxin as determined by LD50 of laboratory mice was 150 mg/kg.

INTRODUCTION

Species of *Alternaira* were known to produce many metabolites mostly phytotoxins, which play an important role in the pathogenesis of plants. But certain species, in particular *A. alternata* are capable of producing several toxic metabolites in infected plants and/or in agricultural commodities, which can contaminate foods and feeds and elicit adverse effects in animals [1, 2].

Mycotoxins are low molecular weight organic compounds produce as secondary metabolites by fungus. Most secondary metabolites are produced after the fungus has completed its initial growth phase and is beginning a stage of development represented by the formation of spores [3].

Mycotoxins that produced by *A. alternata* included alternariol (AOH) and its monomethyl ether (AME), altenuene (ALT), altertoxin I (ATX-I), and tenuazonic acid (TA) [4, 5, 6]. *A. alternata* also produce about (70) types of secondary metabolites back

to different chemical groups such as dibenzobyrons, perylenquinons, tetramic acid, lactons, anthroquinons & cyclipeptides [7, 8, 9, 10, 11]. These mycotoxins known as carsogenic and mutagenic compound [12].

The objective of the present study was focused on the screening for the production of mycotoxin from *A. alternata* isolated from soil.

MATERIALS AND METHODS

1- **Microorganism**:- The isolate of *A.alternata* was isolated from Garmat Ali soil.

2- **Media**:- pH of all media adjusts to (5.5-6.5) in one litter:

A- PDA composed of: 200 g of potato supernatant, 20 g of dextrose & 20 g agar.

B- PCA composed of: 20 g of potato supernatant, 20 g of carrot supernatant & 20 agar.

C- M.E.A. composed of: 15 g of malt extract & 20g agar.

3- **Seed media**: (1 L) contains 10% peptone & 20% dextrose with pH (6).

4- **Fermentation media**: For toxin production PCA, PDA & M.E. broth were used. Corck borer disc (3mm) of the *A.alternata* plate culture were inculated into (100 ml) of seed media in an Erlemayer flask (500ml) and incubated in shaker for 24 hrs at 28-30 C°. Then 3ml of seed media transfer to three Erlemayer flask (500 ml) each one contain 100 ml of PC, PD and ME broth finally incubate in shaker at 28-30 C° for 5-7 days.

3- **Bacterial strains tested**: The following test bacteria were used during this study maintained in wall stopper vails containing nutrient agar (Difco):

1.*Staphylococcus aureus* (NCTC 6571)
2.*Staphylococcus aureus* (ATCC 29213)
3.*Staphylococcus aureus* (R)
4.*E.coli* (NCTC 5933)
5.*E.coli* (R)
6.*Bacillus subtilus* (PCI 219)
7.*B.pumillus* (NCTC 4175)
8.*Proteus vulgaris* (NCTC4175)

9.*Pseudomonas auroginosa* (NCTC 6750)

10.*Streptococcus pneumonia* (R)

*Notice: (R)=Clinical isolate

Assay Method:

1- **Primary Screening**:

The production of mycotoxin was assayed following agar plug method against standard test bacteria *S. aureus* (NCTC 6571) and *E. coli* (NCTC 5933). Mycotoxin titer was determined by the measuring the diameter of inhibition zone (IZ) millimeter (mm) around the plug [13].

2-Secondary Screening:

The potency of the fermentation culture filtrate were measuring by the plate agar diffusion method using the above standard test bacteria [14].

3- Isolation & Purification of Mycotoxin:

Cell free supernatant of ME broth culture (100 ml) was extracted with 100ml ethanol (90%) by separation funnel. Re-extarction the residue twice for complete extraction of mycotoxin. The ethanol extract evaporate and concentrated by a rotary evaporate at room temperature overnight to dryness. Compound was separated in crystalline form. Redissolve in 1ml of ethanol (90%) for recrystallization to get yield 0.6g of hydroscopic crystalline with colorless to yellow-color substance soluble in water [15].

Preliminary Chemical Identification:

1-**Molish Test (Carbohydrate test)**: 1 ml of α-naphthol alcohol was added to 1 ml of the extracted mycotoxin and mixed well. Then 3 ml of concentrated H2SO4 were added through the side of test tube. A purple ring appeared indicates the carbohydrate presence in the sample [16].

2-**Benedict Test**: Eight drops of extracted mycotoxin were added to (5ml) of the reagent (copper sulfate, sodium citrate & sodium carbonate) in the test tube. Test tube placed in the boiling water bath for (5 mins). A green color appeared indicate the positive test [17].

3-**Burite Test**: One milliliter was added to test tube contain 1 ml of extracted mycotoxin soluble in ethanol. After shake the purple color appear indicate the positive test [18].

4-**Ninhydrine test**: One milliliter of 0.2% ninhydrine solution was added to 1 ml of extracted mycotoxin in a test tube and boiled for 2 mins. [17].

5-Alkaloid Test: One to two milliliters of Dragendroff reagent were added to the mycotoxin to get orange sediment indicate the positive test [19].

Thin Layer Chromatography (TLC):

Extracted mycotoxin was dissolved in (90%) ethanol and 5µl were placed in the origin line of the TLC (Silica gel plate 5X20cm) by capillary tube. The mobile phase of the system consist of (water (1.3):formic acid (1):butanol (7.7) v\v). Then the retention flow (Rf) of the mycotoxin extracted were determined [20].

Melting point (M.p.):

Melting point of the extracted mycotoxin was measured by using Melting –point Apparateaus (Eletrothermal_England).

Spectrum Study:

1-Ultraviolet spectrum (UV-spectrum):

The UV of the extracted mycotoxin was measured by using Pye-Unicom SP8-100 Spectrophotometer.

2-Infrared spectrum (IR-spectrum):

Identification of the function groups depending in IR-spectrum by using Pye-Unicom SP3-300 Spectrometer, KBr disk.

Biological Properties:

1-Acute Toxicity:

The acute toxicity of extracted mycotoxin was studied with male mice (Albino-mice BALB/C strain). These divided into five groups with control each one contain (8) animals. Interperitoneal administrations of the doses 100,150,200,250 mg/kg were applied and all animal were examined for 3 days [21].

2-Minimal Inhibitory Concentrations (MICs):

The MICs of extracted mycotoxin was detected depending on the plate agar diffusion method on Mullar-Hinton agar by using arrange of concentration 0.1-10µg/ml against standard test bacteria [22].

3-Cytotoxicity:

A suspension of 1ml human blood in 20ml physiological saline was prepared. A solution of mycotoxin in DMSO (Dimethyl Sulfoxid) with varying concentrations 100ml each were added to 2ml of the erythrocyte (RBCs) suspension and turbidity was read at 10,30 and 60 minutes. The concentration which gave a clear solution due to lysing of RBCs are an indication to the degree of mycotoxin action (test compound) against RBCs [23].

RESULTS

1-Primary screening:

A. alternata able to produced secondary metabolite (Mycotoxin) with activity against both Gm+ve & Gm-ve test bacteria in solid media. Table(1)

2-Secondary screening:

The production of active mycotoxin from *A. alternate* after 5 days of incubation in three different fermentation media ME, PD & PC broth show in table 2, it showed a very action against standard Gm+ve & Gm-ve test bacteria. This lead to conclude that the production of mycotoxin in fermentation media for 5-7 days gave the optimum value in comparison with solid media (table 2).

3-Isolation & Purification of Mycotoxin:

The mycotoxin was extracted from the supernatant of the fermentation media using ethanol (90%). The quality test for the crude extract had shown a positive result with molish & benedict tests while gave a negative result with burite, ninhydrine & alkaloid tests. Using TLC technique which shown on clear spot has (Rf) value equal to (0.15) with brown color. Also this compound has sharp (M.p.). These results indicate the purity of this extracted mycotoxin (table 3).

The spectrum studies such as UV shown on peak of the ethanol extracted mycotoxin with 296 nm. (Table 3, Figure 1). IR-spectrum showed the active groups of this compound (Table 4, Figure 2).

4-Biological Properties:

1-Acute Toxicity:

The median lethal dose (LD50) of the active mycotoxin was 200 mg/kg via intraperitoneal administration. This result showed that the ethanol extracted from fermentation supernatant of *A. alternata* was highly toxic to laboratory mice (Figure 3).

2-Minimal Inhibition Concentration (MICs):

The activity of the extracted mycotoxin against (Gm+ve and Gm-ve) standard test bacteria was shown in the table 5.

3-**Cytotoxicity:**

Preliminary cytotoxicity experiment with human RBCs indicate that the extracted mycotoxin with highly toxic effect by lysing RBCs at 25 ppm as shown in table (6).

DISCUSSION

A. alternata is a frequently occurring species of particular interest because it produces a number of mycotoxin including alternariol (AOH), alternariol monomethyl (AME), altenuene (ALT), altertoxin I, II and III (ATX I, II, and III) and L-tenuazonic acid (TeA) [2, 4, 5, 10, 24, 25].

The results in table 1 and 2 showed that the secondary metabolite produced by *A. alternata* in solid and fermentation media (Primary and Secondary screening) had broad spectrum activity against both Gm+ve and Gm-ve standard test bacteria and causes inhibitory action and this result may due to the production of mycotoxin by *A. alternata* which has inhibitory action against growth of both Gm+ve and Gm-ve bacteria [2, 26].

The pure spot of extracted mycotoxin with (Rf) table (3) indicate to get pure mycotoxin from *A.alternata* depending on Centeno and Calvo [27].

The positive Molish, Benedict, burite and alkaloid tests also the negative results ninhydrine indicating that the extracted compound was carbohydrate mycotoxin with reduction properties depending on the carbohydrate and IR-spectrum [16, 17, 18, 19].

The LD50 of extracted mycotoxin was 150 mg/kg (figure 3) intraperitoneal injection. The mycotoxin has effective against mice, rat and genia pig [2]. The mice which take low concentration appear as located spotted, stomach contraction and sluggish sign that lead to death. In the other side the lysing activity against RBCs 25ppm (table 6) these value of cytotoxicity and acute toxicity (LD50) are comparative to that of other studies which gives to the mycotoxin responsibility to cytotoxic action to human cells line at 6-41 nanogram/ml and erythroleukemia cells and animal [2, 5, 27].Further chemical and biological studies must be done on the isolated mycotoxin to characterizes chemical structure and determine the activity in vivo.

Table (1): Primary screening for the activity of *Alternaria alternata* measured by diameter of inhibition zone (mm)

Test organism	IZ (mm)
Staphylococcus aureus (NCTC 6571)	15.5
E. coli (NCTC 5933)	20

Table (2): Secondary screening of the extracted mycotoxin measured by diameter of the inhibition zone (mm) in three media against standard test bacteria

Broth Media	*Staphylococcus aureus* (NCTC 6571)	*E. coli* (NCTC 5933)
PD	28	30
PC	30	28
ME	28	30

Table (3): The physoichemical properties of the extracted mycotoxin from *Alternaria alternata*

Compound	TLC		M.p. (C)	UV (nm)
	Rf	Spot color		
Mycotoxin	0.15	Brown	140-142	296

Table (4): The important bands that appear in the IR-spectrum of the extracted mycotoxin

Band frequency (cm -1) & shape	Bans assignment	Functional group
3300 (s)	(N-H)	Amine
2900 (s)	CH	Aromatic
2100 (s)	C=C	-------
1630 (s)	C=O	Amide
1340	CH3	---
1040 (s)	C-O	Lactam
900	CH2	Out of plane

s=strech

Table (5): The minimal inhibition concentration of the extracted mycotoxin against standard & clinical test bacteria

Test organism	MIC (μg/ml)
Staphylococcus aureus (NCTC 6571)	0.1
Staphylococcus aureus (ATCC 29213)	0.2
Staphylococcus aureus (R)	0.01
E.coli (NCTC 5933)	10
E.coli (R)	7
Bacillus subtilus (PCI 219)	0.1
B.pumillus (NCTC 4175)	0.2
Proteus vulgaris (NCTC4175)	1
Pseudomonas auroginosa (NCTC 6750)	2
Streptococcus pneumonia (R)	0.2

R=clinical isolate

Table (6): The cytotoxicity of the extracted mycotoxin against human RBCs

Compound	**Concenteration (ppm)**	**RBCs toxicity after 1hr**
DMSO	-	NT (Non Toxic)
Extracted mycotoxin	5 10 **25** 50	NT NT **T (Toxic)** T

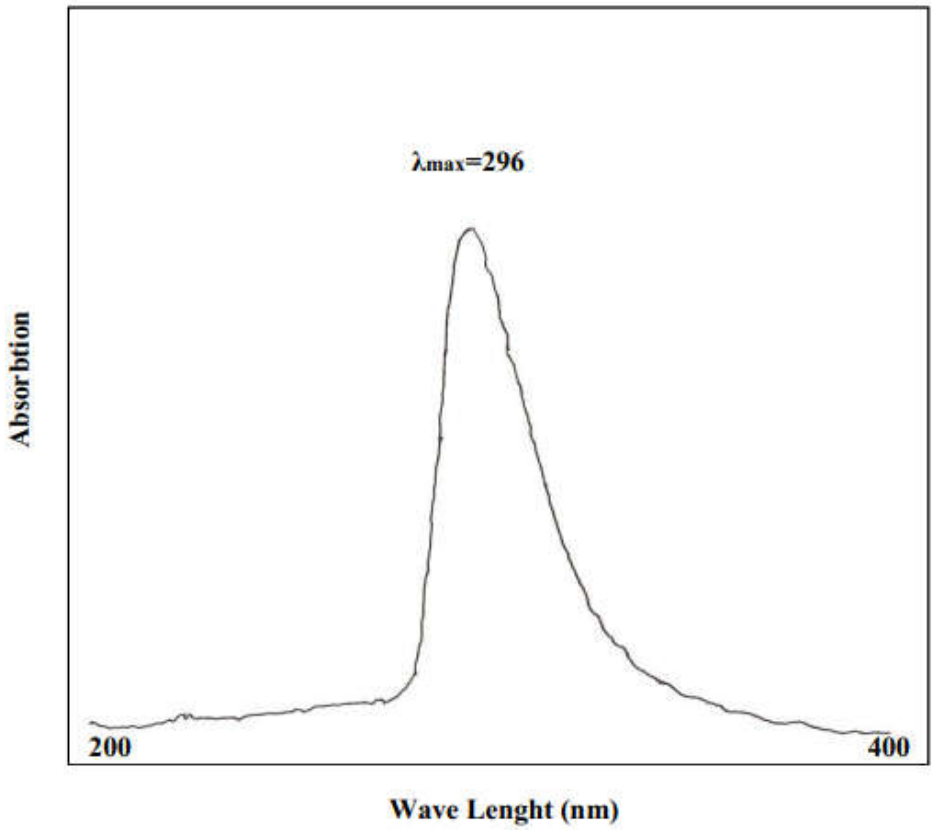

Figure (1): UV-spectrum of extracted mycotoxin

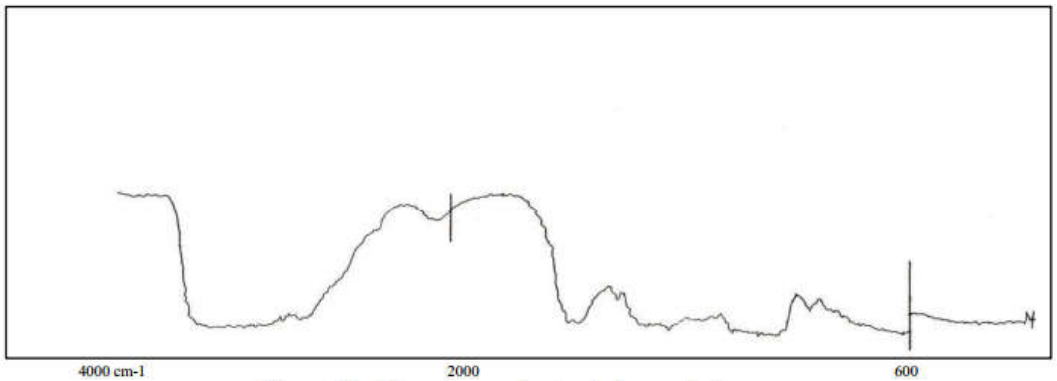

Figure (2): IR-spectrum of extracted mycotoxin

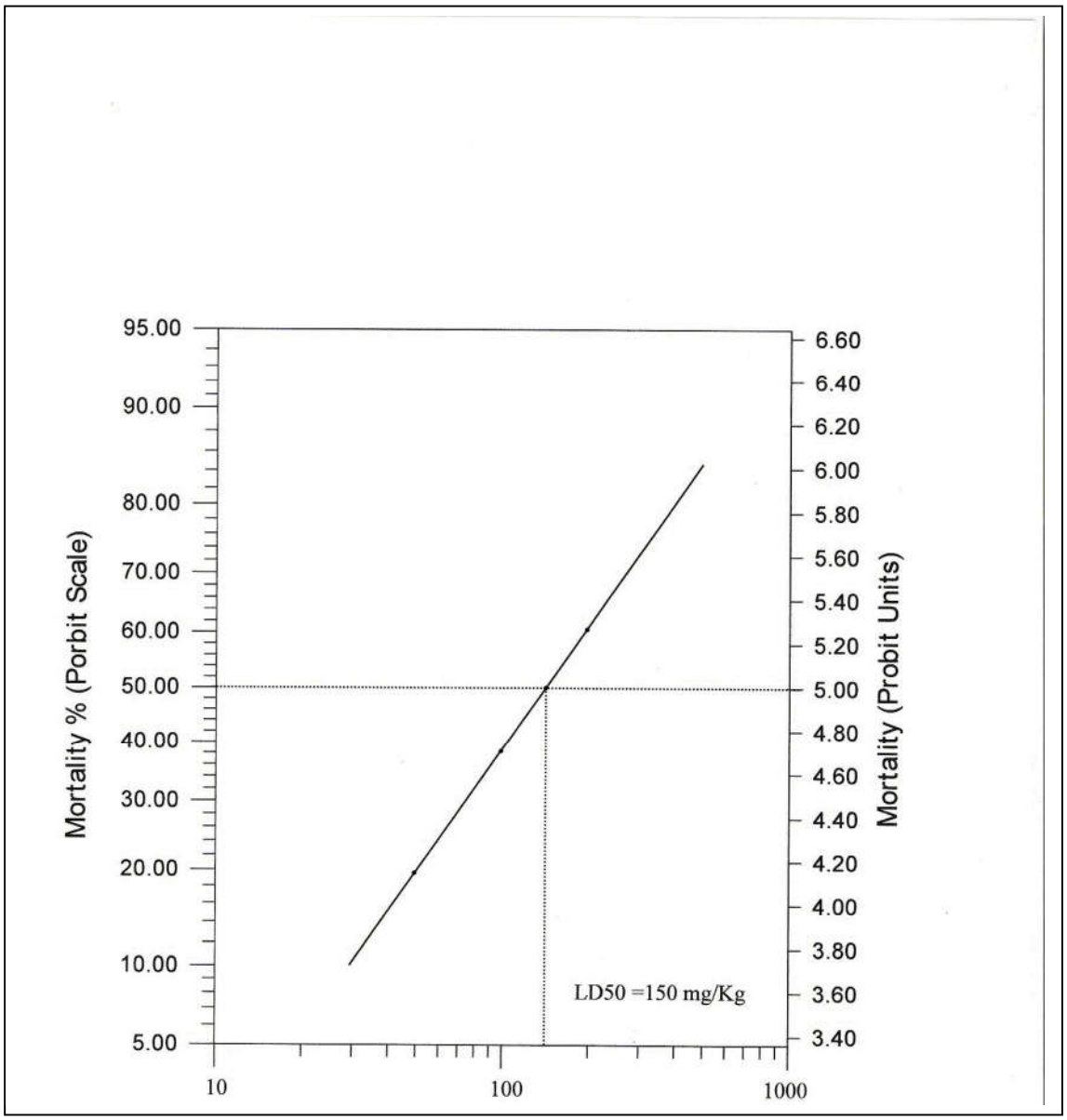

Figure (3): Median lethal dose (LD_{50}) of extracted mycotoxin

عزل وتنقية وتشخيص سم فطري خارج خلوي من الفطر *Alternaria alternata*

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يمتاز الفطر *Alternaria alternata* المعزول من التربة بقابلية على إنتاج أيوض ثانوية خارج خلوية ومنها السموم الفطرية ذات الطبيعة الكربوهيدراتية لها تأثير مثبط لنمو الجراثيم القياسية والمرضية الموجبة والسالبة لملون كرام في الأوساط الصلبة.استخدمت ثلاث أوساط وهي وسط خلاصة الشعير ، وسط البطاطا والدكستروز،وسط الباطاطا والجزر كأوساط تخمرية لإنتاج السم الفطري من العزلة. تم استخلاص السم الفطري من راشح المزارع التخمرية السائلة باستخدام الميثانول بتركيز ٩٠% . درست الخصائص السمية الخلوية للسم الفطري المستخلص ضد كريات الدم الحمر ٢٥جزء من المليون أما السمية الحادة للسم الفطري المستخلص ضد الفئران المختبرية فكانت 150مليغرام لكل كيلو غرام.

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