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RESEARCH ARTICLE

Antioxidant and Antimicrobial Activities of Phenolic Compounds Producing from Local Isolate *Aspergillus niger*

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Abstract

The aim of the present research was to examine the phenolic contents; antioxidant and antimicrobial activities of alcoholic extract for *Aspergillus niger* biomass. The Phenolic compounds were extracted from biomass of isolate by Ethel alcohol, and identified BY using the high performance liquid chromatography analysis (HPLC). The results were compared with the standard phenolic compounds. This study has found that generally, the biomass extract contains gallic acid, rutin and kampferole. However, the phenolic compounds were extracted by different solvents including: ethyl acetate, methanol and ethanol. The highest concentration of phenolic compounds (264.3.µg/ml) was obtained from the ethanolic extract. Ethanolic extract was screened for antimicrobial activity by using the holes diffusion method against five selected pathogenic bacterial strains which include each of *Staphylococcus aureus, Bacillus cereus, Pseudomonase, Escherichia coli*, and *Klebsella pnuemoniae*. We found that compared to G- bacteria, the extract shows high antimicrobial activity against G+ bacteria. On the other hand, Antioxidant activity was determined by measuring the reduction power, and 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging activity. Moreover, we found that the antioxidant activity had been maximizing ethanolic biomass extract (106.1, 92) % respectively, while ethyl acetate and methanol extracts (59.5, 10) % and (49.4, 5.5) % respectively.

Keywords: *Antioxidants, Antimicrobial, DPPH, Reducing power, HPLC, Aspergillus niger.*

Introduction

Recently, there has been renewed interest in the antioxidants. They can act as radical scavengers, and inhibit lipid peroxidation and other free radical mediated processes; therefore, these are able to protect the human body from several diseases attributed to the reaction of radicals. Recently, the using of synthetic antioxidants in order to prevent the free radical damage has been reported to involve toxic side effects, necessitating the search for natural antioxidants and free radical scavengers [1].

Numerous pharmaceutical properties of medicinal mushrooms which have been used in the traditional oriental medicine are known, and they include anticancer, antimicrobial, anti-inflammatory and anti-atherosclerotic. In western civilization, the research on medicinal properties of fungi and yeast are relatively new, and their uses of therapeutic purposes can be new as well. These fungi were considered as a significant source of natural antioxidants due to their production of secondary metabolites. Phenolics or polyphenols, including flavonoids are the main secondary metabolites of medicinal plants, mushrooms and fungi. Moreover, they can be responsible for their antioxidant, antimutagenic and antitumor activity [2, 3]. Screening of biological activity of endophytic fungi showed that they represent a significant source of new bioactive agents with potential use in medicine, agriculture and industry area.

Biologically active ingredients are synthesized in the apex tissue of the hyphal strand of fungi and their extractions are carried out by solvents with different polarity. The biological activity of these substances depends on their chemical structure so that different extraction solvents resulted in various biologically active substances, with different levels of bioactivity [4].The methanolic extracts of many fungi, such as *Fusarium, Aspergillus, Penicillium* and *Mucor* species isolated from *L.*

nicotianifolia showed significant antioxidant potential and the antioxidant nature of the extracts depended on the concentration [5].Many species of fungi isolated from the soil, like *Aspergillus fumigatus*, represent a potential source of natural antioxidants [6]. The potential antioxidant activity of fungal species: *Aspergillus niger* was investigated in the current study. The fungi were isolated from Total phenolic content and determined by using the Folin-Ciocalteu method. The antioxidant activity of alcoholic extract of fermentation broth was carried out by four assays: DPPH free - radical scavenging activity, total antioxidant activity, Fe2+-chelating ability, and Fe3+-reducing power.

Materials and Methods

Mold Isolates

The isolates were obtained from [7].

Screening of Isolates for Phenolic Compounds 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 107 spores. The inoculated flasks were incubated in a shaking incubator of 150 rpm at 30oC for 5 days. When the fermentation process was over, the supernatant was separated from the biomass using a cheese cloth according to [8]. The filtration process was repeated by WattmanNo.1. The biomass was dried at 50oC and grinded.

Phenolic Compounds Extraction

The extraction process was made by using three methods; each one of them has a different type of solvent. In the first method, dried mycelium was extracted with ethanol (1:1, v/v) three times. The supernatant was separated by centrifugation at 5000 rpm for 10 min, fractions were pooled and the ethanolic extract was concentrated by rotary evaporator and stored in dark at 4 °C before being used for the bioactivity test [9]. In the second method, phenolic compounds were extracted according to the method indicated [10] by ethyl acetate and left for a period at room temperature and then filtered under pressure.

At the last method, dried powdered materials (10g) were then extracted with 100ml of methanol by using cold percolation for 24 hours. The obtained extract was filtered by using Whatman No. 1 filter paper, and then concentrated under vacuum at 40C° by using a rotary evaporator [11]. The extracts from the three methods were kept at 4 ° C for use in the following tests.

Determination of Total Phenolic Content

The phenolic compounds were determined as mentioned by [12] 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% Na2CO3 was added to the previous mixture and settled for another 30 minutes at 40°C. Then, the absorbance was measured at 760 nm. While the total phenolic content was estimated by using the standard curve (Fig.1).

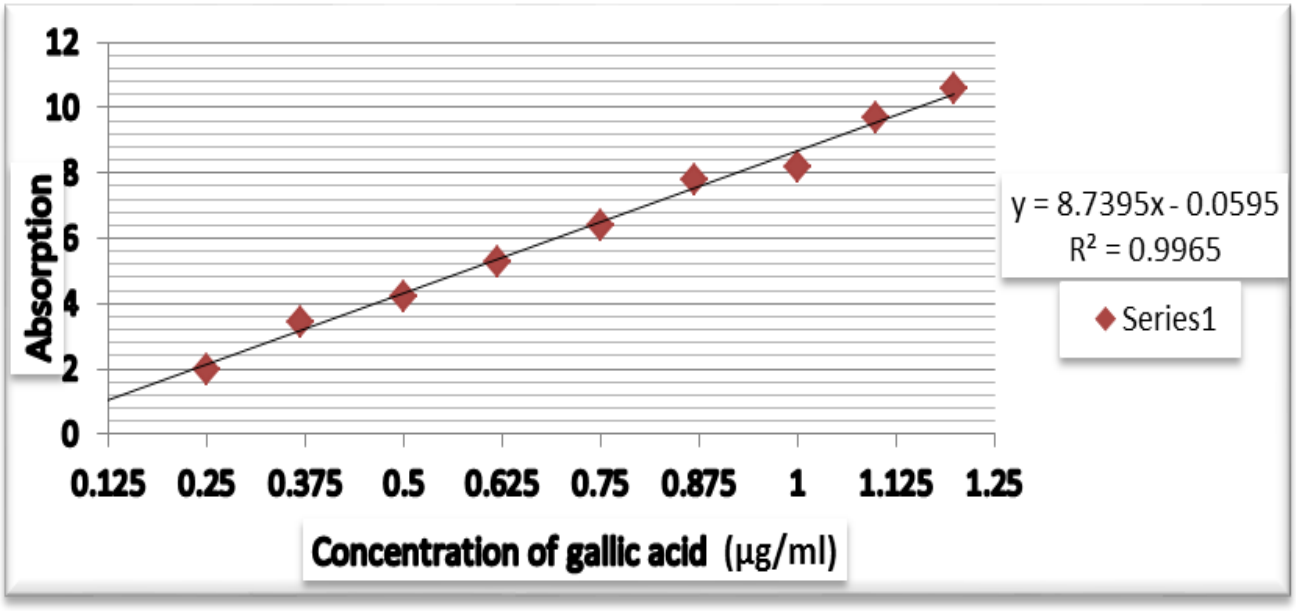

Fig. 1: Standard curve of phenolic compounds

Determination of Phenolic Compounds by HPLC

The Phenolic compounds were extracted by ethyl acetate was determined according to [13] 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° C

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

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

The Inhibitory activity of the Biomass Extract against Some Bacteria

Pathogenic Bacterial Isolates

Six bacterial isolates including: *Bacillus cereuses, Bacillus subtillus, E. coli, Pseudomonas, Staphylococcus aureus, Klebsiella pneumonia, were* obtained from the College of Science / Basrah University for studying the inhibitory activity of ethanol extract. The method of [14] was applied in this experiment depending on the good diffusion method and measurement inhibition Clear Zone area.

Antioxidant Assay

DPPH Free Radical Scavenging Activity

A fresh solution of DPPH 0.15 was prepared, wrapped in aluminum foil and kept in dark to prevent autoxidation. One ml of ethanol extract was added to1 ml of DPPH solution, the mixture was shaken vigorously and allowed to stand in the dark for 1 hour and subsequently, its absorbance was measured at 517 nm.

A blank solution containing only the solvent without the extract. All determination was performed in triplicate and the radical scavenging activities of the test and standard samples expressed as a percentage of inhibition were calculated according to the following equation [15]:

% Inhibition of DPPH activity= A_b- A_a/ A_b ×100

Where: A_b absorbance of the blank (solution without the extract or standard). A_a Absorbance of the extraction.

Reducing Power Assay

The extractive strength of the extract was estimated according to the method of [16], which included mixing 1 ml of an extract with 2.5 ml of Potassium Fericyanide solution, and 2.5 mL of phosphate soluble solution with 0.2 molars at pH 6.6, followed by incubating mixture (TCA) Trichloro acetic acid 10%.

Centrifuge process was carried out at 4000 rpm for 10 minutes, after mixing 2.5 mL of filtrate with 2.5 ml of distilled water 0.5 ml of 0.1% ferric chloride, and a control sample was prepared by add all the substances except the extract, leave the mixture for 30 minutes and then measured the absorption at 700 nm, used a spectrophotometer, and the followed equation was applied to calculate the reduction force [17].

Reducing Power= [(B-A)/B]×100

A: Read the absorbance of the sample.

B: Read the absorption of the control sample**.**

Result

Determination of Total Phenolic Compounds for Biomass Extracts

Fig.(2) Illustrates the effect of using different solvents for all phenolic compounds produced by *Aspergillus niger* B1b which was incubated at 30 C° for 9 days. There was a clear variety in the concentration of phenolic compounds with the different solvents.

The results showed that the highest concentration of phenolic compounds was obtained by using ethanol as solvent

compared to methanol and ethyl acetate, With a concentration of (264.3, 147.5 and 50) µg / mL respectively. This was due to the difference in the chemical nature of the active compounds in the various extracts and the difference in polarity of the solvents [18].

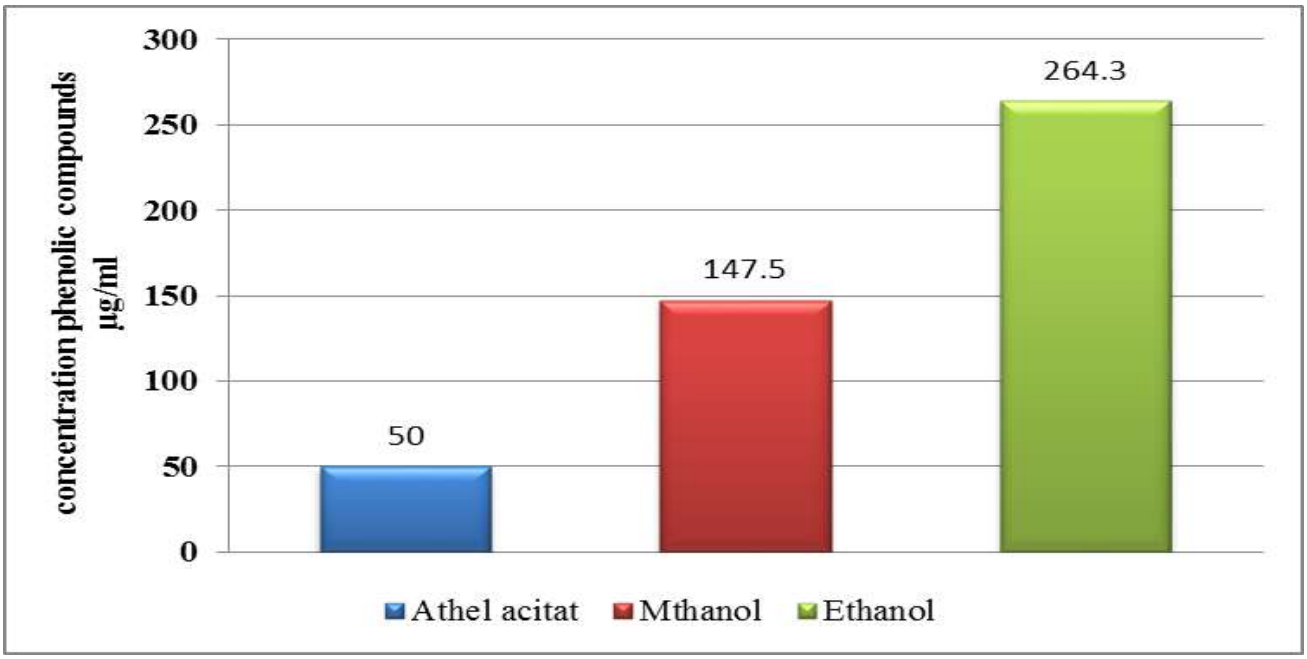

Fig.2: Concentration of total phenolic compounds of biomass extracts

[19] Pointed out that the choice of the appropriate extraction method depends on many factors, including the characteristics of the microorganism, costs of the production process, feasibility, time was taken as well as the purpose of the final product. [20] noted that the use of different extraction methods affects antioxidant effectiveness by influencing the effective compounds or aggregates produced by the mold, he also noted that the use of methanol was very effective in the extraction of phenolic compounds produced with liquid state ferments from the molds Grifola frondosa, Monascus purpureus, Pleurotus spp, Lentinula edodes, Trametes versicolor.

Isolation and Identification of Phenolic Compounds by HPLC

Fig. (3) Shows the isolation and identification of phenolic compounds ethanol extract for the biomass by HPLC. It was found that the biomass extract contains the following phenolic compounds, gallic acid, rutin and kaempferol with concentrations of (28.9, 0, 128.2, 30.6) mcg/ml respectively for biomass extract. µg/ml respectively by comparative with standard phenolic compounds Fig. (4), Fig. (5), Fig. (6).

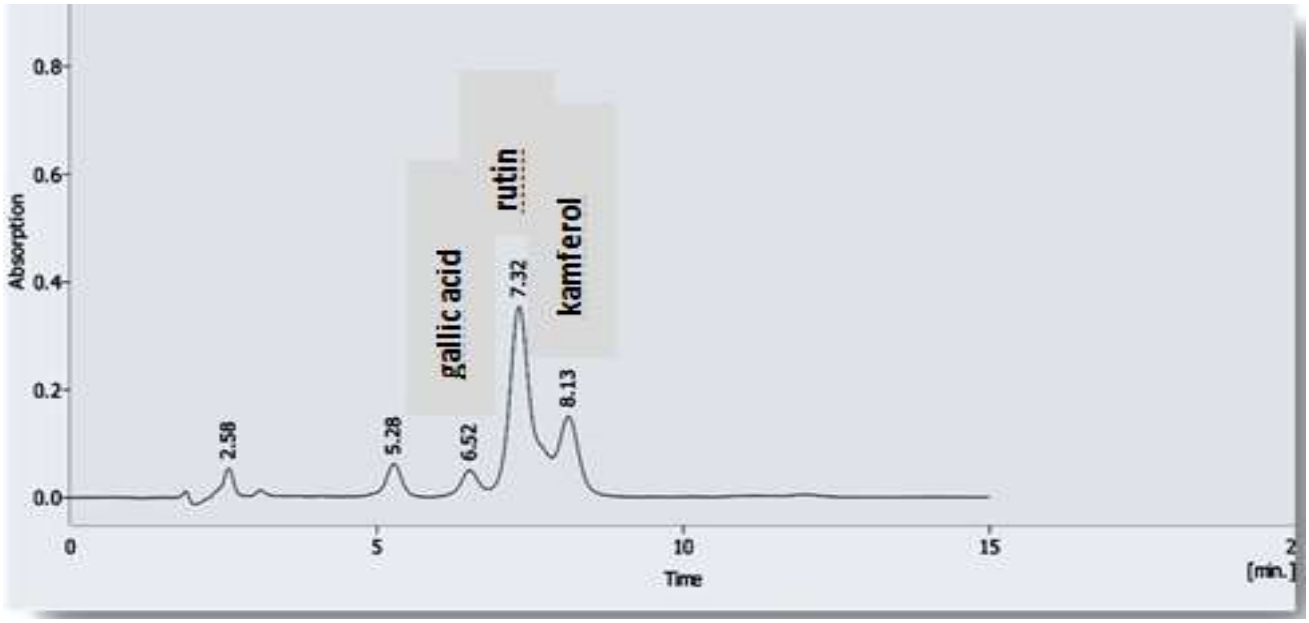

Fig.3: HPLC phenolic compounds for the ethanolic extract of biomass

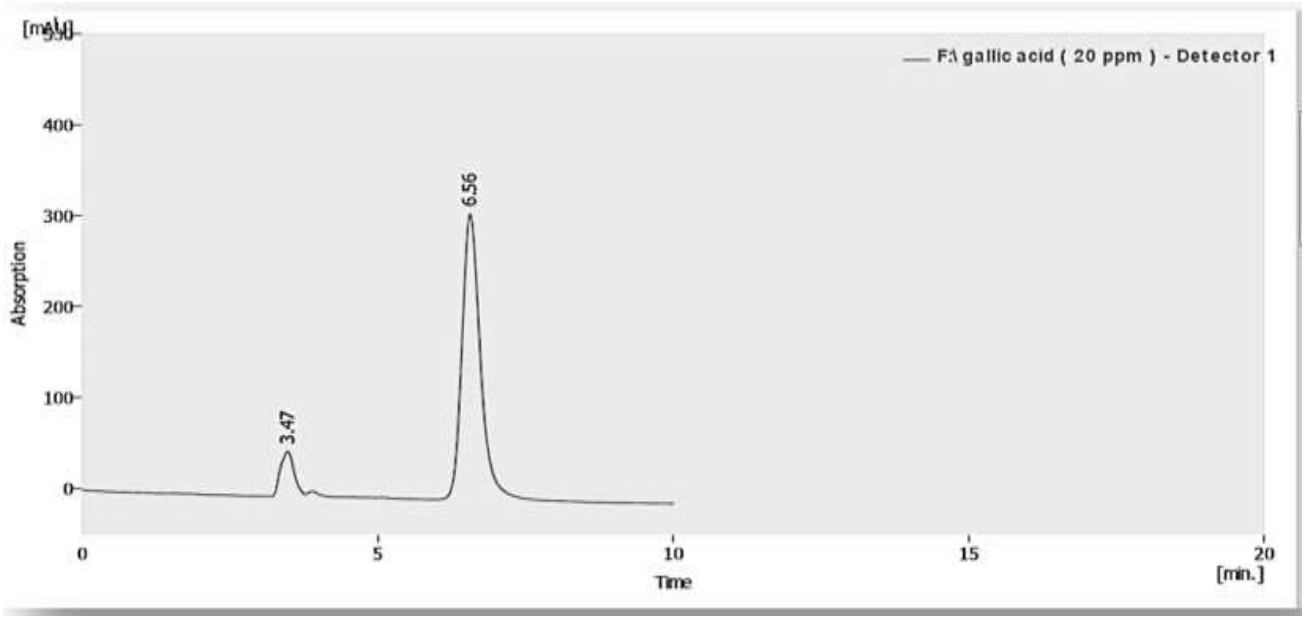

Fig. 4: HPLC diagram of the standard gallic acid

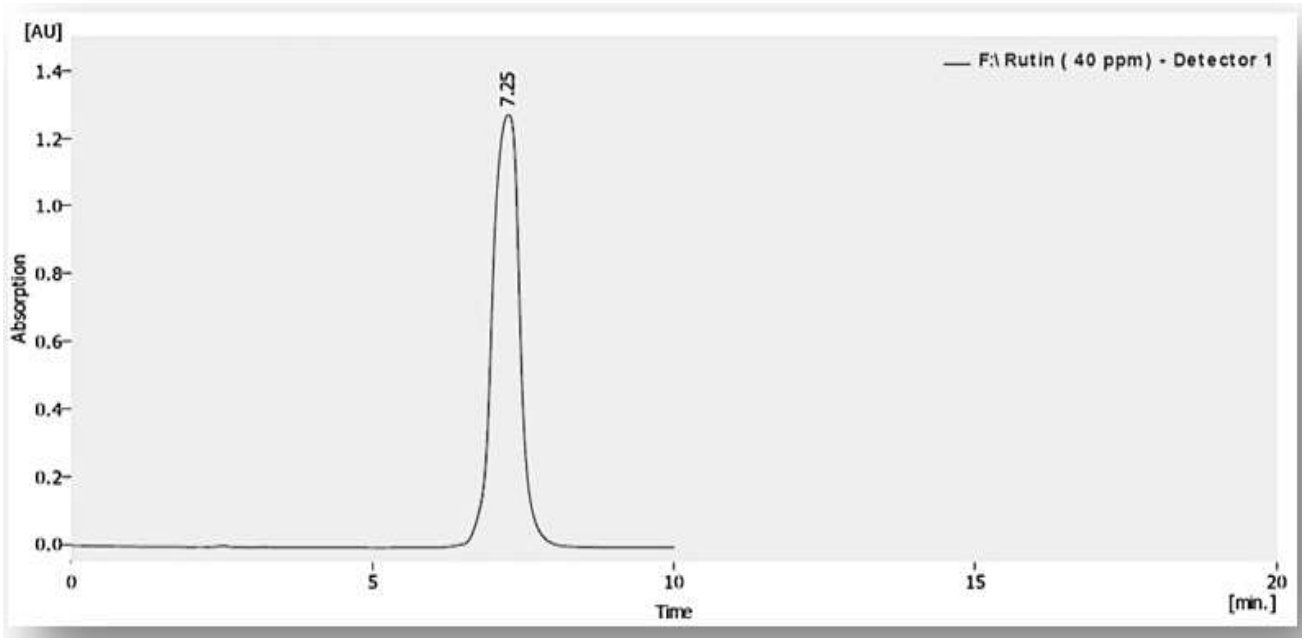

Fig.5: HPLC diagram of the standard rutin

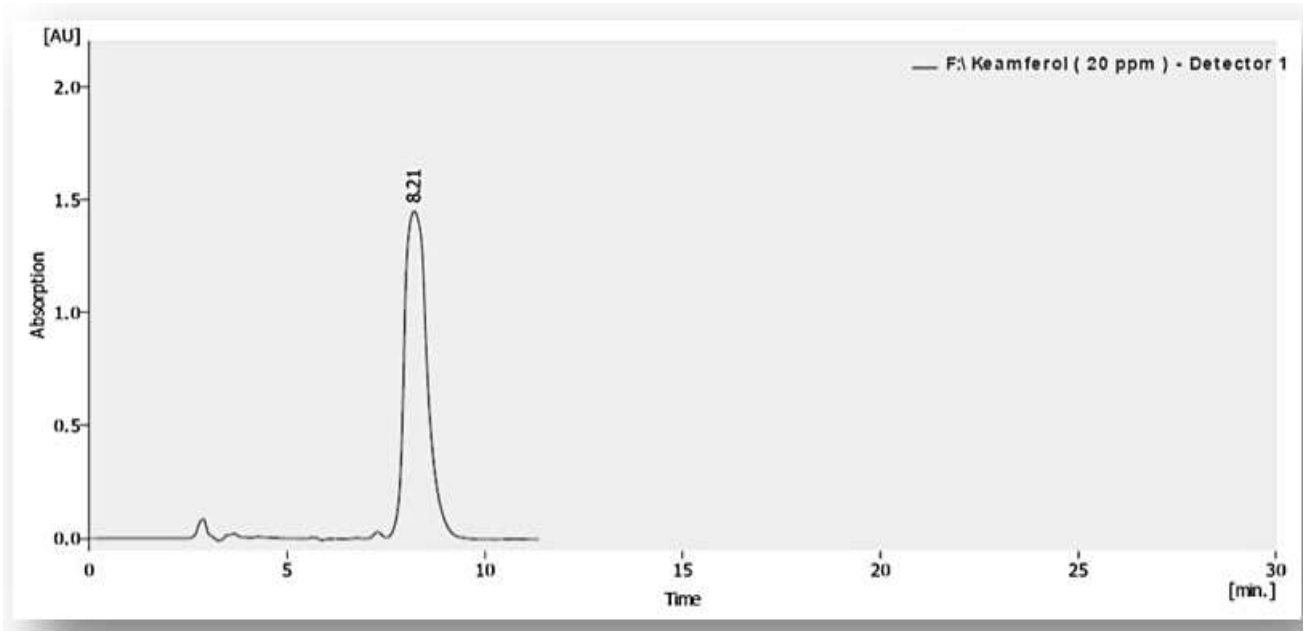

Fig.6: HPLC diagram of the standard kampferole

Determination of Inhibitory Activity against Microorganisms

The results of the present study indicate that the ethanol extract of the biomass is an inhibitory effect of the experimental species of positive and negative bacteria as shown in Table (1) and Fig [7].The results shows highest inhibition for ethanol extract against *S. auruas* and *B. cereus* with diameter of halo inhibition 20 and 19 mm respectively.

Table 1: The inhibitory effect of biomass ethanol extract against some bacteria

	Diameter of halo inhibition (mm) Of the Gram-negative bacteria			Diameter of halo inhibition (mm) For Gram positive bacteria	
Type of extract	*K.pnuemonae*	*E. coli*	*Pseudomonas*	*B. cereus*	*S. auruas*
Ethanolic extract of biomass	7	11	16	19	20

The difference in the sensitivity of Gram-positive bacteria to the Gram-negative bacteria may be due to the difference in cell wall cell structure, which makes *E. coli* more resistant. This is consistent with [21] Gram-negative cell wall is more complex where the lipopolysaccharides have more resistance, as well as the presence of periplasmic space between the cytoplasmic membrane and the cell wall, which is more pronounced in Gram-negative cell.

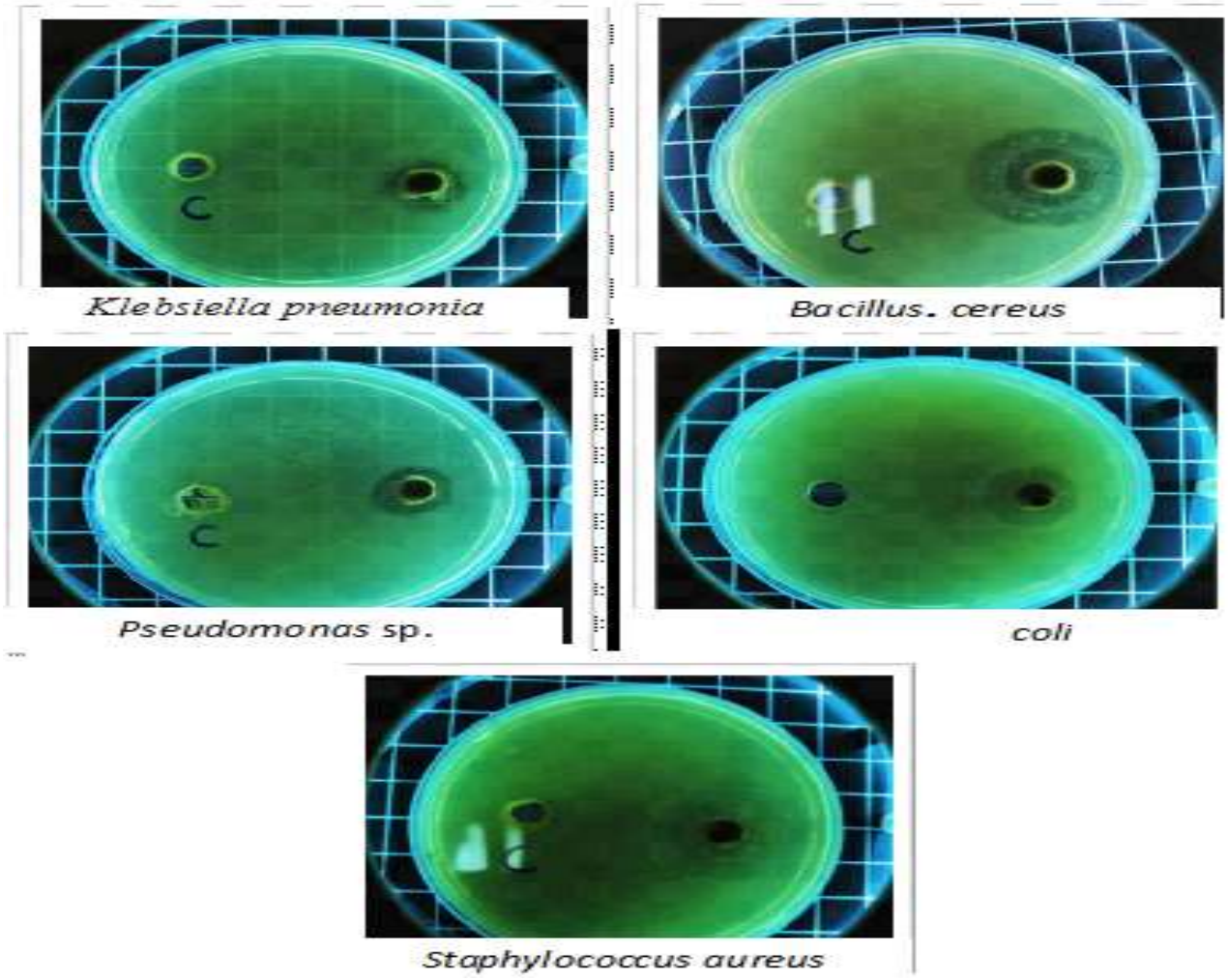

Fig. 7: Shows the inhibitory effect of ethanolic extract on atype of Gram negative and Gram positive

[22] Noted that the secondary metabolites of the *Cochliobolus intermediusdljg* mold possess antimicrobial efficacy such as E. coli, *S. aureus, S. typhi*, and other bacterial species. Also, [23] found that the phenolic and flavonoid compounds of the water extract of the *Aspergillus niger* fermentation medium produced from the pomegranate peel solid state ferments have a high ability to inhibit *Klebsiella pneumonia, Escherichia coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa.* The phenolic compounds have a wide modus operandi towards pathogenic and unsatisfactory microorganisms as they act as antifungal and fungicidal antibiotics [24, 26] and viruses, due to their ability to form

hydrogen bonds or covalent or hydrophobic, which prevents the adhesion of microorganisms or inhibit the construction of DNA due to its association with the rules of nitrogen nucleic acid or by blocking the transport proteins [27].

Determination of Antioxidant Activity of *Aspergillus niger* B1b

The antioxidant activity of the *A. niger* B1b extract is linked to the number of hydrogen atoms available for the leachate by hydroxyl groups [28]. While [29] indicates that the high efficacy of antioxidants arises from the synergistic effect of Extracts, [30] show that the antioxidant effect of the ethyl acetate extract is due to various mechanisms, including the disruption of the reaction starting chain, the bonding of iron ions, the breakdown of peroxides, the discontinuation of hydrogen removal, The effectiveness of antioxidants increases as the extract contains higher concentrations of antioxidant compounds [31].

Determination of Antioxidant activity by Scavenging DPPH:

The use of the free radicals method to estimate the antioxidant efficacy of the extract can be considered as a useful analysis to understand whether the extract has the ability to prevent oxidation in the start-up phase by the ability to neutralize or prevent the formation of free radicals [23]. Fig.(8) Shows the efficiency of biomass ethanolic extract by scavenging DPPH compared with other solvents, with a removing rate of 92% and 49.3 %, 15.5% for methanol extract and ethyl acetate, respectively.

The current results indicate that the antioxidant compounds of the ethanolic extract of the *A. niger* B1b biomass on hydroxylated groups giver of hydrogen, capable of interacting with free radicals, can be converted to more stable products. Thus, it can end the free radical chain reaction. Consequently, antioxidant compounds can play a rule in the theory of the electron giver group which increases the effectiveness of free-radical capture. In addition, the electron-receptor group can reduce the effectiveness of the capture of these roots [33, 34].

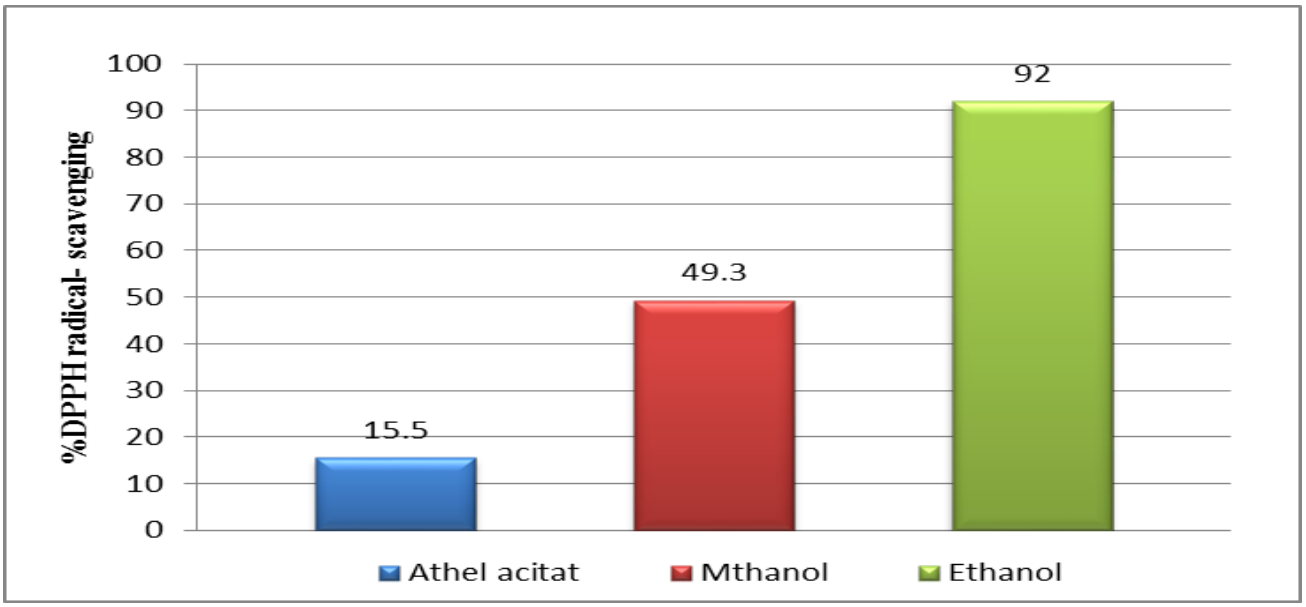

Fig. 8: the antioxidant activity of biomass ethanolic extract by scavenging DPPH

Determination of Antioxidant Activity by Reducing Power Method

Figure (9) shows the susceptibility of the antioxidant compounds of the ethylene extract to the biomass of *A. niger* B1b in reduction of the Fe $^{+++}$ to the Fe $^{++}$ iron in comparison with ethyl acetate and methanol extracts. The reduction power of ethanol extract was 106.1% followed by methanol extract and ethyl acetate extract (59.5%, 10, respectively).

These results are consistent with the findings of [9, 20, 23, 35, 36] which indicate that the cause is due to the possibility of compounds called reductants formed during the fermentation process, which can interact with the free radicals to convert them into more stable products. Then, they can terminate the free root chain reaction. The reduced compounds react with the peroxides and inhibit their formation [37].

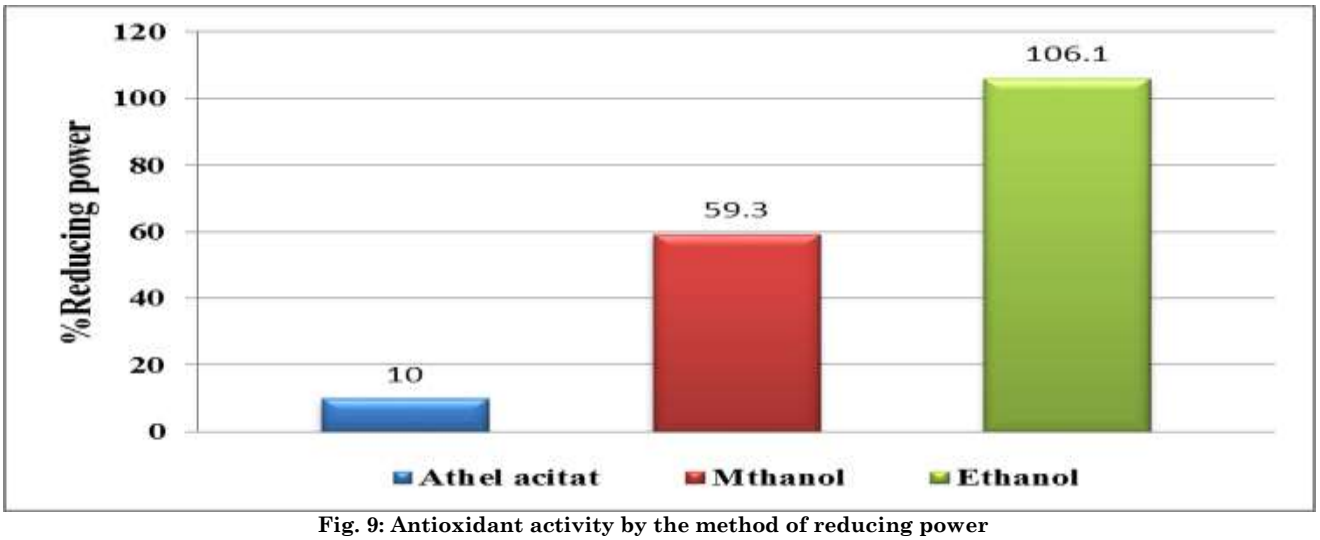

Fig. 9: Antioxidant activity by the method of reducing power

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