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Isolation of aFlavonoid Bioactive compound from *Gymnoascus dankaliensis* Secondary Metabolite

Furdos Noori Jafar[1*], Fatima S.Sabah[2]
[1]*Depatment of Biology, College of Science, University of Basrah, Basrah, Iraq.*
[2] *Depatmen of Chemistry, College of Science, University of Basrah, Basrah, Iraq.*

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Abstract

Flavonoids, a wide range of phenolic compounds produced as secondary metabolites in plants, bacteria and fungi, have highly potent activities. In this study, the fungus *Gymnoascus dankaliensis* was isolated in spring from sheep dung samples, and itsability to produce a secondary metabolite was investigated. The fungal secondary metabolite was extracted, and the antimicrobial and antioxidant activitis were tested. The results showed that *Gymnoascusdankaliensis*, thefirst ever isolated fungus from dung in Basrah, could produce a secondary metabolite that were produced using a Solid State Ferementation SSF medium after 40 days of incubation. The bioactive compounds were extracted from the solid media by using ethyl acetate. The chemical identification of the extracted bioactive compound indicated that the isolated compound belonged to the flavonoid group. The isolated flavonoid compound tested as an antimicrobial agent against three clinical isolates of bacteria,*Escherichia coli* , *Staphylococcus aureus* and and *Bacillus subtilis*, was also tested against two yeast isolates *Candida albicans* and *C. tropicalis*. The results recorded the largest inhibition zone in *S.aureus*. Whereas *C.albicans* was found to be more sensitive toward the isolated flavonoid compound than *C. tropicalis*. The antioxidant activity of the isolated compound was investigated using DPPH (1,1-diphenyl-2-picrylhydrazyl) method where it showed moderate antioxidant activity compared to ascorbic acid.

Keywords: *Gymnoascus dankaliensis*, Antimicrobial, Bioactive compound, Secondary metabolite, Flavoniod and antioxident .

عزل مركب فلافونيدي فعال من الايض الثانوي للفطر*Gymnoascusdankaliensis*

فردوس نوري جعفر [1]* ,فاطمة صيوان صباح [2]
[1]قسم علوم الحياة , كلية العلوم , جامعة البصرة ,البصرة ,العراق .
[2]قسم الكيمياء ,كلية العلوم ,جامعة البصرة ,البصرة,العراق .

الخلاصة

الفلافونيدات هي مركبات فينولية تنتج بشكل مستقلبات ثانوي في النبات،والبكتريا والفطريات، تمتلك هذه المركبات فعالية حياتية عالية ، في هذه الدراسة تم عزل الفطر *Gymnoascusdankaliensis* في

* Email :furdos.jafer@uobasrah.edu.iq

فصل الربيع من روث الخراف واختبرت قابليتة لانتاج الايض الثانوي بعد تنميته على وسط التخمر الصلب، وتم استخلاص المركبات الفعالة من الايض الثانوي للفطر الذي انتج بعد 40 يوم من الحضن، واجريت عملية الاستخلاص باستخدام المذيب العضوي خلات الاثيل، ثم اجريت عليه عدد من الاختبارات الكيميائية التشخيصية للمستخلص الفطري وتم عزل مركب فعال منه والذي بينت الاختبارات النوعية والتشخيصية لاحقا بانه يعود لمجموعة الفلافونيدات .

تميز المركب الفلافونيدي المعزول بفعالية ضد ميكروبية تجاه البكتريا *Escherichia coli* , *Staphylococcous aureus Bacillussubtilis* وفعالية ضد فطرية تجاه الخميرتين *Candida albicans*و*C.tropicalis*.و اختبرت الفعالية ضد التاكسدية للمركب المعزول باستخدام طريقة DPPH، وقد اظهرت النتائج فعالية ضد تاكسدية متوسطة للمركب المعزول مقارنة بالمركب القياسي حامض الاسكوربيك .

الكلمات المفتاحية : *Gymnoascusdankaliensis*, المضادات الميكروبية ,المركبات الفعالة حياتياً ,الايض الثانوي والفلافونيدات , مضادات الاكسدة .

1. Introduction

Fungal secondary metabolites are compounds with a low molecular weight that are considered not essential for the growth, development and reproduction of the fungus that produces them. Fungi can produce a wide spectrum of secondary metabolites many which have been found to have important applications for instance, as antibiotics (penicillin), immunosuppressants (Cyclosporine A), cholesterol-lowering (Lovastatin), food additives, mycotoxins (aflatoxine), antineoplastic drugs, anti-cancer drugs[1]. proteins, vitamins, biofuels, dye effluent treatment [2], and in pigment production [3]. Fungal pigments include carotenoids, melanin, polyketides, azaphilones, etc. Understanding the responsible genes and metabolic pathways involved in fungal pigment synthesis is essential to genetically manipulate the production of specific pigments. Many fungal pigments can be used as drugs to treat cancer, cardiovascular disorders, infectious diseases and Alzheimer's disease [4].

Flavonoids are a wide range of phenolic compounds found in plants, bacteria and fungi. They have a unified structure represented by C6-C3-C6, where C6 units represent the A and B rings, while C3 represents the pyrane ring. According to the degree of addition of hydroxyl groups and the changes that occur at the level of the C ring, the flavonoids are divided into different groups: flavones, flavanols, flavanones and anthocyanins. Most flavonoids are formed by linking ring B to carbon number 2 of ring C. However, in some flavonoids such as isoflavonoids and neoflavonoids, ring B is linked to carbon numbers 3 and 4 respectively [5].

Flavonoids can interfere with many biological activities, as they are called the natural rates of biological responses by inhibiting and reducing various enzymes [6, 7]. They contribute to cellular signal transmission pathways and regulate the cell cycle. These compounds also work to reduce inflammatory cytokines secreted by immune cells. Flavonoids also protect from injury. In vascular and heart diseases, they stimulate programmed cells death and inhibits cells proliferation cancerous [8, 9, 10]. The chemical structure of flavonoids helps to be an antioxidant agent. However, as either endophytic fungi or marine fungi can produce molecules with antioxidant properties which depend on the molecules' structure and the methods of assessment [11].

Antioxidant features of five anthraquinones have been assayed from the deep-sea fungus *Aspergillus versicolor*, through ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) assay [12]. The marine fungus *Acremonium strictum* producesa secondary metabolite as

Acremostrictin. This compound has antioxidant activity through the ability to scavenge DPPH, along with anticancer activity [13].

Gymnoascus dankaliensis is an ascomycetous filamentous fungus isolated from different samples such as clinical samples and soil samples. It can produce secondary metabolites in a fermentation medium. In a study, the fungus was isolated from soil in the vicinity of the Giza pyramids, Egypt, and when used in the solid-state fermentation medium of rice to grow, it resulted in yielding four new compounds including , 11′-carboxygymnastatin N (1), gymnastatin S (2), dankamide (3), and aranorosin-2-methylether, the latter having been reported previously only as a semisynthetic compound [14].

Endophyte fungus *Epicoccum sorghinum*, isolated from the plant *Annona senegalensis*, had high phenolic content with a total antioxidant capacity of about 593.46 ± 1.86 μM CRE [15]. Six strains of flavonoid-producing endophytic fungi that belong to Xylariales, *Chaetomiumglobosum*, and *Daldiniachildiae*, were screened by [16]. These strains exhibited excellent antioxidant and antibacterial activities.

This study aimed to investigate the ability of the fungus *G. dankaliensis* to produce secondary metabolites and evaluate the antibacterial and antioxidant activities of the extracted bioactive compounds.

2. Materials and Methods

2.1 Samples Collection

Sterile paper bags were used to collect sheep dung samples during the period from March to May 2020. The samples were left at room temperature to dry.

2.2 Isolation of the Fungus *G. daneklinsis* from Sheep Dung

Moist chamber method was followed [17] where the samples were placed on a petri dish containing moist sterile filter paper, and then cultured petri dishes were incubated at 27°C for 21–30 days by regularly adding sterile distilled water to the dishes.

2.3 Fungal Growth and Maintains

G. daneklinsis isolate was purified onto potato dextrose agar (PDA) and incubated at 27°C for 14 days, and then preserved in slant tubes for the later experiments.

2.4 Fungal Identification

2.4.1 Morphological Identification

After growing in PDA media for 14 days, the fungus was examined under a compound microscope to investigate its morphological characteristics (ascospores and hyphae)[18].

2.4.2 Molecular Genetic Identification

The isolation of the genomic DNA of the fungus was carried out using the Prestomini gDNA yeast kit (Geneaid). The protocol was performed according to the manufacturer's instructions. The extracted DNA was stored at -80°C for further study. Universal primers ITS1 and ITS4 were used to amplify the conservative region ITS depending on the research by [19]. The quantity and quality of genomic DNA and PCR products were analyzed by Nanodrop and electrophoresis.

The sequencing of PCR products was sent to Macrogen company, in Korea) (http://dna.macrogen.com). The sequencing aligned with the public database to compare the similarity in GenBank by the Basic Local Alignment Search Tool (BLAST).

2.5 Production and Extraction of Bioactive Compound from a Secondary Metabolite

Rice medium (100 g of rice immersed in 100 mL of distilled water and autoclaving) was used as a solid-state fermentation (SSF) medium for the production of the secondary metabolite from the fungus *G. daneklinsis*. Rice medium was inoculated with a fungal growth from a 10-day-old PDB culture and incubated for 30 days at 27°C [20].

Ethyl acetate was used in the extraction of bioactive compounds from the fungal secondary metabolite. Ethyl acetate was added to the solid medium after the incubation period, and the supernatant was filtered using the Whatman No. 1 filter. The extract was dried at room temperature and stored at 4°C until use [20].

2.6 Diagnosis of Effective Groups of theExtracted Bioactive Compound

Preliminary Qualitative Test:

Preliminary tests were carried out on the extracted bioactive compound.

2.7 Chemical Identification:

The chemical identification of the functional groups of the extracted bioactive compound was performed using several tests such as thephenol, alcohol, double bond, and aldehyde and ketone tests[21].

2.7.1 Thin Layer Chromatography (TLC)

TLC tests were carried out on the extracted bioactive compound by using n-butanol : ethanol :formic acid (5:3:1)[22].

2.7.2 Infrared and UV- Visible Spectroscopy

IR spectra using the PyE-UNICAM-30300S infrared spectrophotometer and UV-visible spectra on the JASCO UV- are shown inFigures 3 and 4.

2.8 Antimicrobial Activity Test of the Extracted Bioactive Compound

Antimicrobial activity for the isolated bioactive compound from a secondary metabolite of *G. dankaliensis* extract was carried out using the disk diffusion method against three bacterial isolates: *Escherichia coli, Staphylococcous aureus* and *Bacillus subtilis*. It was also tested against two yeast isolates, *Candida albicans* and *C. tropicalis*. A suspension of each bacterial isolate was prepared at a concentration of 1×10^6 cell /ml. About 10µl of suspension was added on the surface of Mueller-Hinton agar (MHA) which was then spread with a sterile L-shape glass rod. After drying a pore with a 9mm diameter was made in the center of each plate by using a cork borer. The crud fungal extract was applied by adding 100µl in the central pore of plates in the experiments done with duplicate and control plates represented by DMSO. All inoculated plates were incubated at 37°C for 24 h. The corresponding inhibition zone sizes were observed and recorded. For yeasts, the test achieved a similar result as above with substituted the culture media with Saubroud Dextrose Agar (SDA) and an incubation period of 48 h. [23].

2.9 Determination of Minimum Inhibitory Concentration (MIC)

MIC is the lowest extracted bioactive compound concentrationwhich prevents bacteria and yeast growth. It was carried out for the clinical bacterial isolates by adding different concentrations of the isolated bioactive compound to MHA for the bacterial MIC test and SDA for the yeasts MIC of the concentrations were ringing (250,125,62.5,31.2)mg\ml. Suspension from each microorganism was prepared with a concentration of 1×10^6 cell /ml. About 10µl of each suspension was added to the surface of the medium in petri dish. The

cultures were then incubated at 37°C for 24 h.for bacteria and 48 h. for yeasts.Ten % DMSO was used as a control [24].

2.10 Determination of Antioxidant Activities using DPPH

Antioxidant activity was measured by the free radical capture method (1,1-diphenyl-2-picrylhydrazyl ((DPPH) or 1,1-Divinil-2-bicrilliadraz) by capturing free radicals from DPPH and converting its purple color. One ml of 0.135 mM DPPH prepared in methanolic was mixed with 1 ml of different concentrations (100, 200, 300, 400, 500 ppm) of the fungal extract. Shaken well, the mixture was then left in the incubator for 30-60 minutes in a dark place. A control solution was prepared with 1 ml of DPPH solution in 2 ml of methanol. Absorption was measured at 517 nanometers per board and concentration after calibrating the device with methanol. The flavonoid compound was compared to the standard compound ascorbic acid [25]. The inhibition activity percentage of DPPH by the fungal extract was measured using the following equation:

$$DPPH\ scavenging\ activity\ (\%) = [(A - B)] / (A)] \times 100$$

Where A: The absorbance of blank cotrol
B : The absorbance of the sample

2.11 Cytotoxicity Test:

An investigation of cytotoxicity was carried out by preparing the fungal extract against human blood, according to [23]. Four concentrations of fungal extract (10, 50, 100 and 200 ppm) were prepared.From each concentration, 100µl was added to each tube containing the human blood solution. The tubes were left at room temperature before examining them after 15, 30 and 60 min. respectively.The fungal extract was considered toxic if blood solution in tubes became turbid.And still clear fungal extract was taken as being non-toxic. Four concentrations of fungal extract (10, 50, 100 and 200 ppm) were prepared.From each concentration,100µl was added to each tube continuing human blood solution. The tubes, left at room temperature, were examined after 15, 30 and 60 min. respectively. Turbid turned blood solution in tubes containing the fungal extract was considered as being toxic.Still and clear solution meant that the fungal extract was non-toxic .

3. Results and Discussion

3.1 Morphological Identification

There as a slow-growing fungus with an orange-brown colored colony on PDA. The fungus produced globose asci with eight smooth ascospores (Figure 1). The fungi belonging to the Ascomycetous phylum, were isolated from clinical samples as well as soil near the Giza Pyramids in Egypt [14]. It is well known to produce bio-pigments extensively used as colorants, additives, antimicrobials, antioxidants, etc. It is also used in different industries such as food, beverages, cosmetics, textiles and pharmaceuticals [26, 27].

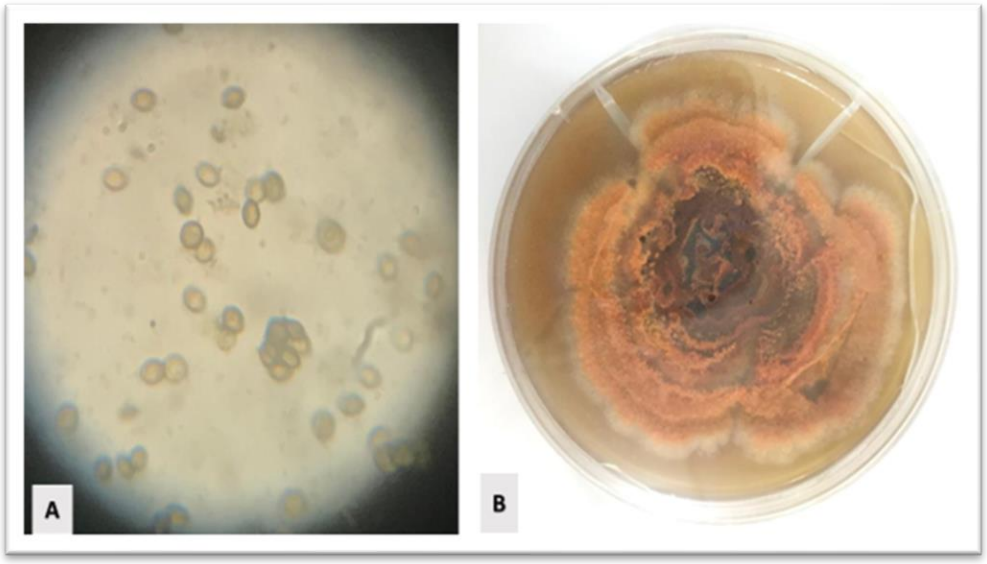

Figure 1: The fungus *G. daneklinsis* A: Ascospore B: Fungal colony after 14 days incubation at 25^{o} C.

3.2 Molecular Identification

G. dankaliensis isolate was identified according to amplification and sequencing, depending on ITS genetic region. The amplified ITS genomic region had a size of 500-600 bp. Upon comparing the sequence of ITSwith the sequences in the public database, a similarity was detected using the program Basic Local Alignment Search Tool (BLAST) (Figure 2). The identified *G. dankaliensis* isolate was deposited at the Gene Bank with the accession number LC647058. An analysis of the sequence explained that this isolate was first ever to have been isolated from dung in Basrah province.

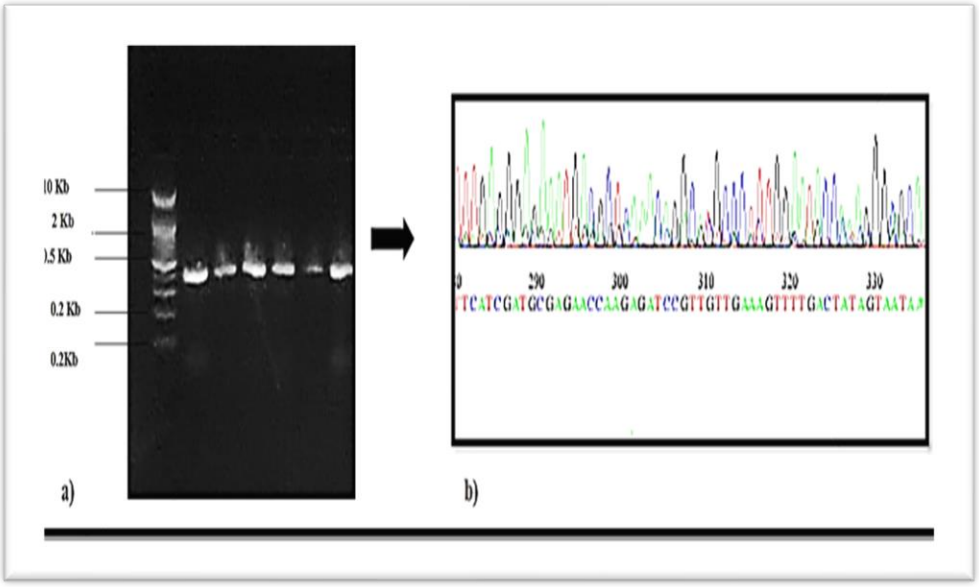

Figure 2: ITS PCR products in agarose gel for sequencing.a: ITS genomic region size about (500-600 bp . b: Peaks of PCR product sequencing.

3.3 Production and Extraction of Bioactive Compound from Secondary Metabolite

The ability of the ascomycetous filamentous fungi *G. dankaliensis* to produce a bioactive compounds as a secondary metabolites was also tested in this study. This fungus produced secondary metabolites in a fermentation medium after 30 days of the incubation period.
This fungus, isolated from soil in the vicinity of the Giza pyramids, Egypt, when used in the solid-state fermentation medium of rice to grow,the fungus resulted in yielding four new compounds including 11′-carboxygymnastatin N (1), gymnastatin S (2), dankamide (3) and aranorosin-2-methylether (4). The latter have been reported previously only as a semisynthetic compound [14].

3.4 Diagnosis of Effective Groups of the Extracted Bioactive Compound

Preliminary tests showed that the isolated compound was from the class of flavonoid dyes. The result was positive with the detection of flavonoids. The chemical groups were confirmed by conducting the tests of the phenol group, the double bond and the ketone group test(Tables1 & 2).

Table 1: Preliminary qualitative test of the isolated flavonoid compound.

Reagents	Result
Benedect	-
Molisch	-
Dragendroff	-
Mayer	-
Frothing test	-
Salkoviski	-
1% $FeC_3$10%	+
Mg ribbon	+

Table 2: The chemical identification of the isolated flavonoid compound.

Reagent	Result
Alcohol test	-
Double bond test	+
Ketone group	+
Aldehyde group	-
Phenol group	+

The result of the kind-layer chromatic class revealed the presence of one spot (RF = 0.65). This result was endorsed by many special statements to prove that this compound belonged to the family of flavonoids (Table 3).

Table 3: The result of the kind -layer chromatic class.

Test	UV lamp	H_2SO_4 4%	Daylight	Folin	Dragndorff	Ninhydrine
Spot	0.65	0.65	0.65	0.65	-	-

The UV spectrum showed an absorption maximum at 265.5 nm because of the two spectrums transition which is π→π*considered to be a distinctive feature of the unsaturated double bond. The visible spectrum also showed an absorption maximum at 445.5nm due to transmission type n→π* to C=O bond (Figure 3).

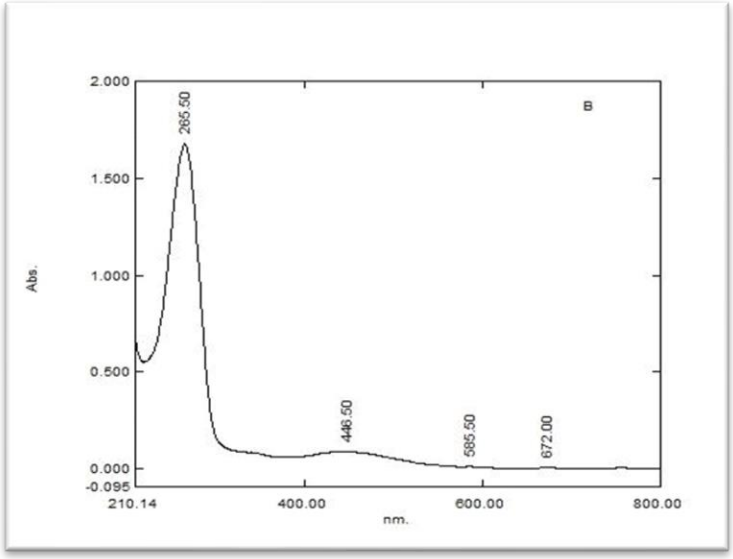

Figure 3: UV –visible spectrum for isolated flavonoid compound.

As for the FT-IR spectrum of the isolated flavonoid compound shown in Figure 4, the appearance of a single peak at 3352. 39cm-1 related to the vibrational extension of the OH bond indicated the presence of a phenol group and 1743cm-1 to a carbonyl group. The band at 3005.2cm-1 is associated with the stretching vibration of the CH = binding of unsaturated hydrocarbons within the structure of aromatic compounds located within the flavonoid synthesis. Preliminary tests showed that the isolated compound from the fungal secondary metabolite extract belonged to the class of flavonoid dyes, where the result was positive with the detection of flavonoids. Many endophytic and marine fungi produce flavonoid compounds such as Xylariales, *Chaetomiumglobosum* and *Daldiniachildiae.* Also *Alternariaalternata* and *Fusariumproliferatum* are endophytic fungi isolated from the plant *Salviamiltiorrhiza* produce flavonoids [28]. *Epicoccumsorghinum* isolated from *Annonasenegalensis* had the highest phenolic content with a high antioxidant capacity [15].

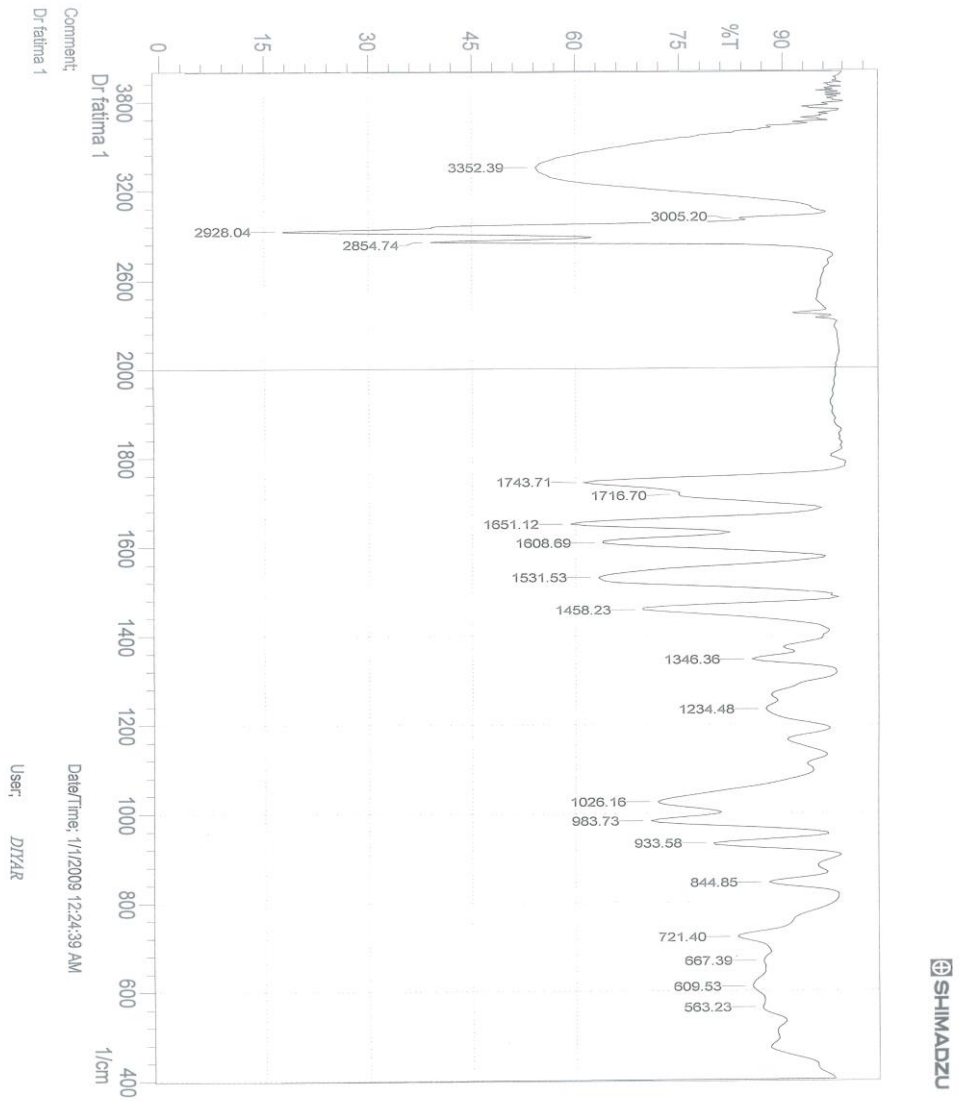

Figure 4: FT-IR spectroscopy for isolated flavonoid compound.

3.5 Antimicrobial Activity Test of aFlavonoid Compound

The isolated fungal flavonoid compound of *G. dankaliensis* showed high activity against both bacterial and yeasts isolates. The discovery of new antibiotics currently is necessary due to the appearance of drug-resistant bacteria so that should discover alternative antibiotics to control bacterial infections. The fungal secondary metabolites are natural compounds that are considered a common source of pharmaceutical drugs such as anticancer, antibiotics, antioxidants and cholesterol-reducing drugs [29].

The isolated flavonoid compound appeared notablly active against gram-positive and negative bacteria. *S.aureus* exhibited the largest inhibition zone of 27mm. The flavonoids have significant antibacterial activity due to their function of inhibiting several bacterial virulence factors [30]. While *E coli* was less affected by the isolated flavonoid compound with a diameter of inhibition zone of 23mm. which was due to the structural difference between gram positive and gram negative cell walls.

Also, the yeast isolates were affected by the isolated flavonoid compounds. *C.albicans* exhibited an inhibition zone with a diameter of 22mm compared with *C.tropicalis* that was 21mm in diameter. Flavonoids compounds have antifungal activity against a wide range of pathogenic yeasts like *C. albicans,C. glabrata*, *C. parapsilosis* and *C. tropicalis* [31].
Many antifungal drugs produce several complications to host tissues. Also, a recent therapeutic research restricted the use of this drug [32]. Hence, it is necessary to investigate novel natural compounds that have greater anti-fungal activity with lesser side effects [33](Table 4& Figure 5).

Table 4: Diameters of inhibition zones in mm for the activity of isolated bioactive compound

Clinical Isolates	Diameter Inhibition Zones (mm)
E.coli	23
Bacillus subtilis	22
Staphylococcous aureus	27
Candida albicans	22
Candida tropicalis	21

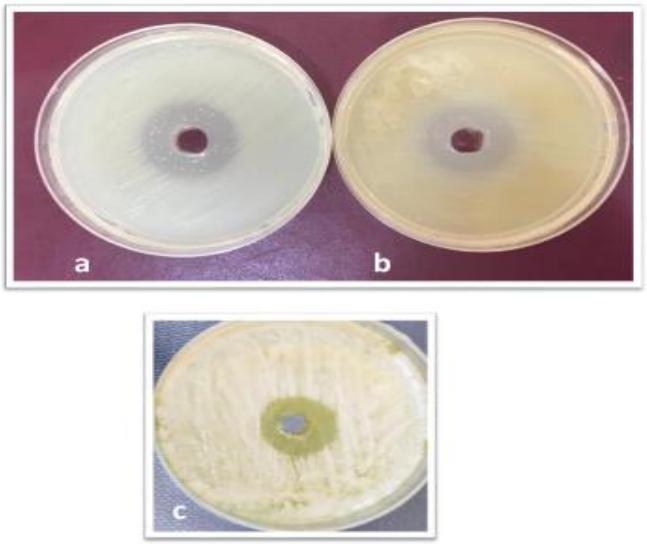

Figure 5: The effect of fungal extract against, a: *S.aureus*, b :*E.coli*, c: *C. albicans*.

3.6 Determination of Minimum Inhibitory Concentration (MIC)

MIC test revealed that all isolates had same MIC values except *S.aureus* which had the lowest MIC value of 75mg/ml(Table 5).
S.aureus is gram positive bacteria that has a less complex cell wall which is composed of only an outer layer of peptidoglycan as compared to the gram negative cell wall that has a lipopolysaccharide layer and periplasmic space. Hence, it can reported that gram positive bacteria are more sensitive to isolated flavonoid compounds [34].

Table 5: Minimum Inhibitory Concentrations (MIC) values for isolated flavonoid compound.

	Concentrations				
Microorganisms	**300mg/ml**	**150mg/ml**	**75mg/ml**	**37.5mg/ml**	**Control**
S. aureus	-	-	+	+	+
B. subtilis	-	+	+	+	+
E.coli	-	+	+	+	+
C. albicans	-	+	+	+	+
C. tropicalis	-	+	+	+	+

Were (-) no growth and (+) growth was present

3.7 Determination of Antioxidant Activities using DPPH

In this research, flavonoid compound was isolated from a fungal secondary metabolite extract and its effectiveness was tested on sniping free radicals using the DPPH method. When compared with ascorbic acid, the fungal extract recorded moderate antioxidant activity, and as compared to the stander treatment, ascorbic acid that has high activity, (Figure 6) shows the percentage of DPPH free radical sniping. Due to their importance in protecting food, the use of antioxidants has become common recently [35]. Therefore, a need to isolate compounds that are characterized by being antioxidants, including flavonoids that were isolated from natural sources, has been emphsized. The results revealed that the free radicals scavenging ratio increased with an increase of the isolated flavonoid compound conecentration. The highest ratio of free radical sniping was 58.7% at 500 ppm, compared to 95.2% at the same concentration for ascorbic acid. Secondary metabolite extracts of marine fungi *G.dankaliensis*, *Nigrosporaoryzae* and *Chaetomium globosum* extracts had high antioxidant and anti-inflammatory capacities. *G.dankaliensis* had the highest total phenolic content. The highest total flavonoids content was also recorded for *Engyodontium album* [36]. Flavonoids are a chemical group of secondary metabolites that have various pharmacological activities such as antioxidation [37]. The high antioxidant activity of flavonoid is due to the molecular structures of polyhydroxyl compounds that it used in preventing and treating various common diseases [38]. In addition to antioxidant activity of flavonoids, it also has antitumor, anti-inflammatory, antibacterial and cardiovascular protection activities [37]. Many studies suggest that large number of fungi are rich in flavonoids [39].

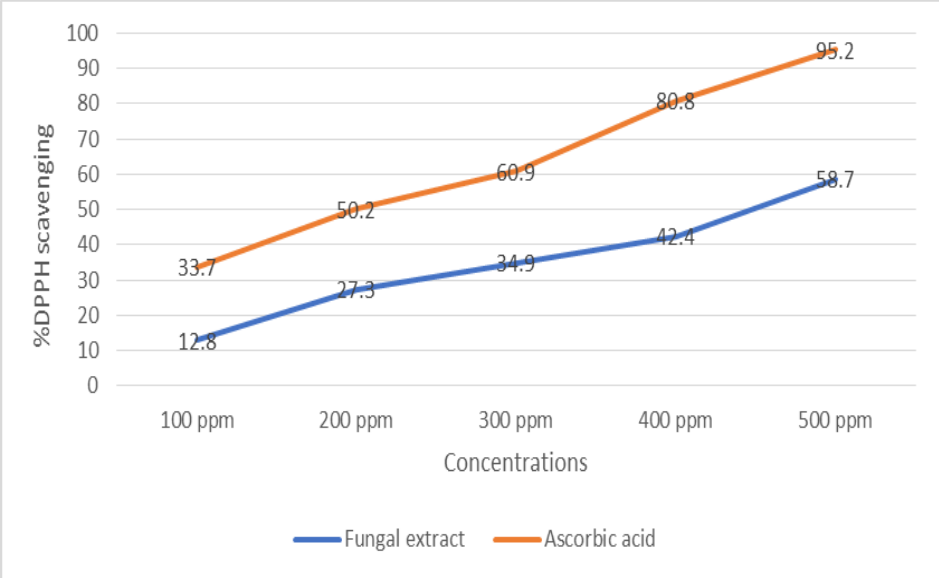

Figure 6: Reducing activity of fungal extract with different concentrations.

3.8 Cytotoxicity Test

This experiment showed that there was no toxic effect of the fungal extract on the human blood solution in all concentrations as the human blood solution was still clear in all tubes. However, some fungal extracts did not have toxic effect. This result was compatible with another study [20]. Hence, this compound could prove to be an important pharmaceutical in future.

4. Conclusion

Fungus *Gymnoascusdankaliensis* successfully produced a secondary metabolite. The preliminary qualitative test and chemical identification of the isolated bioactive compound

from the fungal secondary metabolite clarified that it belonged to the flavonoid group. When tested against clinical bacterial isolates and yeasts, the isolated compoun showed notable activity against them. Also, the isolated compound showed moderate antioxidant activity. Due to this unique bioactivity of the isolated flavonoid compound, it can be a suitable alternative to antibiotics which many bacteria have became resistant to. This novel compound also succeeded in being an antioxidant agent. The most important recommendation of this work was to preform extra identification methods such as H-NMR and GC-mas to detect the chemical structure of this flavonoid compound.

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