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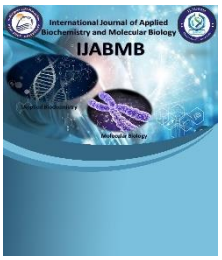

# Detection of the antioxidant activity of fungi and bacteria-derived secondary metabolites

**Ameerah Abdulmunem Yaseen Al-doori[1], Shelan Weli Hasan[2], Israa Abdel Munem Yaseen[3], Shaima Ahmed Hussein Darwish[4], Aqeel Majid Alabbasi[3], Batol Imran Dheeb[2]**

1University of Samarra/College of Applied Sciences/Department of Applied Chemistry, Samarra, Iraq

2University of Samarra/College of Applied Sciences/Department of Pathological Analysis, Samarra, Iraq

3University of Samarra/College of Education /Department of Biology, Samarra, Iraq

4Al-Karkh University of Science, Baghdad, Iraq

***Corresponding author:**

**Batol Imran Dheeb**

University of Samarra/College of Applied Sciences/Department of Pathological Analysis. batoolomran@yahoo.com

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## Abstract

Fungal and bacterial metabolites were utilized to develop a suitable method, which was a prerequisite for scientists to produce natural compounds with strong biological activity from inexpensive sources and to surpass the average efficacy of anti-proliferative agents. Thin-layer chromatography was used to identify fungal metabolites in the current study. The most powerful antioxidant, anti-proliferative, and antibacterial activity was found in one fungal isolate out of ten that underwent bioassay; this isolate was determined to be Aspergillus flavus. With a low IC50 concentration and strong flavonoid content, the fungus extract of A. flavus demonstrated a significant percentage of free radical scavenging activity utilizing Diphenyl-1-picrylhydrazyl (DPPH). The presence of several strong products of A. flavus extract is detected by the results of an instrumental analysis performed using GC. Mass. Furthermore, the extract from A. flavus exhibited encouraging antiproliferative properties. Under these experimental circumstances, inhibitory efficacy against Hepatocellular Carcinoma Cells was found. It is also possible to utilize this approach to extract modern medications.

## Keywords:

Bioactive substances: antimicrobial endophyte, secondary metabolites, TFC, oxidative stress.

## 1- Introduction

Antibiotic-resistant forms of cancer have become more prevalent recently, necessitating the use of other medications to block them [1]. Furthermore, research on the biocontrol of this malignancy is important in order to identify and find a novel way to avoid this disease in vitro [2]. Accordingly, there is a pressing and unavoidable medical need for natural bioactive metabolites with innovative antibacterial and anticancer properties [3], and detection procedures are often concerned with producing reliable and highly effective biocidal products [4]. Safe materials are being used as safe therapies or as biosorptive instruments for antibacterial and anticancer activities, according to recent perspectives worldwide [5]. Natural metabolites from fungi are also utilized in this framework to apply material technologies, industrial processes, and other methods to produce biocidal products that are both reliable and highly effective [6]. Fungi have the greatest diversity of secondary metabolites, and various primary extracts have a significant impact on the biological activity of species [7]. Various fungi have recently been the subject of research techniques that have proved their role in therapeutic sectors [8]. New medicines and industries are characterized by their inherent outputs, which set derivative extracts from fungal strains apart [9]. Many of the bioactive compounds that were separated from Aspergillus species have been reported in various publications [10]. These metabolites have demonstrated medicinal significance due to their anticancer, antibacterial, and antioxidant properties [11]. The goal of this study is to identify the antibacterial, anticancer, and antioxidant properties of Aspergillus flavus bioactive metabolites.

## 2- Capacity to scavenge free radicals and antioxidants

Using a variety of techniques, including the six fungal pigments were examined for lipid peroxidation,

reducing power, superoxide radical anion, hydroxyl radical, nitric oxide, and metal chelation ability, as well as antioxidant and free radical scavenging capacity. In each assay, the pigment extract exhibited concentration-dependent radical scavenging activity [6]. The reducing power assay showed that the pigment extract had significantly higher scavenging activity than the other approaches. Thermomyces pigment extract had the highest scavenging effect across all trials, followed by Chaetomium at all doses. Reactive oxygen species are produced by ultraviolet light [9]. Many pathological effects, including lipid peroxidation, protein peroxidation, DNA damage, and cellular degeneration linked to cardiovascular disease, aging, cancer, inflammatory diseases, and a host of other disorders, are caused by reactive oxygen species, which are UV light, ionizing radiation, chemical reactions, and metabolic processes [10]. Chemical entities with high reactivity are known as free radicals. Thus, one of the key ways hazardous chemicals affect cells is by generating free radicals during xenobiotic metabolism. Aerobic organisms produce reactive oxygen species (ROS), which can cause damage to proteins, lipids, carbohydrates, and DNA. ROS consist of single molecular oxygen, hydrogen peroxide, hydroxyl radicals, and superoxide anion radicals. [11].

**3-A system of both enzymatic and non-enzymatic antioxidants regulates the effects of these ROS**

These antioxidants scavenge free radicals and eradicate pro-oxidants. An increase in oxidative metabolism, which generates many ROS molecules leads to oxidative stress. Antioxidants can have positive health effects and help prevent oxidative stress [12]. A high dietary intake of proven antioxidants can dramatically reduce the risk of a number of chronic illnesses, including cancer, cataracts, and heart disease. Due to their antioxidant properties, capacity to prevent or postpone degenerative illnesses, and ability to boost both human and animal immunological responses, fungal carotenoids are of great interest in

nutrition [13]. Animals cannot synthesize carotenoids; thus, they must be provided in their food as colorants, a source of vitamin A, and possibly as nutraceuticals [14]. Unlike antioxidant capacity, antioxidant activity is a kinetic-based assessment of the rate constant of redox reactions or radicals, scavenging activities over time [9]. Antioxidant capacity, on the other hand, measures how well different antioxidants stop biological macromolecules from breaking down oxidatively [15].

Total antioxidant capacity calculates the sum of the antioxidant capacity that each component of a complicated combination or biological system contributes, either antagonistically or synergistically [16]. Since different antioxidant components have varying modes of action, determining the entophytic metabolites TAC may not be simple. Some may completely stop ROS generation, while others scavenge free radicals. In order to repair oxidative damage, some metabolites may also trigger signalling pathways [17]. Accurately calculating the TAC of a biological system is difficult due to the variety of antioxidant activity pathways [18]. Individual differences in metabolism and the contributions of endogenous antioxidant enzymes make calculating TAC in vivo even more challenging. Because of this, the typical TAC ranges of several in vivo models lack standardized reference values [19].

**The characteristics, origins, and metabolites of endophytic fungi**

The varied collection of microorganisms known as endophytes, which live asymptomatically in a variety of plant tissues [12], are found in terrestrial, mangrove, and marine environments [2]. Many bioactive metabolites with a wide range of pharmacological characteristics can be found in plants. Due to a lengthy evolutionary link and genetic transfer, the investigation of entophytes within both plants produces comparable chemicals [14]. Wide scale and contributes to the preservation of biodiversity. Additionally, endophytic fungi, endophyte bacteria, or

actinomycetes are recognized producers of natural chemical structures with antioxidant activity [8]. As well as new compounds with important biological properties, the great potential of endophytes as a secondary metabolite repository is shown by the confirmation that several fungi produce beneficial secondary metabolites [9]. These organisms may live in a variety of organs of known higher plants, producing a broad range of bioactive compounds that may be used as treatments for several illnesses [7]. Endophytic fungi are notable for being the main producers of a variety of metabolites with strong biological properties immunosuppressive, anticancer, antidiabetic, antibiotic, and antioxidant qualities [9]. Numerous bacteria that can synthesize new metabolites and chemicals are present in their cohabitation with host plants, which are primarily higher [11].

**4- Endophytic fungal metabolite extraction, purification, and characterization**.

A crucial first step before characterizing and using an endophytic fungus is the extraction of its bioactive metabolites. Pure endophyte cultures are first grown in a specific liquid medium, and then, depending on the target metabolites' solubility, all metabolites of the microorganism are extracted with the proper solvents or mixtures of them. [12]. Liquid–liquid extraction is a frequently used technique in which the organism and the organic solvent are cultured in a broth medium. Bioactive metabolites from endophytic fungi have been demonstrated to be quickly extracted using a variety of solvents, such as dichloromethane (DCM), ethanol, hexane, ethyl acetate, and methanol [11]. For example, an equimolar concentration of ethyl acetate and methanol was used to extract bioactive metabolites from the endophyte and Pestalotiopsis neglecta. A rotating vacuum evaporator was then used to dry the resultant crude extract that contained the active fraction [12]. In a related investigation, a fungal isolate in a liquid medium was incubated with

ethyl acetate for six weeks at room temperature. A rotary evaporator was used to dry the resultant crude mixture after it had been vacuum-filtered [13]. Furthermore, a crude metabolite extract was produced by simultaneously extracting secondary metabolites from two Cladosporium species using ethyl [14]. Additionally, only ethyl acetate was used to extract metabolites from endophytic fungi that were separated from Salvia abrotanoides. Glass beads were used to mechanically break apart the resultant ethyl acetate mycelia combination to improve cell wall lysis. Following centrifugation of the homogenate, the supernatant was used for additional research [15]. On the other hand, frequently used ethyl acetate to extract secondary metabolites from Penicillium citrinum in order to improve the extraction efficiency of the endophytic fungus.

On the other hand, bioactive metabolites from Curvularia sp, the endophytic fungus T12 were extracted using methanol alone, which, after concentration and drying, produced a brown crude extract [16]. The extraction processes create crude metabolites that must be processed further to be separated into component fractions for further analysis. Pure metabolites must be extracted before they may be comprehended and characterized structurally. To purify entophytic fungal metabolites, a variety of chromatographic methods have been explored, including column chromatography and high-performance liquid chromatography (HPLC) [17]. Thin-layer chromatography was used to fractionate a crude metabolite extract from the endophytic fungus Penicillium citrinum into 11 bands, with a mobile phase of chloroform, methanol, and ethyl acetate and a stationary phase of silica gel. [18]. Comparably, employing silica gel column chromatography, the entophytic fungi crude metabolite extract of isolated from Ocimum basilicum was split into two white solid fractions [19]. A two-step purification procedure was used, consisting of silica gel Sephadex LH-20, column chromatography and gel filtration. The bioactive chemicals that are present

must be identified and described once the metabolites from fungal cultures have been extracted and purified. Reproducible results and less time-consuming secondary metabolite characterization are the consequence of the advancement of analytical techniques for characterizing and detecting bioactive metabolites from more complex and simpler sensitive and quick methods [20]. Characterizing recovered active fractions is frequently accomplished using methods including mass spectrometry (MS), nuclear magnetic resonance (NMR), and gas chromatography [21]. used high-resolution MS to separate cryptotanshinone, a bioactive chemical, produced from the endophytic fungus of the native Salvia abrotanoides plant. Fusarium oxysporum is a fungal endophyte. was used to extract vinblastine and vincristine, which were then characterized using the molecular weight is determined using tandem mass spectrometry (MS-MS) and electrospray ionization mass spectrometry (ESI-MS), while structural features were elucidated using 1H NMR [22]. TLC, GC-MS, 1H NMR, and 13C NMR experiments were used to detect a purified fraction of bioactive compounds from the company, endophytic fungus P.citrinum. milbemycin, one of the bioactive compounds that was discovered, demonstrated antibacterial action against bacteria and fungi that are harmful. Since the metabolite extracts of endophytic fungi are complex mixtures of several chemicals, the separation and characterisation of these compounds have been accomplished using appropriate hyphenated procedures. To analyse a variety of endophytic metabolites, methods like LC-PDA and LC-MS are frequently used. from tiny, non-polar substances to many polar components. Additionally, multiple-hyphenated methods such as LC-PDA-NMR-MS and LC-NMR have also become more popular [23]. Techniques for calculating the endophytic fungal metabolites' total antioxidant capacity (TAC), total phenolic content (TPC), and total flavonoid content (TFC) One interesting source of antioxidants is

endophytic fungi that are obtained from medicinal plants The measurement of a sample's total antioxidant capacity (TAC) offers a comprehensive evaluation of its capacity to absorb, scavenge, neutralize, or free reactive, radicals relative quantities of common antioxidants like ascorbic acid or trolox [24]. TAC can be estimated using the , scavenge technique, Numerous studies indicate that techniques used for metabolites plant are frequently comparable to those used for fungi endophytic, despite the fact that these approaches may differ in their underlying concepts, procedures, and sensitivity to various antioxidants Furthermore, a number of studies have documented the presence of novel metabolites with Phenolic Content (TPC) or the Total Flavonoid Content (TFC), despite the fact that plants and endophytes may create identical metabolites[23]. The components of flavonoids, cumulative, other polyphenols, determine phenolic acids, and the total phenolic content, which represents antioxidant capacity [24]. TPC is often measured by the Folin–Ciocalteu method, which forms a blue-colored complex when phenolic components of the extracts reduce [25]. In a similar vein, Total Flavonoid Content (TFC) gives details on a sample's antioxidant potential based only on the flavonoid (a subclass of phenolic compounds) content [26]. The chloride aluminium colorimetric method, which specifically generates a yellow-orange combination with flavonoids, is used to assay TFC. The flavonoid concentration, which may be quantified as absorbancy at the maximum spectrum of 415 nm, is proportional to the intensity of the colorful complex that forms. A standard curve made from either quercetin or catechin (as flavonoid standards) is used for extrapolation to obtain a quantitative value from absorbance readings of the TFC assay. Accordingly, TFC results are usually reported as equivalents of quercetin [27]. 2,3-diphenyl-4-picrylhydrazyl radical scavenging using the phosphomolybdenum method. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical with a purple colour

that turns yellow when electrons neutralize or reduce it [28]. Because of this feature, DPPH is a useful reagent that is frequently used to assess an antioxidant's or metabolite's Total Antioxidant Capacity (TAC)—the ability to donate an electron. At 515 nm, the amount of colour change in the presence of an antioxidant can be measured spectrophotometrically and is proportional to the antioxidant capacity [29]. How to: The method used to estimate the TAC of endophytic fungal metabolites using DPPH is quite like that used for plants. Direct extrapolation from a standard curve created with common antioxidants, like ascorbic acid or Trolox, is how TAC is measured. Additionally, the absorbance of the sample and the absorbance of the control (which only includes DPPH solution) can be used to estimate the percentage scavenging activity of antioxidant metabolites. Like patterns seen in research on plant phytochemicals, the DPPH assay has become more and more popular in recent studies using the metabolites and extracts of endophytic fungi. An overview of a few studies that have used the DPPH method to assess endophyte metabolites' antioxidant potential [30].

**5-Phenolic chemicals isolated from the fungal endophyte Cladosporium velox extract were measured using the DPPH technique.**

For example, using the DPPH method, phenolic compounds obtained from the extract of the fungal endophyte Cladosporium velox, isolated Tinospora cordifolia stem, had a value of 22 µg/mL, somewhat lower than ascorbic acid [30]. Similarly, the DPPH assay was used to examine the antioxidant activity of endophytes from five plants: Trigonella foenum, graecum Calotropis proceri, Catharanthus roseus, Euphorbia prostrate, and Vernonia amygdalina [31]. Trigonella foenum-graecum seeds had the highest IC50 value (17.0 µg/mL) among the isolated fungal strains. Endophytic fungi from Cinnamomum loureiroi leaves, Neo Pestalotiopsis sp. and Diaporthe sp., exhibited considerable scavenging activity, with IC50 values of 22.92 and 37.61 µg/mL, respectively. Eugenol,

acid, myristicalhyde, and caprylic acid were identified as the major antioxidants in both fungal extracts [32]. Additional specific illustrations with data demonstrating antioxidant capacity determined using the DPPH assay.

**6- The antioxidant power of ferric reducing**

The Reducing Ferric and Antioxidant Power assay measures the overall antioxidant capacity of a sample through reducing ferric tripyridyl triazine to its ferrous analog in the presence of antioxidants at a low pH. [33]. Ferric tripyridyl triazine, cf antioxidants, cause the ferric complex to be reduced further, which in turn causes a higher loss of the blue hue. Total antioxidant capacity (TAC) is quantitatively extrapolated fr the decline in absorbance values and is represented as reducing equivalents of a standard antioxidant. The antioxidant power of ferric reducing [2].

**7- A variety of bioactive chemicals can be produced by bacteria**

Numerous bioactive substances, such as those reducing antioxidant properties, can be produced by bacteria [34]. Some bacterial antioxidants, however, have different chemical structures and biological activities than those derived from plants and animals. For example, bacteria alone can produce certain antioxidant chemicals, such as phycobiliproteins, prodigiosin, and violacein, but plants and fungi cannot [35Numerous studies on antioxidants generated from the bacterial phylum Actinobacteria have been published; however, the bulk of them have concentrated on a specific type of Actinobacteria-derived antioxidant molecules, such as pigments, polysaccharides, peptides, or phenolic compounds. In contrast to Actinobacteria, there have only been three reviews [36]. Given the current knowledge gap, the purpose of this study is to evaluate the literature on antioxidant metabolites derived from diverse phyla within the domain Bacteria and the phylum Actinobacteria. It is also worth noting that the current study concentrated on

the many antioxidant compounds found in these phyla, such as pigments, polysaccharides, peptides, phycobiliproteins, mycosporine-like amino acids, phenolics, and alkaloids [37].

**8- Stress from oxidation and antioxidants**

Free radicals are mostly produced by oxygen (ROS) and nitrogen (RNS) in living systems Superoxide (O2•−), hydroxyl (•OH), alkoxyl (RO•), peroxyl (ROO•), hydroperoxyl (HO2•), oxygen peroxide (H2O2), singlet oxygen (1O2), ozone (O3), and hypobromous acid (HOBr) radicals are examples of ROS. Nitrogen and nitric oxide (NO•) are examples of RNS. dioxide (NO2·), nitrous acid (HNO2), nitroxyl anion (NO−), nitrosonium cation (NO+), Peroxynitrite (ONOO−) and both. According to [9], reactive oxygen species like O2•− and H2O2 combine with NO• to create RNS. The order of RNS reactivities is as follows: No• < NO2• < ONOO−. When NO• reacts with O2•−, it produces the highly poisonous and reactive species ONOO −, which destroys DNA, protein, and lipid molecules, even though NO• is less likely to be chemically reactive [10]. The chemical makeup of metabolites produced by microorganisms that have antioxidant activity [13].

**9- Similar amino acids as mycosporins**

Colourless, water-soluble, nitrogenous macromolecules are known as mycosporin-like amino acids (MAAs). In addition to stabilizing free radicals and absorbing UV-A and UV-B rays, they can also release the energy as heat. Numerous species are capable of synthesizing them, such as cyanobacteria, lichens, fungi, and algae. The MAAs include mycosporin-glycine, Palythene, palythine, asterina-330, shinorin, and porphyra 334. The ability of MAAs to absorb radiation and stabilize free radicals makes them photoprotective and antioxidant substances in these species [22].

**10- The Peptides**

Numerous bacterial and fungal species produce lipopeptides, commonly referred to as microbial surfactants, either extracellularly or as a component of the cell membrane. These peptides are mostly produced in bacteria by non-ribosomal peptide synthetases (NRPS), which contribute one amino acid to the expanding peptide chain in a ribosome-independent way [11]. Potential lipopeptide manufacturers include Streptomyces, Pseudomonas, and Bacillus species [23]. Lipopeptides have strong antibacterial qualities that are well known. Additionally, their antioxidant properties are significant. In tests for ferric-reducing capacity, lipid peroxidation inhibition, and DPPH radical scavenging, the lipopeptides from Bacillus methylotrophicus DCS1 demonstrate in vitro antioxidant properties [28]. They also discovered that the lipopeptides have strong anti-adhesive, antifungal, and antibacterial properties. experiments for peroxidation inhibition. In a rat model, the lipopeptide-gel formulation can also speed up wound healing and lead to full wound closure, as shown by the in vivo tests. The pure peptide YD1 from Bacillus amyloliquefaciens CBSYD1 showed antibacterial efficacy, ferric-reducing capacity, and radical-scavenging (DPPH and ABTS radicals) properties [29]. ROS levels in cells exposed to LPS-induced oxidative stress and to increase the activities of antioxidant enzymes.

The peptides are composed of six distinct amino acids, according to the structural analysis, and have an epoxy fatty acid side chain in their chemical structure. The in vitro tests showed that the lipopeptides were capable of scavenging DPPH and ABTS radicals, indicating their antioxidant efficacy. In one study, lipopeptides from B. subtilis strains (surfactin, mycosubtilin, and plipastatin/fengycin) were assessed for their antiradical and antioxidant properties. All of the peptides were found to have antioxidant activity against DPPH, O2•-, and OH• radicals [33]. However, plipastatin had a greater capacity for scavenging than the others.

The fermentation produces a cyclic lipopeptide called epichlicin, which has antifungal and antioxidant qualities. It can also scavenge DPPH radicals and reduce ferric acid. A distinct research team. The peptide extracted from Bacillus velezensis culture broth demonstrates high antioxidant activity in both metal-reducing (ferric and cupric-reducing) and radical-scavenging (DPPH and ABTS) tests. By increasing the activities of antioxidant enzymes and lowering intracellular NO• and [33]. In a new study, scientists wanted to maximize the synthesis of surfactin from SOPC5 in a medium based on soybean meal and then assess the surfactin's in vitro antioxidant capacity. Optimization studies showed that temperature, incubation duration, mineral content, and soybean concentration all had a major impact on surfactin formation. DPPH and ABTS radicals can be scavenged by the pure surfactin, according to in vitro antioxidant tests [37.

**Conclusion and future perspectives**

Numerous human health issues are associated with oxidative stress, which is caused by an excessive buildup of reactive oxygen species (ROS) and nitrogen species (RNS) in the cells. In addition to being obtained from other natural sources, endogenous and exogenous antioxidants work in concert to remove excess ROS and RNS. According to this review, five phyla of the domain Bacteria, Actinobacteria, Cyanobacteria, Bacteroidetes, Firmicutes, and Proteobacteria can produce a wide variety of exogenous antioxidant metabolites, such as polysaccharides, pigments, phycobiliproteins, mycosporin-like amino acids, peptides, phenolics, alkaloids, and polyketides. Fungi are the exclusive source of certain of these antioxidants, like phycobiliproteins, prodigiosin, and violacein, which are not generated by other species like plants or fungi. Metabolites and extracts from bacteria exhibit antioxidant properties both in vitro and in vivo.

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