ANTIOXIDANT PRODUCTION FROM LOCAL FUNGAL ISOLATE

N. Sh. Ahmed **E. I. Al – Shamary**
Researcher **Assist. Prof.**
Dep. of Food Sci, Coll. of Agri. University of Baghdad
noora_sha85@yahoo.com **Elhamfadhil@yahoo.com**

ABSTRACT

This study aim was antioxidant production from local fungal isolate, Twenty six purified isolates of *Aspergillus* sp. were obtained by using different sources (soil, Spoiled fruits, wheat, barley, maize and Spoiled bread). The isolates were examined for their aflatoxins production, Non-aflatoxins producing isolates were used as phenolic compounds producer. The highest phenolic compounds (45.3 µg/ml) was obtained from *Aspergillus* sp.BP1 isolate. This isolate was identified by 5.8 rRNA gene sequecing as *Aspergills niger* (accuracy of 100%), which was matched with the sequence of *Aspergills niger strain* YMCHA 55 recorded in Gene bank under the ID: JF 436883.1.The Phenolic compounds were extracted from filtrate of selected isolate by ethyl acetate and identified using high performance liquid chromatography analysis (HPLC) and the results were compared with that for standard phenolic compounds, It was found that the filtrate extract contains gallic acid, cumarin, rutin and kampferole with concentrations of 47.69, 6.60, 218.30 and 22.00 µg/ml respectively.

Keywords: phenolic compound, HPLC analysis, molecular identification, *Aspergillus niger* B1b, free radical.
Part of M.Sc. thesis of the first author

إنتاج مضادات الأكسدة من عزلة فطرية محلية

نور شكر احمد الهام اسماعيل الشمري
باحث أستاذ مساعد
قسم علوم الأغذية، كلية الزراعة، جامعة بغداد

المستخلص

هدف البحث الى انتاج مضادات الأكسدة من عزلة فطرية محلية، تم الحصول على 26 عزلة نقية من *Aspergillus* sp. من مصادر مختلفة شملت التربة والفواكه التالفة والحنطة والشعير والذرة والخبز التالف. تم اختبار قابلية العزلات على انتاج سموم الافلاتوكسين. واستعملت العزلات غير المنتجة للافلاتوكسين كمصادر لإنتاج للمركبات الفينولية، و تفوقت العزلة *Aspergillus* sp. B1b بانتاج اعلى تركيز للمركبات الفينولية وبواقع 45.3 مايكروغرام/مل . شُخصت العزلة *Aspergillus* sp.B1b بواسطة تتابعات الجين SrRNA 5.8 على انها عزلة *Aspergillus* niger بنسبة تطابق بلغت 100% مع تتابعات السلالة *Aspergillus niger strain* YMCHA 55 والمسجلة في بنك الجينات العالمي تحت ID: JF 436883.1 . استخلصت المركبات الفينولية من راشح العزلة المحلية بواسطة خلات الاثيل وشخصت باستعمال تقنية كروموتوكرافي غاز سائل عالي الاداء (HPLC) وقورنت النتائج مع المركبات الفينولية القياسية، وجد ان مستخلص الراشح يحوي على حامض الكاليك والكيومارين والروتين والكامفيرول وبتراكيز 47.69، 6.6، 218.3 و22 مايكروغرام/مل على التوالي.

. الكلمات المفتاحية:المركبات الفينولية، الكشف بـHPLC ، تشخيص جيني، الجذور الحرة، *Aspergillus niger* B1b .
بحث مستل من رسالة ماجستير للباحث االاول

***Received:12/6/2018, Accepted:4/9/2018**

INTRODACTION

Reactive oxygen species (ROS) are continuously formed by normal cellular processes endogenously and environmental factors exogenously (2),ROS include nonradical species such as H_2O_2, hypochlorous acid ($HOCl^-$), singlet oxygen (^1O), and free radicals such as superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical (OH^*), and hydroperoxide (ROO^*) (17), (22). Levels of ROS higher than physiological concentrations cause oxidative-antioxidant imbalance and oxidative stress (23). When enzymatic or non-enzymatic endogenous antioxidants are inadequate to remove ROS from the body, it becomes important for the body to receive exogenous natural antioxidants such as phenolic compounds(16).The phenolic compounds are a family of secondary metabolites common to all higher plant.These compounds can be further divided into primary and secondary metabolites (28). Phenolic compounds are the most widely distributed secondary metabolites, ubiquitously present in the plant kingdom. Plant phenolics" and "polyphenols" are secondary natural metabolites arising biogenetically from the shikimate/phenylpropanoid pathway, which directly provides phenyl propanoids, or the "polyketide" acetate/malonate pathway, which can produce simple phenols, or both, thus producing monomeric and polymericphenols and polyphenols, which fulfill a very broad range of physiological rolesin plants (8). Two methods for the phenols to exert their biological effects have been proposed: the phenols can act either locally in the gastrointestinal tract, or as absorbable metabolites. As the phenolic compounds are common to all higher plants, and with more than 8000 phenolic structures of varying complexity reported, they are typically classified based on the number and arrangement of carbon atoms (9). Phenolics are uncommon in bacteria, fungi, and algae. However, Filamentous fungi produce several secondary metabolism materials which have effect on the society by exploiting them as antibiotics and anticancer materials which have an antioxidant effect (27). The fungi that belong to *Aspergillus* genus are known for producing effective secondary metabolism (11). The aim of this study is to get *Aspergillus* isolates from local sources which produce appreciable amounts of phenolic compound and have an antioxidant effect

MATERIALS AND METHODS

Mold isolates sources

Mold isolates were collected on July of 2017from many places and isolation sources in Baghdad and other governorates as shown in (table1)

Table 1. Mold isolate sources

Source	Location of isolate
Furat wheat type	Al-Zaafaraniyah
Abo Ghraib wheat type	Al-Zaafaraniyah
IbaaWheat type	Al-Zaafaraniyah
local markets wheat	Baghdad
local markets barley	Baghdad
local markets white maize	Baghdad
local markets yellow maize	Baghdad
Spoiled bread	Baghdad
Spoiled apples	Baghdad
Spoiled pears	Baghdad
Spoiled oranges	Baghdad
Spoiled tomatoes	Baghdad
Desert soil	Baghdad
Agricultural soil	Baghdad
Garden soil	Baghdad
Desert soil	Thi'Qar
Agricultural soil	Thi'Qar
Garden soil	Thi'Qar

2- Culture media

Potato dextrose Agar (PDA)

The PDA media was prepared as described by the manufacturing company. The media was autoclaved at 121^o C and 15 pound/inch^2 for 15 min. The prepared media was preserved by adding antibacterial chloramphenicol (0.025%).

Potato dextrose broth (PDB)

The media was prepared as described by the manufacturing company. The media was autoclaved and used for fungi isolated growth.

Coconut extract agar (CEA)

The media was prepared according to(29).100gm of dry powder coconut was mixed with 600 ml boiling distilled water and filtered with a piece of cloth. The semi clear extract was transferred to separated funnel. 1.5% agar was added to the lower layer (450ml). The mixture (media) was autoclaved. The chloramphenicol (0.025%) was added as antibacterial at 59^o C. The media was cooled

to 50°C to inhibit the bacterial growth. The prepared media was used for aflatoxins assay.

Czapek doxs broth

The media was prepared according to(25): 3 gm sucrose, 0.2gm $NaNO_3$,0.1gm K_2HPO_4,0.05g Mg SO_4, and 0.05gmKCl were dissolved in 100 ml distilled water.The media was autoclaved and used for screening the antioxidants producing fungi. The same media was used for screening the antioxidants producing molds.

3- Methodology

Isolating and purifying

Many isolated samples (Table 1) were collected from various locations and sources.The samples were transferred to the laboratory in clean polyethylene bags prior to isolating processes.A pour plate method was used as described by (4) for isolating processes. After preparing the decimal dilution for the samples appeared in (Table 1).1 ml of each dilution was poured in a dish containing PDA media. The dishes were incubated at 30°C for 5 days. To isolate molds from cereals and breads, the samples were directly incubated at 30°C for 5 days in sterilized petri dishes containing PAD media. The purification process of isolates was carried out by sequential transferring into PAD media and incubation at 30°C for 5 days.

Identification of molds isolates

The cultural and morphological characteristics of mold isolates on PDA media, were carried out by using lactophenol dye and examined under microscopic field. The isolates which not belonging to the genus *Aspergillus* (according to the taxonomic keys) were discarded, (10) (14) .

Detection of aflatoxin

The ammonia vapor method, according to (21)(29), was used to examine the ability of molds isolates to produce aflatoxin, using coconut media (which was inoculated by stabbing method from young culture for 5 days).The plates were incubated at 25°C for 5 days, then filter paper was immersed in ammonia solution 25% and placed on the internal surface of the growth plate cover . The plates were incubated upside-down at 25°C and the results were recorded after 24 hours.

4- Screening of isolates

Spore suspension preparation :The spore suspension of 26 non-producing aflatoxin isolates was prepared as described by (26). The spores were accounted according to (7), using hemocytometer slide and the amount of spores for all isolates was adjusted to 10^7 spore/ml.

Screening of isolates for antioxidant production

Czapek Doxs broth was used for this purpose. 250 ml flask contains 50 ml of broth media at pH 5 and inoculation volume of 2ml spore suspension was prepared. Each ml of the spore suspension contains 10^7 spores.The inoculated flasks were incubated in a shaking incubator of 150 rpm at 30°C for 5 days. when the fermentation process was over, the supernatant was separated from the biomass using a piece of cloth according to(19)**.** The filtration process was repeated by WattmanNo.1. The biomass was dried at 50°C and stored.

Determination of total phenolic content

The phenolic compounds were determined as mentioned by (5),(6) with some modifications. Folin -Ciocalteu indicator was added to 0.5 ml of the supernatant and settled for 10 minutes at room temperature. Two ml of 7.5% $Na_2CO_3$was added to the previous mixture and settled for another 30 min at 40°C. then the absorbance was measured at 760 nm. The total phenolic content estimated using the standard curve (Fig.1).

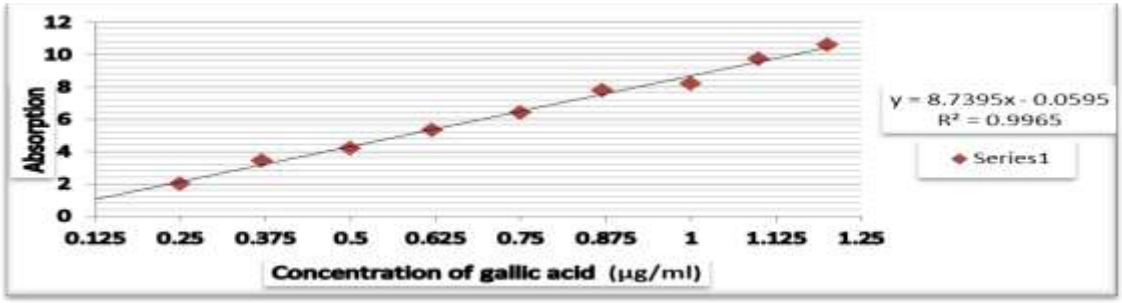

Fig. 1. standard curve of phenolic compounds

5- Molecular identification **DNA extraction**

The local *Aspergillus* sp. isolate (B1b) was cultured on PDB using shaker incubator of 150 rpm at 30°C for 5 days. 3 ml of the broth media culture was centrifuged at 13000 xg for 1 min. (the supernatant was ignored). 100-500 mg of precipitated biomass cells was taken and smashed under liquid nitrogen using a mortar. The smashed sample was transferred to a 1.5 ml Eppendorf tube. A molecular biology kit (from bio basic Inc, Canada) was used to extract the DNA from the mold isolate according to the kit extraction method (EZ-10 Spin Column Fungal Genomic DNA Mini-Preps, no.FT82012).DNA purification was estimated by Nano drop.

Primer	Sequence	GC%	Temp. C°
Forward (5.8s)	CGC TGC GTT CTT CAT CG	58.5	55.7
Reverse (ITS1F)	TCC GTA GGT GAA CCT GCG G	63.2	60.7

Polymerase chain reaction (PCR)

PCR was used to amplify the Internal Transcribed Spacer (ITS) of the rRNA 5.8s ribosomal gene in order to confirm the selected isolate type which includes the two zones ITS1and ITS2 (using the primers in table (2) as described by (13)**.**The concentration of the primer was 100 pecomole and the numbers of nitrogen bases were 17 in forward primer and 19 in the reverse one. The amplification was done in 20 µl which then added to the master mix that was supplied by the Bioneer Company as shown in Table(3)

Table 2. The sequence of used primers

Table 3. The compounds in the master mix

Compounds in the master mix	Vol.(µl)
10 P mole Forward primer	2
10 P mole Reverse primer	2
DNA extract	4
Deionized water	12
Total volume	20

The master mixture was mixed for few seconds using vortex. The tube was placed in PCR thermo cycler. The device was programmed according to table (4) and the amplification was taken place to amplify the extracted DNA.By the end of the reaction time, 5 µl of 5.8S rRNA amplifying product was withdrawn for electrophoresis assy.

Table 4. Conditions of the PCR master mixture

No.	Step	Temperature	Time	Cycles
1	Initial Denaturation	94°C	5 min.	One cycle
2	Denaturation	94°C	30 sec.	35 cycle
3	Annealing	53°C	40 sec.	
4	Extension-1	72°C	40 sec.	
5	Extension -2	72°C	5 min.	One cycle
6	Holding	4°C	∞	

The Electrophoresis of DNA amplification products on agarosegel

The PCR products were loaded on 1.5% agarose gel using a horizontal electrophoresis. 5 µl of PCR products and 2 µl of loading buffer for every each 5 µl of DNA extract. The mixture was mixed well and located into the sample gel wells. The sample was subjected to electrophoresis assay for 1 hour, 5 volt \ cm and 70 milliamps in order to initiate the movement toward negative and positive poles. The DNA bands were detected by UV light Tran's illuminator device

Determination of nitrogen bases sequence.

The gene amplifying products have been sent to korean Macrogen Company in order to determine the nitrogen bases (DNA sequencing) for the pure isolate ITS zone. The sequencing was compared with the available information on that gene with NCBI website using BLAST Nucleotides software to identify the species of the chosen isolates

6-Determination of phenolic compounds by HPLC.

he Phenolic compounds were extracted from growth media filtrate (of locally isolate) by ethyl acetate and dried. the phenolic content of dried extract was determined according to (15) using SYKMA HPLC (Germany) under the conditions below:

Column : C18-ODS (25 cm x 0.46 cm)
Mobile phase: A- Methanol: Water: Acetic acid (85: 13: 2)
B- Methanol: Water: Acetic acid (25: 70: 5)
Flow rate : 0.8 ml/min
Injection volume : 10 µl
UV Detector : 360 nm
Oven temperature : 25^{o} C

The concentration of phenolic compounds was accounted according to the equation below:

The concentration of the

$$\text{Conc.= } \mu g/ml = \frac{\text{standard x Peak area of sample}}{\text{Peak area of sample x Dilution factor}}$$

RESULTS AND DISCUSSION

Thirty four fungal isolates were obtained from different sources (Table 5). Twenty six isolates were identified as *Aspergillus* (Table 6) according to the following cultural and morphological characteristics (Table 7 and Fig, 2). It was observed that the fungal isolate formed black to gray colonies on potato dextrose agar media. It was distinguished by their heavy growth when it was incubated at 30^{o} C for 5 days. In the optical microscope field (Fig. 3), a septated mycelium was observed (10)(14). According to previous observations, the isolate was identified as *Aspergillus*. It has been reported (20) that the isolation process for any microorganism need to know the most important characteristic which represents the main target of this process. Generally, the desired characteristic is a restricted factor to isolate the microorganism from its natural media (soil, water, air, etc)ز

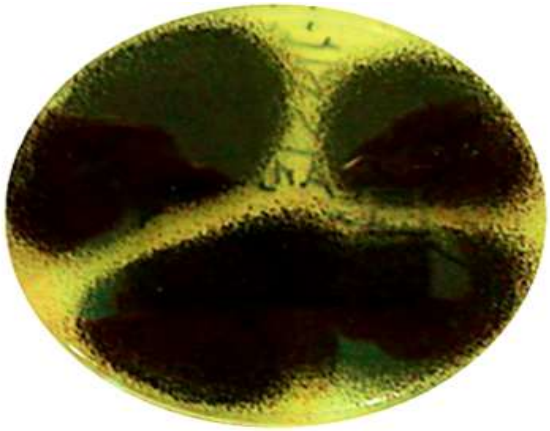

Fig. 2. The cultural characteristics of *Aspergillus*

Table 5. The sources of isolated fungi and their places and number's

Source	Isolation place	Symbol	No.
Abo Graib wheat type	Al-Zaafaraniyah	A1 (a, b, c)	3
Furat wheat type	Al-Zaafaraniyah	A2	-
Abaa wheat type	Al-Zaafaraniyah	A3	1
Local market wheat	Baghdad	A4	1
Local market barely	Baghdad	A5	1
Local white corn	Baghdad	A6	1
Local yellow corn	Baghdad	A7 (a, b, c, d)	4
Local spoiled bread	Baghdad	A8	1
Spoiled bread	Baghdad	A9	1
Garden soil	Thi' Qar	B1 (a, b, c, d, e, f, g)	10
		B2 (a, b, c)	
Agricultural soil	Thi' Qar	B3	-
Desert soil	Thi' Qar	B4	1
Garden soil	Baghdad	B5	1
Desert soil	Baghdad	B6	-
Local market spoiled apple	Baghdad	C1	-
Local market spoiled plum	Baghdad	C2 (a, b, c, d,e)	
		C3	6
Local market spoiled tomato	Baghdad	C4	1
Local market spoiled orange	Baghdad	C5, C6	2

Table 6. Number of isolates belonging to the genus *Aspergillus* sp. and their source, code and number's

Source	Isolation place	Isolated Type	Symbol	No.
Abo Graib wheat type	Al-Zaafaraniyah	*Aspegillus*	A1 (a, b, c)	3
Abaa wheat type	Al-Zaafaraniyah	*Aspegillus*	A3	1
Local market barely	Baghdad	*Aspegillus*	A5	1
Local spoiled bread	Baghdad	*Aspegillus*	A8	1
Spoiled bread	Baghdad	*Aspegillus*	A9	1
Garden soil	Thi' Qar	*Aspegillus*	B1 (a, b, c, d, e, f, g)	9
			B2 (a, b)	
Desert soil	Thi' Qar	*Aspegillus*	B4	1
Garden soil	Baghdad	*Aspegillus*	B5	1
Local market spoiled plum	Baghdad	*Aspegillus*	C2 (a, b, c, d, e)	
			C3	6
Local market spoiled orange	Baghdad	*Aspegillus*	C5, C6	2

Table 7. The most important cultural and morphological characteristics of the local *Aspergillus* sp. Isolates

Characteristic	Observations
Colonies	Black, heavy growth on PDA at 30^{o} C after 5 days
Mycelium	Septated
Conidial heads	Black
Vesicle	Spherical shape
Conidia	The mature is large, immature is small

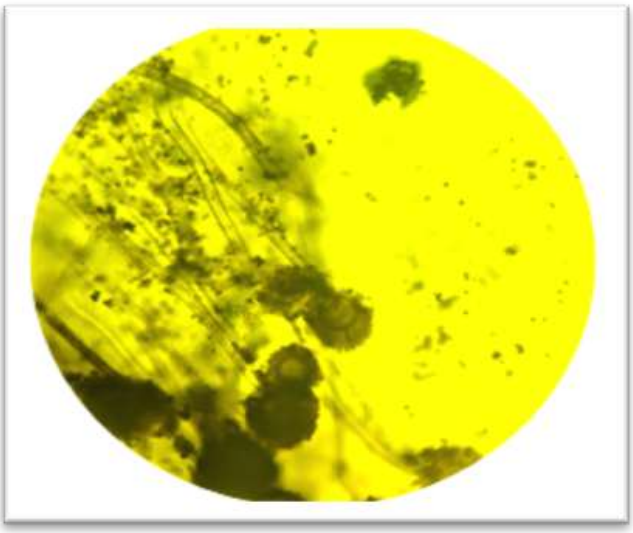

Fig. 3. The morphological characteristics of *Aspergillus* as shown in the microscope field

Examine mold isolates for aflatoxins production

The ability of the previous 26 *Aspergillus sp*. isolates was examined for aflatoxins production. The results show a negative response through no color was changed on coconut extract agar media. The positive response is accompanying with color changing from white to pink. The results were in agreement with what were found by (1),(3) that the results of aflatoxins production were negative for *A. niger*, *A. fumigates*, *Alternaria sp*.; *Fusarium sp*.; *Trichoderma sp*.; *Penicillium sp*.; but were positive for *Aspergillus flavus* and *Aspergillus terreus*.

Screening of *Aspergillus* isolates

A screening for 26 *Aspergillus* isolates in Czapek Dox broth (Table 8)was carried out depending on the total phenolic compounds concentration. The results showed that all the isolates were phenolic compounds producier. The *Aspergillus sp*. B1b isolate showed high phenolic compounds concentration (45.3µg/ml, after 5 days of incubation). The results of the present study were in agreement with what was found by (18), that the genuses of *Aspergillus*, *Penicillium* and *Rhizopus* have the ability to produce antioxidant compounds. Also found, that the varieties which were belong to the same species are differs in their

metabolism to give antioxidant compounds. Astudy (30) reported that *A. candidus*, *P. oxalicum* and *Aspergillus sp*. are good sources of highly active antioxidants (with preference to *A. candidus*).(12), others have found that *A. saitol* had high ability to give phenolic compounds as compared with 30 isolates of *Aspergillus*. Researcher (8) stated that *A. niger* A-12 has the ability to produce antioxidant compounds with high activity.

Table 8. A screening for 26 *Aspergillus* isolates in Czapek Dox broth depending on the total phenolic compounds concentration

	Isolate symbol	Total phenolic Compounds, µg/ml
1	B1a	16.50
2	B1d	23.40
3	B1f	28.10
4	B5	22.20
5	A1	27.20
6	B1b	45.30
7	B1g	26.40
8	B1e	18.00
9	A9	17.70
10	A8	22.70
11	B4	22.80
12	B1c	20.60
13	C3	16.50
14	A5	22.70
15	A1a	26.80
16	A1b	25.50
17	B2a	20.50
18	C2e	22.14
19	C5b	19.14
20	A3	13.80
21	B2b	20.40
22	C2b	19.30
23	C2c	19.60
24	C2a	20.90
25	C2d	24.00
26	C5a	15.80

Molecular Identification

DNA extraction.

The DNA was extracted from *Aspergillus sp***.** B1b and the purity of DNA was examined by Nano Drop with a purity of 1.99 which is adequate for Polymerase Chain Reaction (PCR) process. Green and(24) reported that the PCR did not need a large quantity of DNA which may instead produce unlimited amplifying products. On other hand, an adequate quantity of DNA may reduce the accuracy.

Polymerase Chain Reaction (PCR)

A PCR for the local *Aspergillus sp*. B1b for the internal transcribed spacer (ITS) which were represented by ITS1 and ITS4 that were located within the ribosomal gene (5.8S ribosomal RNA-rRNA) was carried out. The electrophoresis on 1.5% agaros show (by using U.V detector), that there was a clear band represents the genes amplifications (fig,4). The molecular size of gene amplification band was 300bp comparing with ladder size at the same conditions, which refers to the prime binding to the complete sequence in DNA pattern. The interference space, ITS1and ITS4 ribosomal unit 5.8s rRNA was used to distinguished the various species of fungi, with accuracy identification result

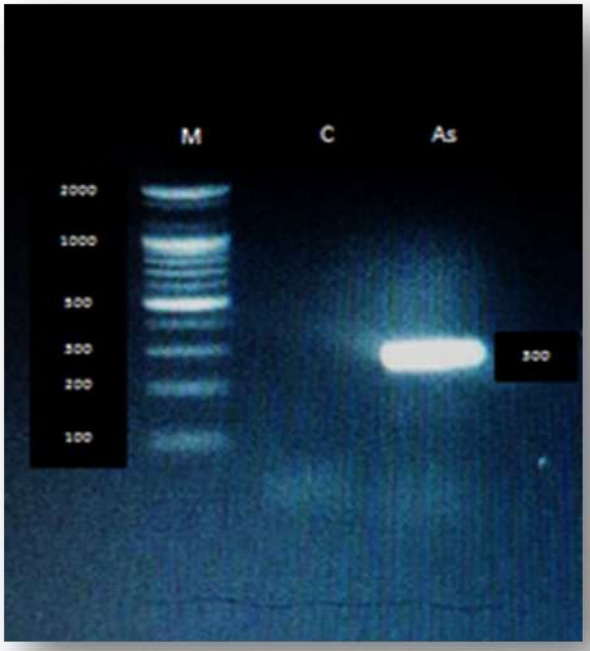

Fig. 4. Electrophoresis for the local *Aspergillus* sp. B1b for the internal transcribed spacer (ITS) on agaros gel.

Sequence analysis of amplification products

The sequence of nitrogen bases, of the internal transcribed spacer ITS1, for the local mold isolate (*Aspergillus sp*. B1b) was studied by sending the amplification products to the Korean company Macrogen. The nitrogen bases sequence (240 base-pair) which was taken from the local isolate sequence (of the present study) is shown in (Fig, 5) . The PLAST program has been used to find out the

similarity of gene with the bank information (NCBI). The results showed that there is a match between isolation and 100% with global isolation sequences global ID: JF 436883.1 Registered on the NCBI website and registered in the United States of America Which belong to *Aspergillus niger strain* YMCHA 55. (Fig, 6)

CCCTGTTGCTTCGGCGGGCCCGCCGCTT
GTCGGCCGCCGGGGGGGCGCCTTTGCC
CCCCGGGCCCGTGCCCGCCGGAGACCC
CAACACGAACACTGTCTGAAAGCGTGC
AGTCTGAGTTGATTGAATGCAATCAGT
TAAAACTTTCAACAATGGATCTCTTGGT
TCCGGCATCGATGAAGAACGCAGCGA

Fig.5. Sequence for the local *Aspergillus sp*. B1b for the internal transcribed spacer (ITS)

FORWARD (5.8s):
GCTTCGGGAGTGCGGGGTCTTTGGGCC
ACCTCCCATCCGTGTCTATTATA

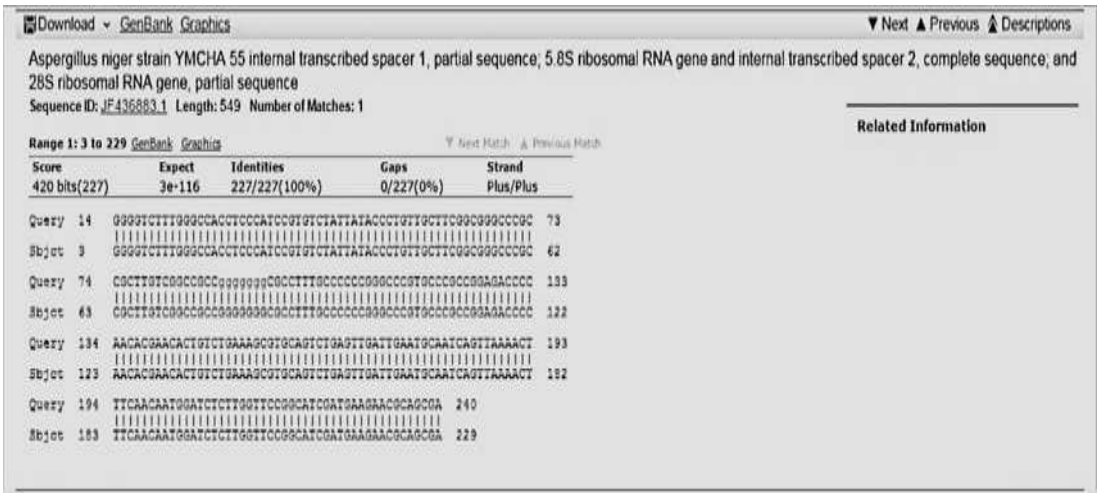

Fig. 6. Match the sequence of nitrogen basesfor internal transcribed spacer (ITS) for the local *Aspergillus sp*. B1b within the ribosomal gene (5.8S ribosomal RNA-rRNA) with global isolate *Aspergillus niger strain* YMCHA 55

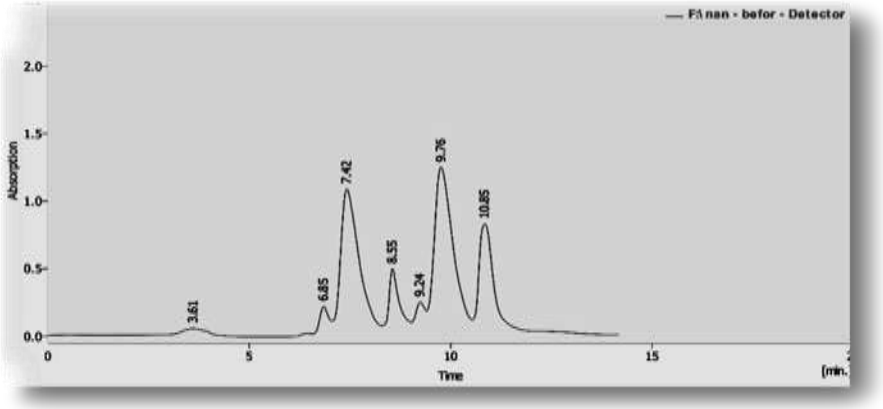

Fig. 7. HPLC diagram of the extract phenolic compounds

Isolation and identification of filtrate phenolic compounds by HPLC

Fig.7, show the isolation and identification of phenolic compounds ethyl acetate extract for the filtrate by HPLC. It was found that the filtrate extract contains the following phenolic compounds, gallic acid, cumarin, rutin and kampferole with concentrations of 47.69, 6.60, 218.30, and 22.00µg/ml respectively by comparative with standard phenolic compounds Fig 8. ,Fig. 9 ,Fig.10 ,Fig.11

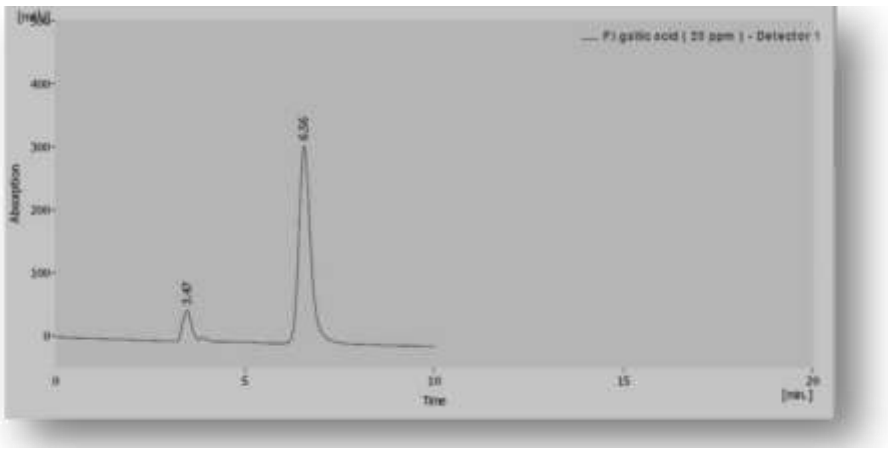

Fig. 8. HPLC diagram of the standard gallic acid

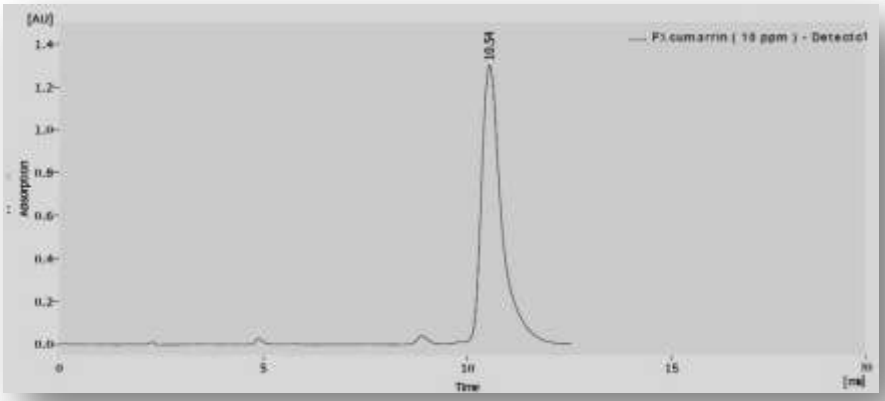

Fig. 9. HPLC diagram of the standard cumarin

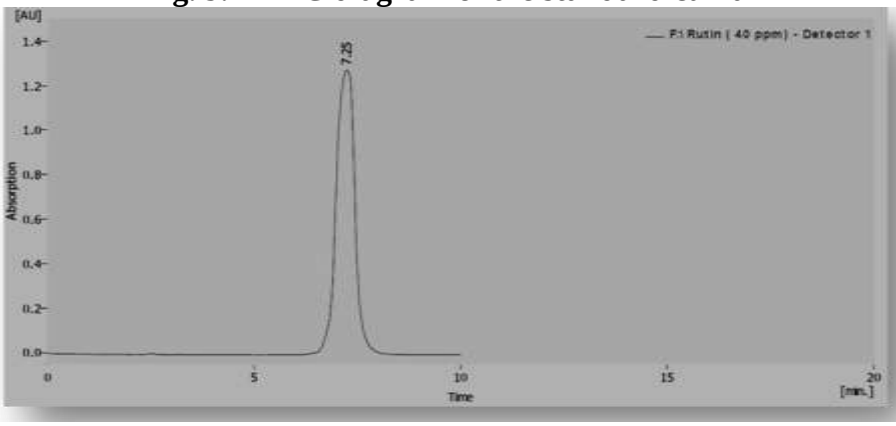

Fig. 10. HPLC diagram of the standared rutin

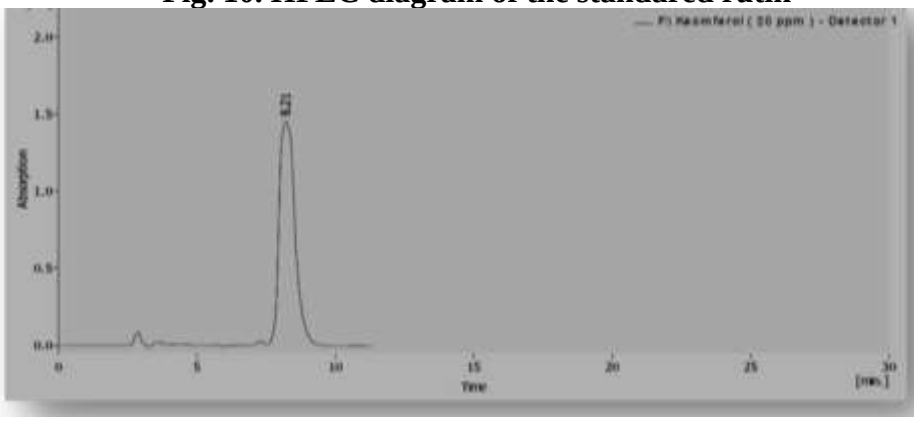

Fig. 11. HPLC diagram of the standared kampferole

REFERENCES

1. Abdul Karim, N. F. 2011. The Purification and Characterization of An Enzyme Glutaminase Product of Local Isolation *Aspergillus niger*. M.Sc. Theses. college of Agriculture. University of Basra. pp:68

2. Aksu, K.; B. Özgeriş; P. Taslimi; A. Naderi; Gülçin, İ.; and S. Göksu. 2016. Antioxidant activity, acetylcholinesterase, and carbonic anhydrase inhibitory properties of novel ureas derived from phenethylamines. Archiv der pharmazie. 349(12): 944-954

3. Al-Dwahery, N. H. K. 2011. Production, Purification and Characterization of Inulinase from Local Fungal Isolate of *Aspergillus niger*.PH.D. Dissertation College of Agriculture. University of Basra.pp:85

4. APHA: American Public Health Association. 1978. Standard Methods for the Examination of Dairy Products. 14th Ed. Washingtion, DC, USA. pp.449

5. Arora, D. S. and P. Chandra. 2010. Assay of antioxidant potential of tow *Aspergillus* isolates by different methods under various physo-chemical condition. Brazilian Journal of Microbiolog . 41: 765-777

6. Ayoola,G.A.; S.S. Ipav; M.O. Sofidiya; A.A. Adepoju Beello; H.A. Coker; and T.O. Odugbemi; 2008. Phytochmical screening and free radical scavenging activities of the fruits and leaves of allanblackia floribuna oliv (Guttiferae) . International Journal of Health Research. 1(2):87-93

7. Bind, A., S. K. Singh; V. Prakash; and M. Kumar. 2014. Evaluation of antioxidant through sold state fermentation from pomegranate peels using *Aspergillus niger* and it's antibacterial properties. International Journal of pharmacy and biological science. 4(1): 104-112

8. Cheynier, V.; G. Comte; K. M. Davies; V. Lattanzio; and S. Martens. 2013. Plant phenolics: recent advances on their biosynthesis, genetics, and ecophysiology. Plant Physiology and Biochemistry. 72: 1-20

9. Crozier, A.; I. B. Jaganath,; and M. N. Clifford. 2006. Phenols, polyphenols and tannins: an overview. Plant secondary metabolites: Occurrence, structure and role in the human diet, chap.1, 18. pp:25

10. Diba, K. and G. Buzsáki. 2007. Forward and reverse hippocampal place-cell sequences during ripples. Nature neuroscience. 10(10): 1241

11. Dulf, F. V.; D. C. Vodnar; E. H. Dulf ; and A. Pintea. 2017. Phenolic compounds, flavonoids, lipids and antioxidant potential of apricot *(Prunus armeniaca L.)* pomace fermented by two filamentous fungal strains in solid state system. Chemistry Central Journal. 11(1): 92

12. Esaki,H.; H. Onozaki; S. Kawakishi; and T. Osawa. 1997. Antioxidative activity and isolation from soybeans fermented with *Aspergillus* spp. J. Agric. Food Chem. 45(6): 2020 -2024.

13. Fierer, N.; J. A. Jackson; R. Vilgalys; and R. B. Jackson. 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Applied and environmental microbiology. 71(7): 4117-4120

14. Gugnani, H. C. 2003. Ecology and taxonomy of pathogenic aspergilli. Front Biosci. 8(1-3),pp 346

15. Gupta, M.; S. Sasmal; S. Majumdar; and A. Mukherjee; 2012. HPLC profiles of standard phenolic compounds present in medicinal plants. International Journal of Pharmacognosy and Phytochemical Research. 4(3):162-167

16. Huyut, Z.; Ş. Beydemir; and İ. Gülçin, . 2017. Antioxidant and antiradical properties of selected flavonoids and phenolic compounds. Biochemistry research international. 2017:10

17. Ibrahim, M. M.; G. A. Mersal; A. M. M. Ramadan; S. Y. Shaban; M. A. Mohamed; and S. Al-Juaid. 2017. Synthesis, characterization and antioxidant/cytotoxic activity of oxovanadium (IV) complexes of methyliminodiacetic acid and ethylenediaminetetracetic acid. Journal of Molecular Structure. 1137: 742-755

18. Ishikawa,Y. 1992. Development of new types of antioxidants from microbial origin. J. Jpn. Oil Chem. Soc. 41: 762 -767

19. Jakovljević, V. D.; J. M. Milićević; J. D. Stojanović; S. R. Solujić; and M. M. Vrvić. 2014. Antioxidant activity of ethanolic extract of Penicillium chrysogenum and Penicillium

fumiculosum. Hemijska industrija. 68(1): 43-49

20. Jernejc, K. and A. Cimerman. 2001. Morphological characteristics, extracellular and intracellular protein and enzyme patterns of five *Aspergillus species*. Food technology and Biotechnology. 39: 333-40

21. Kumar, S.; M. Shekhar; K.A. Ali and P. Sharma. 2007. A rapid technique for detection of toxigenic and non-toxigenic strain of Aspergillus flavus from maize grain. Ind. Phytopathol. 60(1): 31-34

22. Prauchner, C. A. 2017. Oxidative stress in sepsis: Pathophysiological implications justifying antioxidant co-therapy. Burns. 43(3): 471-485

23. Rahal, A.; A. Kumar; V. Singh; B. Yadav; R. Tiwari; S. Chakraborty; and K. Dhama. 2014. Oxidative stress, prooxidants, and antioxidants: the interplay. BioMed Research International. 2014: 9

24. Sambrook, J. and R. M. Green. 2012. Molecular Cloning: A Laboratory Manual, Fourth Edition. CSHL Press

25. Shedbalkar, U.; R. Dhanve; J. Jadhav. 2008. Biodegradation of triphenylmethane dye cotton blue by Penicillium ochrochloron MTCC 517. Journal of Hazardous Materials. 157(2): 472-479

26. Silveira , S.T. ; M.S. Oliveira ; J.A.V. Costa; and S.J. Kalil. 2006 Optimization of glucoamylase production by *Aspergillus niger* in Solid state fermentation . Applied Biochemistry and Biotechnology. 128:131-139

27. Takaidza, S.; F. Mtunzi; and M. Pillay. 2018. Analysis of the phytochemical contents and antioxidant activities of crude extracts from Tulbaghia species. Journal of Traditional Chinese Medicine. 38(2) : 272-279

28. Wu, S.; and J. Chappell. 2008. Metabolic engineering of natural products in plants.; tools of the trade and challenges for the future. Current Opinion in Biotechnology. 19(2): 145-152

29. Yazdani, D.; A. M. Zainal; Y. H.Tan; and S. Kamaruzaman. 2010. Evaluation of the detection techniques of toxigenic Aspergillus isolates. African Journal of Biotechnology. 9(45): 7654-7659

30. Yen,G.C. and C.A. Lee. 1996. Antioxidant activity of extracts from molds. J. Food Prot. 59(12):1327-1330.