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GC-MS Analysis of Fungal Filtrate Crude Extracts Produced by Two Species of *Aspergillus*

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Abstract

The species of the genus *Aspergillus* have an ability to produce secondary metabolites, however, not all filamentous fungi can form the metabolites. The current study showed that two species of the genus grew at 27 °C on potato dextrose agar (PDA). *Aspergillus terreus* appeared brown colonies on the PDA but *A. niger* grew as black colonies. Potato dextrose broth (PDB) used to be a fermenting medium by which the fungal filtrate of both fungi separated from the mycelia. Residues of the extracted filtrates gave the blackish brown crude extracts. The extract weights recorded 300 mg/L obtained from *A. niger*, and 200 mg/L of *A. terreus*. GC-MS analysis revealed the extract of *A. niger* consists of three compounds are {[5-methyl-2-phenylindolizine],[thiocarbamicacid,N,N-dimethyl,S-1,3-diphenyl-2-butenylester], and [22-beta.-acetoxy-3.beta.,16.alpha.-dihydroxy13,28 epoxyolean-2]}. *A. terreus* extract possesses four compounds are {[2.2] paracyclophane, [5-methyl-2-phenylindolizine],[2H-1-benzopyran-6-ol,3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltride)] , and [dI-.alpha.-tocopherol]}. 300 µL taken from 10 mg/ml of *A. niger* crude extract led to hemolysis of red blood cells while 800 µL of the same concentration from *A. terreus* extract gave the hemolysis. The present study concluded that 5-methyl-2-phenylindolizine was detected a similar compound in crude extracts of both *A.niger* and *A.terreus* with the same retention time. It may be attributed to the presenting same gene within the genome of both fungi led to producing 5-methyl-2-phenylindolizine.

Keywords: GC-MS Analysis, *Aspergillus*, Tocopherol, Secondary Metabolites.

Introduction

The genus *Aspergillus* is considered as cosmopolitan fungi which are anamorphic members of the ascomycetes. The genus classified in Trichocomaceae (family), and its order is Eurotiales (Pitt *et al*., 2000; Klich, 2002). *Aspergillus* species have economic importance due to their producing primary metabolites possess the benefit products such as enzymes, examples, α-amylase, amyloglucosidase, hemicellulase, and glucose as well as citric acid is given by *A.niger* (Carlile *et al*.,2001; Deacon, 2006; Júnior *et al*., 2012). *A.terreus* produces itaconic acid which used in the paint manufacture as a co-polymer. Relating to the secondary metabolites, *A.terreus* is able to give medically used products such as a cholesterol-lowering drug, example, statins. (Carlile *et al*.,2001; Deacon, 2006). However, *Aspergillus* species can give the benefit products, including secondary metabolites, there are a hazard and toxic secondary metabolites. Mycotoxins such as aflatoxin and cyclopiazonic acid obtaining from *A. flavus* and *A. parasiticus* besides ochratoxin by *A. ochraceus* (WHO,2011).

The current study aimed to detect and identify some chemical compounds produced in the fungal extracts obtained from two species of *Aspergillus*.

Materials and Methods

Collection of Fungal Species

Two fungal species were used in this study. One of them was *Aspergillus terreus* isolated from a water sample obtained from the marsh areas in the south of Iraq. The water samples were collected using a sterile disposable tube. Aseptically, the tube opened and closed under the water where collecting the sample. Half ml of the sample was cultured on potato dextrose agar (PDA) and incubated at 27 °C for 7 days. The second species was *A. niger* isolated as a contaminating fungus on PDA in the Laboratory of Veterinary Medicine College, University of Thi-Qar, Iraq. Both species were subcultured on PDA, malt extract agar (MEA), and Czapex Dox Medium (CDM) under the same conditions. The identification performed depending on the morphological appearances as well as lactophenol blue used to the microscopic examination.

Process of Fermentation and Extraction

Aspergillus terreus and *A. niger* grew on PDA Petri dishes at 27 °C for 7 days. Two discs (7 mm diameter for each disc) were separately cut from each growing species, placed in a sterile flask containing 400 ml of potato dextrose broth (PDB), and incubated at 27 °C for 7 days. After completing incubation, the mycelia separated from the filtrate by filter paper. According to (Rao and Renn, 1963) and (Hanson, 2008), the filtrate poured on a filter paper covered by 2 grams of an activated charcoal as a film until the residue adsorbed on the charcoal while the filtrate discarded. After dryness of the charcoal, 100 ml of methanol-HCL (Normality was 0. 36) poured on it for getting a filtrate which evaporated at 37 °C until obtaining a brown extract and kept at the fridge. The activated charcoal used to remove proteins from the secondary metabolites that formed in a process of the fungal fermentation.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The fungal crude extract was dissolved in DMSO and filtered through µM 0.45 filter syringe (Millipore) by which the filtrate submitted to the GC-Mass spectrometry carried out by gas chromatography-mass spectrometry, MSDCHEM\1\METHODS\MUAFAQ.M for the determination of negative ions(m/z) by using a column characterized by HP-5MS, 5% Phenyl methyl Sillox(1629.5), 30m × 0.250 µm I.D. x 0.25 µm, SS., Inlet He, then application of the parameters in (Table 1).

Table 1. GC-MS analysis parameters used to detect the compounds in the fungal crude extracts of *A. terreus* and *A. niger*.

Analysis Parameters	
EMV mode	Gain Factor (1.00)
Resulting EM voltage	1306
Power capacity	70 EV
Low Mass	28.0
High Mass	441
Threshold	150
Minimum quality for all narcotics	97%-90
Flow rate	1ml/min
Runtime	24 min
Hold up time	1.5288 min
Solvent delay	3.00 min
Average velocity	36.796 cm/sec
Temperature	Initial 70 °C to Maximum 375 °C
Pressure	8.81 Psi

Preliminary Toxicity Test

According to Nair *et al*., (1989) with some modification, dissolving 1 ml of human blood in 20 ml of a normal saline to form the blood solution while the fungal crude extract dissolved in distilled water to prepare 10 mg/ml (10000 µg/ml) of each fungal extract. 100 µL-1 ml of the concentration added to 2 ml of the blood solution tube for 10 min., 30 min., and 1 hr. at room temperature. The changing blood solution color into pale color indicates to the positive result (hemolysis of the red blood cells). The test was compared with control tube contained 2 ml of the blood solution with 100 µL of distilled water.

Results

Identification of Fungi

Aspergillus terreus appeared in the various colored colonies had irregular growth on the potato dextrose agar (PDA), malt extract agar (MEA), and Czapex Dox medium (CDM). It is important to note that the exudates were produced on the PDA only. Microscopically, conidial heads are seen as biseriate, compact and columnar. Conidiophores observed hyaline and smooth-walled. Conidia have a shape as globose to ellipsoidal are seen as long chains in addition to the single and double ones. Conidia are slightly smooth-walled (Table 2), (Fig.1). *Aspergillus niger* produced black colonies on the mentioned media. The sporulation of *A. niger* was little on the CDM. The microscopic appearance revealed heads of the conidia are black while globose to subglobose conidia have a rough wall and dark blue in color. Additionally, they founded as single or double besides chains (Table 2) and (Fig.2).

Table 2. Growth characteristics of *A. terreus* and *A. niger* on three media at 27 ºC for 7 days.

Characteristics	*Aspergillus terreus*		
	The Used Media		
	PDA	MEA	CDM
Colony	Brown	Yellowish white	Whitish brown
Reversible colony	Deep orange-brown.	Similar to PDA	Yellowish orange
Colony edge	White	White	Brown
Colony grooves	Present	Present	Absent
Exudates on colony	Present	Absent	Absent
Topography	Irregular	Irregular and flat	Irregular and flat
Aerial growth	Slightly	Absent	Absent
Aspergillus niger			
Colony	Black	Grayish black	Black
Reversible colony	Brownish black	Blackish brown	Blackish brown
The color of colony edge	White	White	Black
Colony grooves	Present	Absent	Absent
Exudates on colony	Absent	Absent	Absent
Topography	Irregular	Irregular	Irregular and flat
Aerial growth	Present but not more	Present	Absent

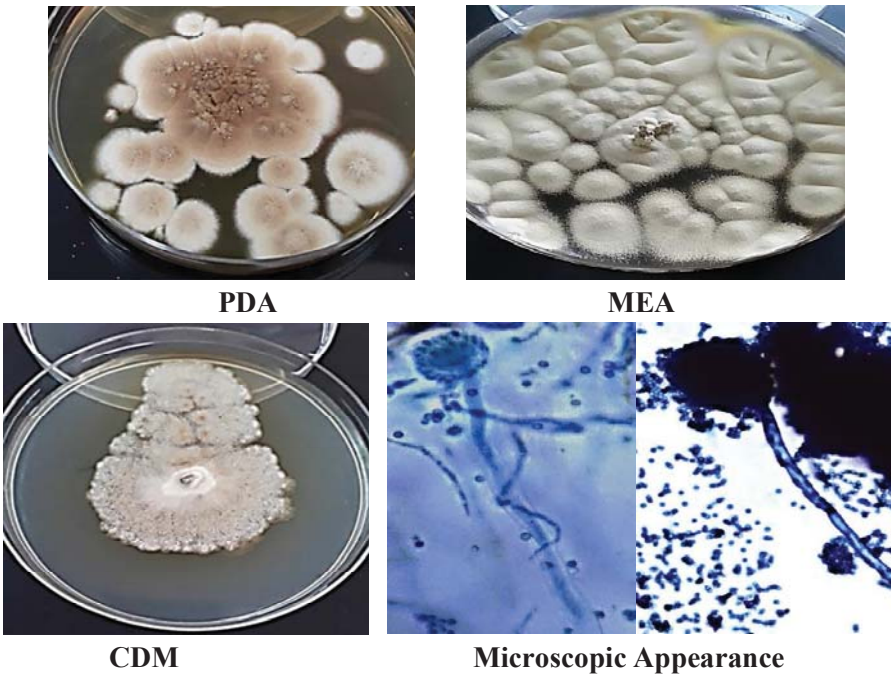


Fig. 1. Morphology of *A. terreus* at 27 °C. PDA: Potato dextrose agar. MEA: Malt extract agar. CDM: Czapex Dox medium.

PDA

MEA

CDM

Microscopic appearance

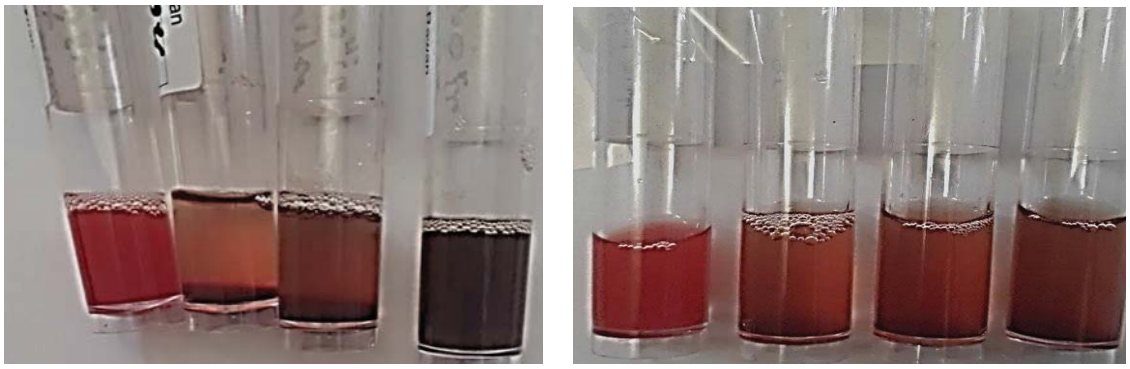
Fig. 2. Morphology of *A. niger* at 27 °C. PDA: Potato dextrose agar. MEA: Malt extract agar. CDM: Czapex Dox medium.

Preliminary Toxicity Test

Three hundreds µL of the crude extract produced by *A.niger* changed a normal color of the blood solution as hemolysis evidence after 5 minutes of the testing time period that the volume added to the solution, while 500 µL gave the hemolysis during 1 min. Relating to *A.terreus*, 800 µL of its crude extract had a hemolytic result after 31 minutes but 1000 µL resulted in the hemolysis after 19 min. (Table 3) and (Fig. 3).

Table 3.Preliminary red blood cellular toxicity of the fungal crude extracts at room temperature during 1 hour as the time period of the test.

Red Blood Cellular Toxicity of *A. niger* Crude Extract		
Volumes	**Results**	**The time period of the test (1 hr.)**
100 µL of 10 mg/ml	No hemolysis	During 1 hr of the test
200 µL of 10 mg/ml	No hemolysis	After 1 hr of the test
300 µL of 10 mg/ml	Hemolysis	After 5 min. of the test
400 µL of 10 mg/ml	Hemolysis	After 3 min. of the test
500 µL of 10 mg/ml	Hemolysis	After 1 min. of the test
Red Blood Cellular Toxicity of *A. terreus* Crude Extract		
100 µL of 10 mg/ml	No hemolysis	During 1 hr of the test
200 µL of 10 mg/ml	No hemolysis	During 1hr of the test
300 µL of 10 mg/ml	No hemolysis	During 1 hr of the test
400 µL of 10 mg/ml	No hemolysis	During 1 hr of the test
500 µL of 10 mg/ml	No hemolysis	During 1 hr of the test
600 µL of 10 mg/ml	No hemolysis	During 1 hr of the test
700 µL of 10 mg/ml	No hemolysis	During 1 hr of the test
800 µL of 10 mg/ml	Hemolysis	After 31 min. of the test
900 µL of 10 mg/ml	Hemolysis	After 25 min. of the test
1000 µL of 10 mg/ml	Hemolysis	After 19 min. of the test



A B

Fig.3. Preliminary red blood cellular toxicity of the fungal crude extracts at room temperature.

A: Toxicity of *A. niger* extract against RBCs, from left to right: First is a control tube (a mixture of the blood, normal saline and 100 µL of D.W), second, third and fourth tubes are 300 µL, 400 µL, and 500 µL of the fungal extract added to the blood solution respectively. **B:** Toxicity of *A. terreus* extract against RBCs, from left to right: First is a control tube, second, third and fourth tubes had 800 µL, 900 µL, and 1000 µL of the fungal extract added to the blood solution respectively.

Extraction of Fungal Filtrates and Their GC-MS Analysis

The extracts were blackish-brown crude extracts for both species. 300 mg/L of the extract produced by *Aspergillus niger* while 200 mg/L of *A. terreus* extract. GC-MS analysis revealed the extract of *A. niger* consists of three compounds (Table 4) and (Figures: 4a and 4b) while five compounds within the extract of *A. terreus* (Table 4) and (Figures: 5a, 5b, and 5c). It is important to note that 5-methyl-2-phenylindolizine has detected a similar compound found in both fungal extracts. Additionally, the extracting solvent (DMSO) was also detected.

Table 5. GC-MS analysis of the filtrate extract produced by *A. terreus*.

Compounds	**Retention Time (min.)**
DMSO (sample solvent)	3.111
5-Methyl-2-phenylindolizine	18.897
Thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester	21.263
22-Beta.-acetoxy-3.beta.,16.alpha.-dihydroxy-13,28epoxyolean-2	21.891

Table 4. GC-MS analysis of the filtrate extract produced by *A. niger*.

Compounds	**Retention Time (min.)**
DMSO (sample solvent)	5.962
Dimethyl sulfone	6.033
[2.2] Paracyclophane	11.076
5-Methyl-2-phenylindolizine	18.969
2H-1-Benzopyran-6-ol,3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltride)	22.352
dI-.Alpha.-tocopherol	22.424

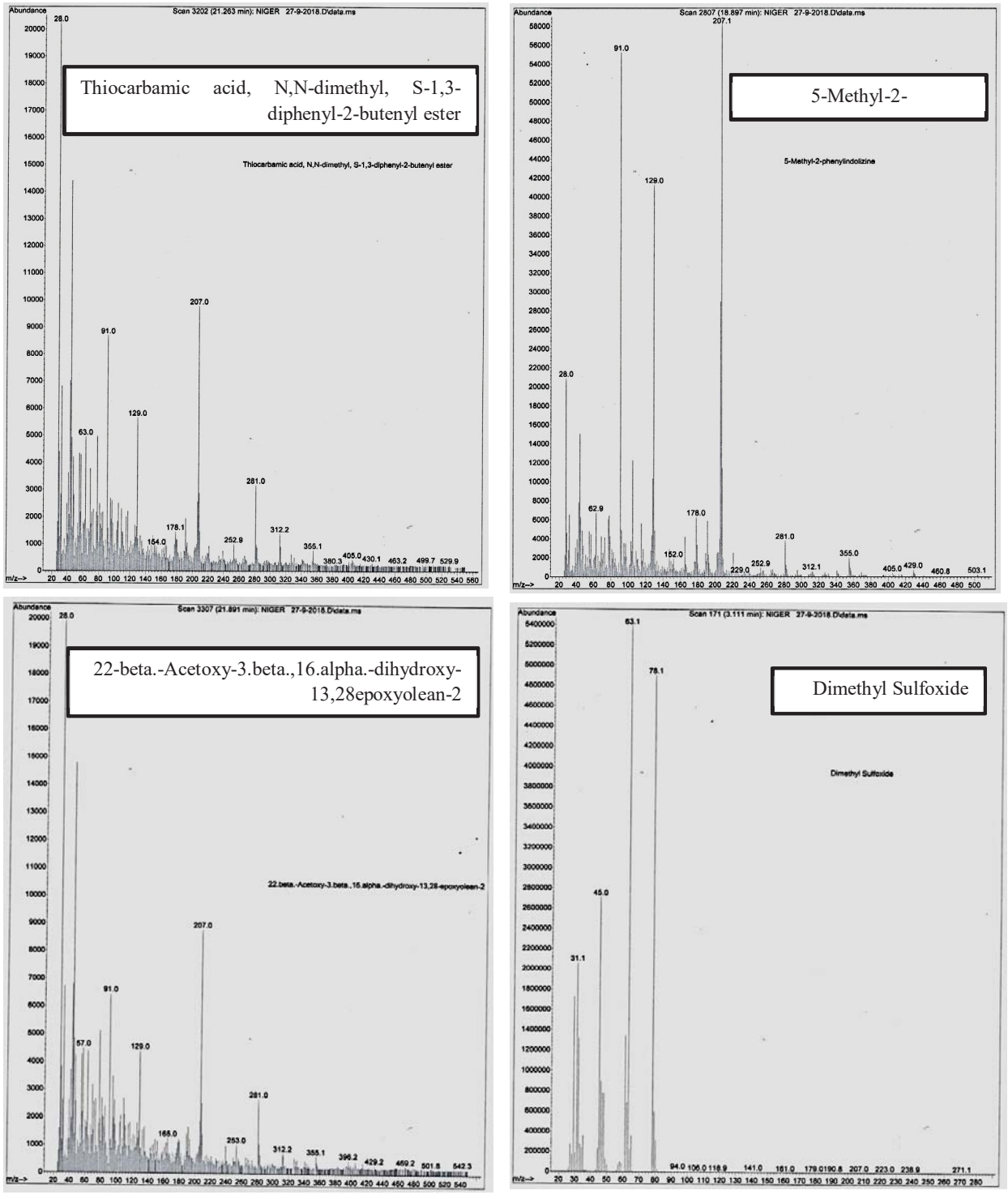


Fig.4. (5-Methyl-2-phenylindolizine), (thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester) and (22-Beta.-acetoxy-3.beta.,16.alpha.-dihydroxy-13,28epoxyolean-2) produced by the filtrate crude extract of *A.niger* besides DMSO (extract solvent) detected by GC-MS.

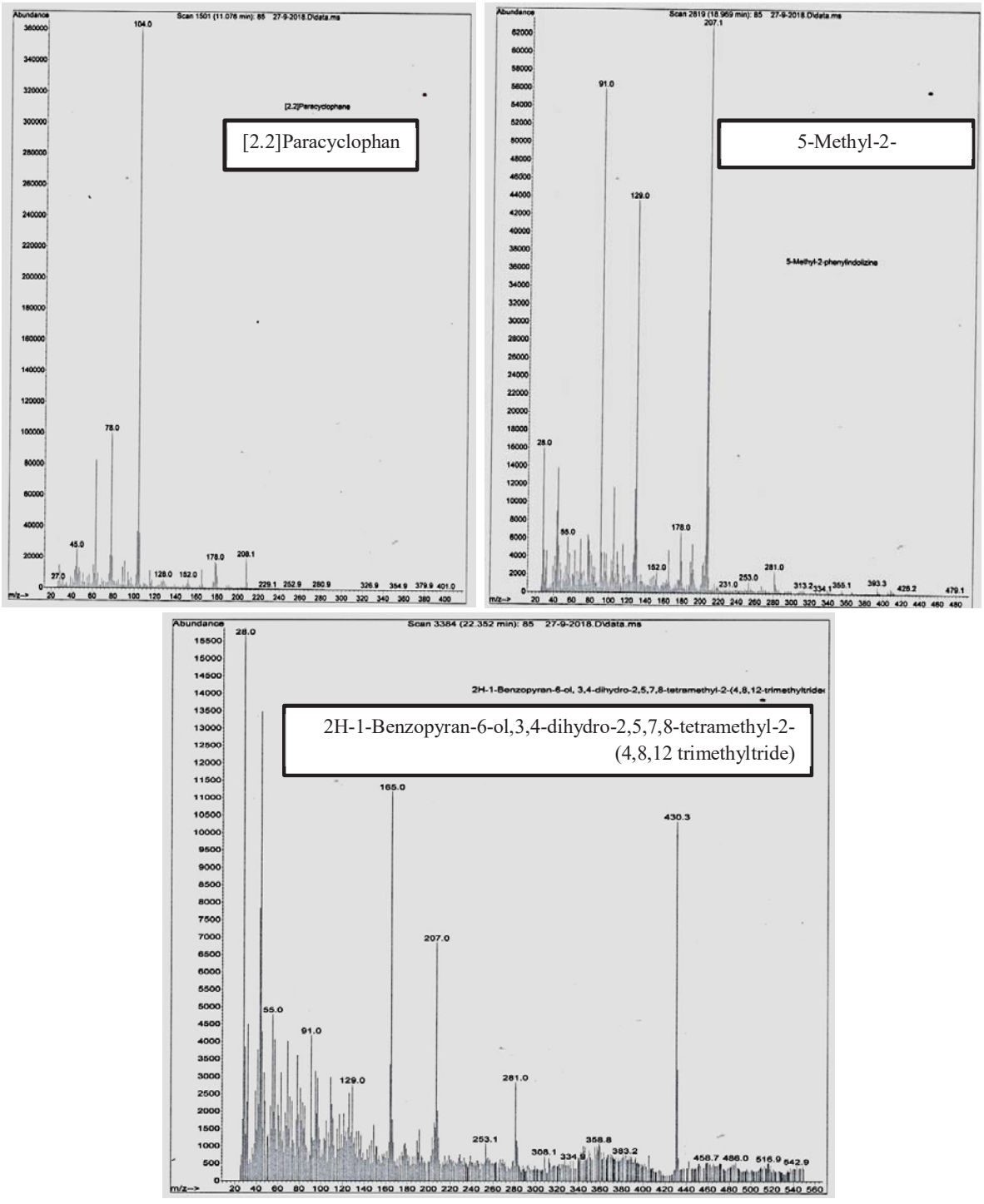


Fig.5a. ([2.2]Paracyclophane), (5-methyl-2-phenylindolizine) and 2H-1-benzopyran-6-ol,3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12 trimethyltride) of filtrate crude extract produced by *A.terreus* detected by GC-MS.

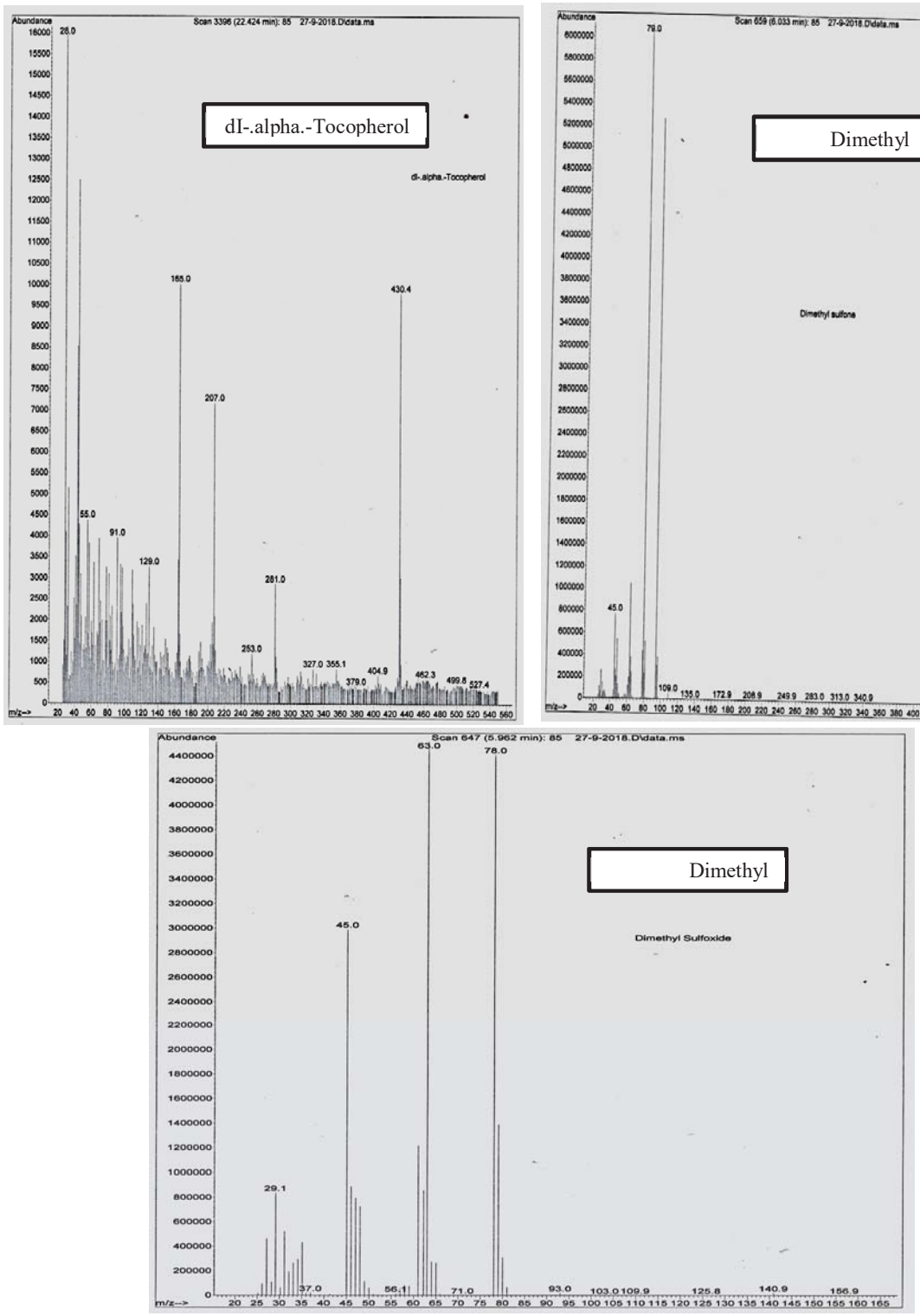


Fig.5b.(dI-.alpha.-Tocopherol), dimethyl sulfone of filtrate crude extract produced by *A.terreus* detected besides DMSO (extract solvent) detected by GC-MS.

Discussion

Secondary metabolites represent the compounds have no roles and necessity relating to the normal growth or development (Fox and Howlett, 2008). They are given by limited members of the genus, family, order even phylum (Frisvad *et al.*, 2007). When a fungus inoculated in a fermenting medium, the fungus will adapt for getting the lag phase, then fungal growth rapidly increases to reach the tropophase. If a nutritional depletion occurs, a fungus has idiophase (living cells equal to the dead cells). When the fungal dead cells increase, the decline phase will be given. All phases occur in the process of fermentation, and the secondary metabolites can be formed through idiophase (Hanson,2008).

The GC-MS analysis of our study revealed the crude extract of *A.niger* contains{[5-Methyl-2-phenylindolizine],[thiocarbamic acid,N,N-dimethyl,S-1,3-diphenyl-2-butenylester],and[22-beta.-acetoxy 3.beta.,16.alpha.dihydroxyl3,28 ep- -oxyolean-2]} while the crude extract of *A.terreus* has {[2.2] paracyclophane, [5-methyl-2-phenylindolizine],[2H-1-benzopyran-6-ol,3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltride)], and [dI-.alpha.-tocopherol]} (Fig. 4) and (Figures: 5a, and 5b).

Secondary metabolites regulated by gene clusters possess the transcription factors which control the genes in the clusters. For example, the transcription factor *afl*R regulates a gene that responsible for the clusters of aflatoxin in *Aspergillus flavus* and *A. parasiticus*. Additionally, sterigmatocystin cluster within *A.nidulans* possessing a regulation of three genes outside the aflatoxin gene cluster (Chang *et al*., 1993; Woloshuk *et al.,* 1994; Brown *et al*., 1996; Yu *et al.,* 1996; Fernandes *et al*., 1998; Price *et al*, 2006). The present study concluded that 5-methyl-2-phenylindolizine was detected a similar compound in crude extracts of both *A.niger* and *A.terreus* with the same retention time (Table 4) and (Table 5).It may be attributed to the presenting same gene within the genome of both *A.niger* and *A.terreus* led to producing 5-methyl-2-phenylindolizine.

Relating to the GC-MS analysis of the fungal extracts, our study showed detecting thiocarbamic acid, N, N-dimethyl, S-1,3-diphenyl-2-butenyl ester was detected in the crude extract of *A.niger* (Table 4). Interestingly, the crude extract of *A.terreus* has dI-.alpha.-tocopherol is one of the extract constituents (Table 5). Peng *et al*.,(2011) characterized 5-methyl-2-phenylindolizine in plant Bamboo (*Phyllostachys pubescens*). Also (Oloyede *et al*., 2011; Musini *et al*., 2013; Moronkola *et al*., 2017) isolated the compound from plants, *Dieffenbachia picta*, and *Blighia unijugata*, as one of the essential oil phenolic compounds. Schledz *et al*.,(2001) showed phytyltransferase obtained from *Synechocystis* species possesses a role in the biosynthesis of tocopherol. In our study, these compound appeared in the extracts of *A. niger* and *A. terreus* which may be attributed to existing of a same gene in the genomes of all mentioned sources led to producing these compounds, however, producers of the compounds are different.

The biotransformation can be performed by a fungus has a growth if a substrate such as dimethyl sulfoxide, acetone, and ethanol added to a medium. The fungus can oxidize dimethyl sulfoxide into a crystalline dimethyl sulfone (Hanson, 2008). GC-MS analysis of the present study showed that the crude extract of *A.terreus* contained dimethyl sulfone while *A.niger* extract did not contain it(Tables 4), (Table 5) and (Fig.5b). The sample solvent used in the analysis was dimethyl sulfoxide in the extracts of both fungi. The result of our study may be attributed to an ability of the one or more of compounds in the *A.terreus* extract made dimethyl sulfoxide to be oxidized into dimethyl sulfone while extract of *A.niger* has no those oxidizing compounds.

Tocopherol prevents a hyper-aggregation of the platelets and platelet clumping (Rizvi *et al*.,2014). This study showed the crude extract of *A. niger* resulted in a hemolysis during a short period using few volumes of the same concentration compared with the extract of *A.terreus* (Table 3) and (Fig. 3). The result may be

due to tocopherol found in the extract of *A.terreus* (Fig.5 b) led to protect the human red blood cells (RBCs) more than *A.niger* extract which has no tocopherol in its structure (Fig.4). It may be said tocopherol made affinity of the hemolytic compound within extract of *A. terreus* less than *A. niger* extract for binding blood proteins.

Conclusions

The crude extracts of *Aspergillus niger* and *A.terreus*, growing in PDB at 27 °C for 7 days, contain the different compounds. So, they needs to separate and purify these compounds, then testing them for implementing. As well as, there was 5-methyl-2-phenylindolizine was detected in both fungal extracts. It may be indicated to present a same gene in the genome of *A. niger* and *A. terreus*. The gene can be studied in the fungal molecular identification.

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