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Antibacterial and Antioxidant Activites of Secondary Metabolites of Endophytic Fungus *Stemphylium radicinum* (Meier, Drechs and Eddy)

Rashid Rahim Hateet*
Department of Biology, College of Sciences, University of Misan, Iraq

Abstract

Endophytic fungi are gaining interest as sources of novel bioactive metabolites. The study was carried out to screen and isolate endophytic fungus.An endophytic fungus isolated from root of calyptous plant .The fungus was identified as *Stemphylium radicinum* (Meier, Drechs and Eddy)based on morphological characterization. Fungal secondary metabolites was carried out by ethyl acetate solvent.The antibacterial activity was tested against five bacterial isolates. *Esherichia coli*, *Staphylococcus aureus, Proteus vulgaris, Klebsiella pneumonia* and *Streptococcus pyogenes* by using a disc diffusion technique. The inhibition zones exhibited by Fungal secondary metabolites were ranged between 22.5-35.5 mm. Minimum inhibitory concentration MIC test revealed that the extract of *S. radicinum* exhibited a minimal inhibition values ranging between 25.0-100 ug/ml against bacterial strains .A verification of non toxicity of the fungal secondary metabolites against human blood revealed a negative test. Active compounds of the fungal secondary metabolites involves Tannins, phenols and amino acid .The antioxidant activity was analyzed using2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assays .The metabolite produced by the fungi could be an alternative source of antimicrobial against clinical pathogens.

Keywords: Endphytic fungi, Bacteria, Antibacterial, Secondary metabolites.

الفعالية الضد بكتيرية والضد تاكسدية للأيض الثانوي لفطر النابوت الداخلي ***Stemphylium radicinum*** **(Meier ,Drechs and EddY)**

رشيد رحيم حتيت*
قسم علوم الحياة، كليه العلوم، جامعة ميسان، العراق

الخلاصه

تضمنت الدراسة عزل وتشخيص الفطر *Stemphylium radicinum* من داخل النسيج النباتي لجذور نبات اليوكالبتوز Endophytic fungus . واستخلص الراشح باستعمال المذيب اثيل استيت (ethyl acetate) للحصول على الايض الفطري الثانوي. وقد اظهر هذا الايض فعالية واضحة تجاه خمسه من العزلات البكتيريه المرضيه *Esherichia coli, Staphylococcus aureus, Proteus vulgaris* and *Streptococcus pyogenes* *Klebsiella pneumonia*, باستخدام تقنيه الانتشار بالأقراص وكانت اقطار مناطق التثبيط قد تراوحت بين22.5-35.5 ملم. وكشف اختبار التركيز المثبط الادنى قيم تثبيط تراوحت بين 100-25.0 ميكروكرام/غرام.كما لم يظهر الايض الفطري الثانوي أي سميه تجاه كريات الدم الحمر للإنسان. اظهرت الكشوفات الكيميائيه للمستخلص الفطري احتواءه على التانينات والفينولات و احماض امينيه وعدم احتواءه على الفلافونيدات . وقد اختبرت الفعالية الضد تأكسدية للايض الفطري الثانوي باستخدام 2, 2-diphenyl-1- picrylhydrazyl (DPPH) وقد أظهرت نسب تثبيط عالية عند التراكيز المختبره أن الايض المنتج من فطريات Endophytic يمكن ان تكون مصدرا بديلا جيدا للمضادات البكتيرية.

*Email:biorashed2@yahoo.com

Introduction

Endophytes are microorganisms that are present in living tissues of various plants, establishing mutual relationship without apparently any symptom of diseases [1].They reside inside the tissues of nearly all healthy plants. The relationship that they establish with the plant varies from symbioticto bordering on pathogenic [2].Studies on endophytic microbes over the past 25 years indicate that they inhabit a unique ecological niche and are thought to influence plant distribution, ecology, physiology and biochemistry.

There is a general call for new antibiotics, chemotherapeutic agents and agrochemicals that are highly effective, possess low toxicity, and will have a minor environmental impact. Endophytic fungi are relatively unexplored producers of metabolites useful to pharmaceutical and agricultural industries [3].Some studies showed that endophytes could promote the growth of the host plant, inhibit the growth of pathogens, and improve stress tolerance e.g. [4,5].

Secondary metabolites are small biomolecules considered to be non-essential for the life of the producer organism [6]. Few types of microorganisms produce the majority of secondary metabolites. Secondary metabolites are produced when the cell is not operating under optimum conditions for example when primary nutrient source is depleted. Secondary metabolites are synthesized for a finite period by cells that are no longer undergoing balanced growth [7].

The production of economically important metabolites as antibiotics by fermentation of microbes are one of the major activities of the bioprocess industry. Secondary metabolites such as penicillin are produced during the stationary phase of cell growth. Most of the knowledge concerning secondary metabolism comes from the study of commercially important microorganisms [8].

Secondary metabolites are sometimes bioactive, usually of low molecular weight, and are produced as families of related compounds at restricted parts of the life cycle, with production often correlated with a specific stage of morphological differentiation [9].several antibiotics have been discovered from the secondary metabolites produced by actinomycetes and fungi [10].

Antioxidants act as radical-scavengersand inhibit lipid peroxidation and other free radical-mediated processes; therefore, these are able to protect the human body from several diseases attributed to the reactions of radicals.The natural antioxidants were characterized from the fungal compounds [11]. both natural and synthetic are gaining broad significance in prevention of diseases. To date, many kinds of bioactive compounds have been isolated from various fungi [12].

Materials and Methods

Collection of plant samples

The roots of calyptus collected from Misan city south of Iraq. Healthy roots were collected and processed separately within 48 h of collection.

Isolation and identification of endophytic fungus

The root samples were surface sterilized by [13]. The surface sterilized roots segments were evenly spaced in Petri dishes (9 cm diameter) containing potato dextrose agar (PDA) medium (amended with chloramphenicol 150 mg-l). The Petri dishes were sealed using Parafilm TM and incubated at 26°C in alight chamber with 12 hours of light followed by 12 hours of dark cycles. The Petri dishes were monitored every day to check the growth of endophytic fungal colonies from the root segments. Identified of fungus was confirmed according to the available taxonomic literature.

Extraction of secondary metabolites

Five discs (0.5mm diam.) were cut from the fungal culture of isolate by using a cork borer were edited into PD liquid medium in 500 ml flasks (triplicates) and incubated at 27 ◦C for 14 days on a rotary shaker. Fungal cultures were filtered on Whatman No 1 filter paper and the pH was adjusted at 3 by HCl for fungal filtrate. Filtrate was extracted with ethyl acetate (1:1 v:v) by using separating funnel. The organic layer was collected by dehydration of water by using Na2 SO4. The filtrate was filtered. Again and placed in Petri dishes then left to be dray at room temperature. 100µg of the dried secondary metabolites was dissolved in 1 ml ethanol as stock secondary metabolites solution to be used for further experiments.

Microorganisms Test

The test bacteria used in this study were *Esherichia coli, Staphylococcus aureus, Proteus vulgaris, Klebsiella pneumonia* and *Streptococcus pyogenes*. All bacterial isolates were obtained from the Microbiology Laboratory in the College of Science University of Misan.

Antimicrobial Bioactivity Assay

Filter paper discs (0.6 mm) after being sterilized by autoclave were socked in secondary metabolites solution for 5 min., filter paper discs with extract were placed on the surface of Muller-Hinton agar medium in Petri-dishes streaked with 0.2 ml of bacterial suspensions. Plates were incubated at 37 ◦C for 24 hr, an appearance of inhibition zones around the filter paper disc indicating the bioactivity of secondary metabolites of tested fungal isolate [14].The diameters of the clear zones were measured and compared with control agar plates containing discs with solvent only (control), triplicates were made.

Minimal Inhibitory Concentration

The minimal inhibitory concentration (MIC) values were determined by the standard serial dilution assay [15]. The inhibitory test was carried out on Muller-Hinton agar medium.

Chemical Analysis of Secondary Metabolites

Fungal secondary metabolites of endophytic fungus *S.radicinum* was chemically analyzed for alkaloids, phenols, amino acids, flavonoides and tannins according to the method described by [16].

Toxicity Test

Cytotoxicity of the fungal secondary metabolites was examined by using human RBC following a previously described method [17].

Antioxidant Activity

The DPPH radical scavenging capacity was measured according to [18].1 ml of fungal secondary metabolites was mixed with 0.5 ml of 0.2 mM methanolic DPPH solution. The reaction was allowed to stand at room temperature in the dark for 30 min and the absorbance was recorded at 517 nm against a blank(methanol solution).Dilutions were made to obtain concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90 and 1.95 µg/ml. Tests were carried out in triplicate. The ability to scavenge the DPPH radical was calculated using the following equation:

Scavenging effect (%) = [(A0-A1)/A0] × 100, where A0 and A1 are the absorbance of the control and the sample, respectively.

Statistical Analysis

Data were analyzed using Analysis of Variance (ANOVA) between any pair of variables.

Results and Discussion

Antimicrobial activities of secondary metabolite of endophytic fungus *S. radicinum* against the five bacterial pathogens was screened by paper disc diffusion method [19]. The antibacterial effect was highly variable, ranging between 22.5-35.0 mm .The antibacterial effects of the fungal secondary metabolite is shown in Table-1, Figure-1.

The fungi in general are a good source for produce antimicrobial agents (Janes *et al*., 2007). Some reports regarding the secondary metabolites produced by *S.radicinum* are available. It has been investigated that some species of this genus are sources for a potential secondary metabolites such as phytotoxins, StemphyloxinI,$C_{12}H_{32}O_5$. Nevertheless, the production of secondary metabolites substances by fungi, in general, is often affected by various growth conditional factors mainly the fermentation medium [20].

In the present study a liquid state fermentation medium used is efficient for a mass production of bioactive secondary metabolites by the fungus. The secondary metabolite of the examined of *S.radicinum* exhibited an inhibitory action against bacteria. The MIC secondary metabolites of *S.radicinum* exhibited the minimal values of MIC ranging between (25.0-100ug/ml) against test bacterial isolates Table-1.

A verification of non-toxicity of secondary metabolites against human blood revealed a negative test.

Table 1- Growth inhibition zones (mm diam) exhibited by the Fungal secondary metabolites

Bacterial strains	Inhibition zones (mm)	MIC(ug/ml)
E. coli	35.5	50.0
S.aureus	25.5	25.0
K. pneumoniae	24.0	100
P.vulgaris	22.5	100
Strep. pyogenes	23.0	50.0

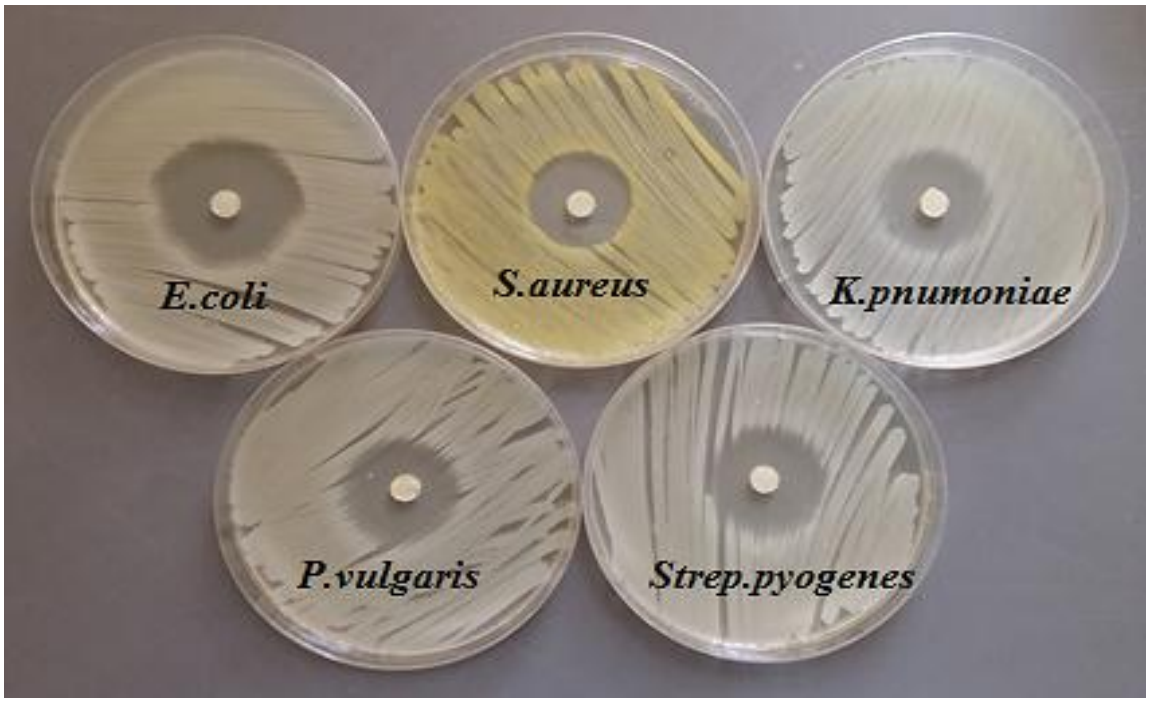

Figure 1- Inhibition zones exhibited by Fungal secondary metabolites

The chemical analysis of secondary metabolites of *S.radicinum* showed that contains Tannins group, phenol compounds and amino acid and absent flavenoide Table-2.

The antimicrobial inhibitory impact of secondary metabolites can be related to the bioactivity of these compounds. It has been reported that several phenolic compounds including tannin are potent inhibitors of microbial enzymes [21].

Studies showed that the inhibitory action of alkaloids against both G-negative and G-positive bacteria has also been demonstrated. Nonetheless, the inhibitory mechanism has been related to the inhibition of DNA synthesis by a specific alkaloid compounds [22].On the other hand, that tannin inhibits the growth of bacteria and has been attributed to the mechanism of tannin binding with the protein of the bacterial cell walls [23].

Antioxidants are compounds that inhibit or delay the oxidation process by preventing the initiation or propagation of oxidizing chain reactions. DPPH radical scavenging assay was observed that the scavenging activity of secondary metabolites from endophytic fungus *S. radicinum* at all concentrations from1.95 to 1000µg/ml is rather strong 18-77%.The extract improved 77% inhibition at higher concentrations,indicating lesser antioxidant capacity than positive control Figure-2.

DPPH radical scavenging assay is a swift and sensitive method for the antioxidant activity. to determination of free radical scavenging activity using the stable 2, 2- diphenyl-1-picryl-hydrazyl radical (DPPH) has received the utmost attention owing to the ease of use and its convenience [24]. A conclusion can be derived from this preliminary screening of *S.radicinum* that this fungus possessing a potential secondary chemical compounds that can be of significance and a promising as antimicrobial agents.

Table 2- Chemical compound of secondary metabolites from *S. radicinum.*

Indicators	Results
Alkaloids	+
Amino acids	+
Flavonoids	-
Phenols	+
Tannins	+

+ Present – absent

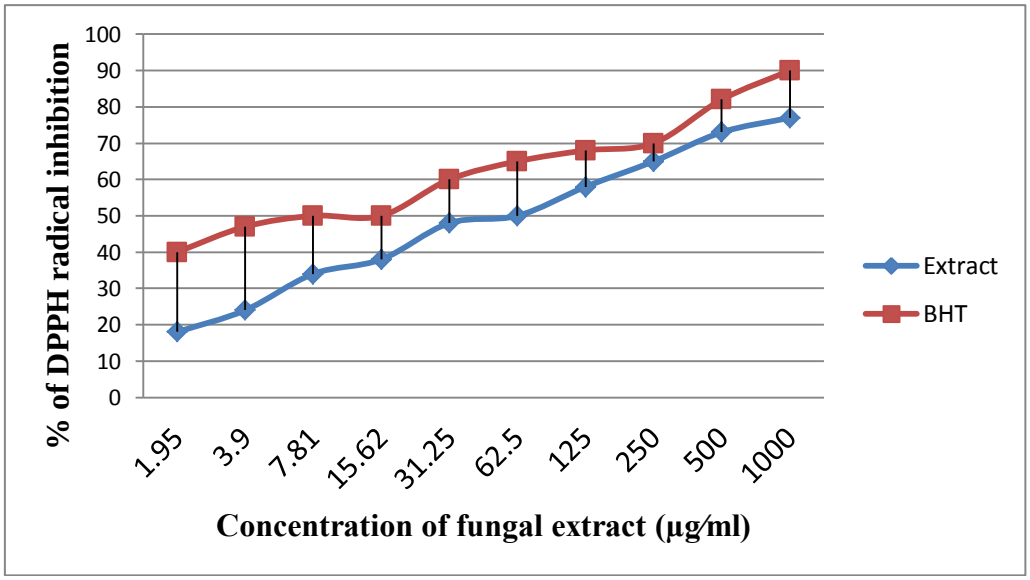

Figure 2-Antioxidant (DPPH scavenging) activity of investigated fungal secondary metabolites.

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