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Biological Activity of an Endophytic Fungus Phomopsis sojae Ay05 isolated from Glycine L. max

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Abstract: In the present study, the endophytic fungal isolate of *Phomopsis sojae* associated with *Glycine L. max* was investigated and identified according to its cultural and morphological characteristics, in addition to 18srRNAsequencing and phylogenetic analysis. The biological activity of ethyl acetate, aqueous and methanol extracts of fungal growth, i.e. antimicrobial, antioxidant and antitumor was evaluated. The potentiality of fungal extracts against some of G+ and G- bacteria such as *Staphlococcus* ,*Bacillus, Klebsiella* and also *Candida* has been investigated. In other words, the antioxidant potentiality of aqueous extract was superior to other ones, being 0.25351 µg/ml. Interestingly, the ethyl acetate of fungal extracts showed a moderate activity against tumor cell line of breast cancer with IC50=22.9 µg/ml. Ultimately, these a aforementioned results indicated the role of some microorganisms in production of new bioactive compounds, with feasibility and could be eco-friendly.

Keywords: Endophytic fungus, antioxidant, *Phomopsis sojae*, *Glycine L. max.*, antitumor

1.Introduction

Endophyte refers to the microorganisms that live asymptomatically in tissue of all parts of plant either intercellularly or intracellularly [1, 2]. Endophytic fungi are a heterogeneous group found everywhere as intercellular fungi belonging to the Ascomycotina, Deuteromycotina, Basidiomycotina and Oomycetes [3, 4].

There is an urgent need to find a new ecological niche for sources of bioactive agents for many pharmaceutical, agriculture, and industrial uses; these could be renewable, eco-friendly and easily to get [5].

The biotic impact on fungal growth by other organisms, predators, competitors and/or pathogens, induces production of fungal bioactive metabolites to maintain their growth. Recent papers provide the information that fungi produce a number of chemicals against predation to survive hustle situation [6].

There are several reports and studies on the biological activities of endophytic fungi as antiviral, anticancer and antimicrobial effects [7, 8]. Endophytic fungi may help the host plant to remain healthy by providing bioactive secondary metabolites; therefore they may have good potential to be sources of new naturally made bioactive or pharmaceutical products that increase continuously. Endophytic fungi are synthesizing bioactive compounds which help plants in defense against pathogens and some of these metabolites have been proven useful for new drug discovery[9]. Fungal metabolites include alkaloids, phenols, terpenoids, quinones, peptides, polyketides and acids[10].

Genus *Phomopsis* (Diaporthe) includes more than 1000 species which classified basically on its plant host, and is often identified as an endophyte [11].

Because of endophytic fungi, *Phomopsis* sp is wide distributed, it has been attracted much interest in the last years for its ability to produce different bioactive secondary metabolites [12, 13]. These secondary metabolites including antimicrotubule phomopsidin [14], antimalarial and antitubercular phomoxanthones [15], antifungal phomoxanthone A [16] and phomodiol [17], antifungal[18], herbicidal [19], algicidal [20], antimicrobial, and plant growth regulatory activities [21]. Fungus *Phomopsis* produces taxol which is one of potent antitumor compounds [22]. Phenolic and flavonoid compound could have a great role in stabilizing lipid oxidation, associated with antioxidant activity also extracted from *Phomopsis* [23].

Herein, the investigated study aims to (i) isolate and identification of the fungus *Phomopsis sp* associated with Glycine L.max[24] evaluation of the biological activity,i.e.antimicrobial, antioxidant and antitumor of some extracts of the fungus, *Phomopsis* sojae.

2.Materials and methods

Isolation and identification of the Endophytic Fungus

Adult and healthy leaves of *Glycine* L. max (soybean) plant were collected from agricultural farmlands Talkha cities, Dakahlia governorate, Egypt and sterilized superfially [25]. The sterilized leaves were fragmented into pieces 5×5 mm and plated on potato dextrose agar (PDA) contains 100 µg/ml of penicillin and streptomycin sulphate to inhibit growth of bacteria, then left for incubation at 28 °C for one week. The emerged hyphae from segments were carried to new PDA Petri dish for purification process. The isolated endophytic fungi were identified initially depending on the morphology of colony on PDA dishes, then they were stored on a slant of PDA

18S rRNA gene sequencing

The DNA was extracted using the FastDNA® Spin Kit. The amount of DNA was evaluated by using a Nano Drop spectrophotometer at absorbance 260 nm. The ITS1 sectors from DNA extracts was amplified in triplicate by universal primers that specific for fungi 18FITS1 (5'- CTTGGTCATTTAGAGGAAGTAA-3') and 18RITS4 (5'- TCCTCCGCTTATTGATATG

C-3'). The PCR reaction was carried out according to[26, 27]. The sequencing reaction is performed by [28]method. DNA similarity searches were performed by [29].

The phylogenetic and molecular evolutionary analyses for 18S rRNA gene nucleotide sequences results sequence alignments by the computer programs ClustalW and BioEdit 7.0.5.3 and implement in MEGA software version 6. The phylogenetic trees were formed using the Neighbor-Joining [30, 31] algorithm method.

Enumeration and Extraction of Secondary Metabolities of Endophytic Fungus Phomopsis sojae

The endophytic Phomopsis sojae was cultured under non shaking conditions for 25 days at 28 °C in 4 × 500 ml conical flasks with Potato dextrose broth (200ml/flask). The mycelia then air-dried to a constant weight. Nearly 1 g of the dry mycelia was extracted by methanol, by 1:50 (w/v) ratio overnight then filteration. The extract solution was concentrated by evaporation at room temperature [25]. The filterate was extracted by ethyl acetate with ratio (1:1) to obtain the organic phase extract, then concentrated by evaporation at room temperature, while the aqueous phase is collected separetlly. The dry residue was dissolved in 1 ml DMSO (10%) [32].

Antimicrobial test

Microbial susceptibility testing is carried out for the mycelia, organic and aqueous phases of culture filtrate. In triplicate The antimicrobial activity was evaluated by qualitative biologically analysis, by the disk diffusion method. The microorganisms that are used in this evaluation were the human pathogenic bacteria that characterized by drug resistant Gram-negative bacteria; *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris* and *Enterobacter cloacae* and Gram-positive bacteria; *Bacillus subtilis, Streptococcus pyogenes, Staphylococcus epidermidis* and *Staphylococcus aureus*, besides one fungal

strain *Candida albicans*. To evaluate the

antibacterial activity, 100 µl inoculum of 106 cells/ml bacteria were spread on Petri dishes with solid LB medium [33]. Disks of sterile filter paper Whatman no. 4 (6 mm) were placed in each dish equidistantly saturated with 10 µl of extracts. As negative controls, paper disks were inoculated with 10% DMSO(Dimethyl Sulfoxide). As positive control, Ciprofloxacin (50 µg/ml) was used. The dishes then incubated at 37° C for 24 h. The antibacterial activity was estimated by measuring inhibition zone, according to Souza *et al.* [34].

Antioxidant test

The free radical scavenging activity of the three extracts.i.e. aqueous extract ,ethyl acetate extract of organic phase of filterate and the methanolic extract of mycelia of the endophytic fungus *Phomopsis sojae* depends on the stability of 1, 1-diphenyl-2- picrylhydrazyl (DPPH). Free radical scavenging test was determined by the method of the Braca *et al.*[35]. 3ml of a 0.004% methanol solution of DPPH was prepared, the crude extract (0.1 ml) was added to it. After 30 min, the absorbance was determined at wave lenghth of 517 nm and calculated the percentage inhibition activity by [(A0–A1)/A0] x100 relation, where A0 is the absorbance of the control, and A1 is the absorbance of the extract/ standard. The inhibition curves were prepared and IC50 values were calculated.

Antitumor test

Organic fungal extract is used in the evaluation of antitumor activity that was performed according to the MTT (tetrazolium salt[3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay, which was described first by Mosmann to measure quantitation of mitochondrial dehydrogenase activity[36]. The human tumor cell lines breast (MDA-MB231) were used in the present study. The growth inhibition rate in percentage (I %) was calculated using the following equation: I %= [(Ablank –Asample)/Ablank] ×100, where Ablank is the absorbance of the control reaction (containing all reagents except the test extract and using "RPMI-1640 supplemented with 10 % fetal bovine serum" instead of "the test extract"), and Asample is the absorbance of the tested extract. Adriamycin was the positive control.

3. Results and Discussion

Morphological characterization of the isolated endophytic Ay05 fungus

The isolated endophytic fungus Ay05 gives its mycelia that covered the whole plate from the second day without pigments, and the pigment gradually appear until became yellow at the sixth day**(Figer 1) Molecular characterization:**

The isolated endophytic fungus Ay05 was identified via analysis of the 18S rRNA gene sequences and comparing to the closest type strains obtained from the database. The analysis showed that isolate could be identified as *Phomopsis sojae* species **(Figure 2).**

Antimicrobial activity of *Phomopsis sojae* extract

Antimicrobial test by discs diffusion assay of the aqueous, organic and mycelia extract of the endophytic fungus *Phomopsis sojae* against 12 different pathogenic bacteria and only one fungal strain which is *Candida albicans* with

Tetracycline and Nystatine as +ve control for bacterial and fungal strains respectively showing various values of inhibition zones (mm) (Table 1). Gram-positive bacteria *Staphylococcus aureus* (AQU=10mm, ORG=9mm and MYC=8mm), *Staphylococcus epidermidis* (AQU=8mm, ORG=8mm and MYC=7mm), *Bacillus subtilis* (AQU=10mm and ORG=8mm) and Gram-negative bacteria *Klebsiella pneumonia* (AQU=10mm, ORG=10mm and MYC=12mm).

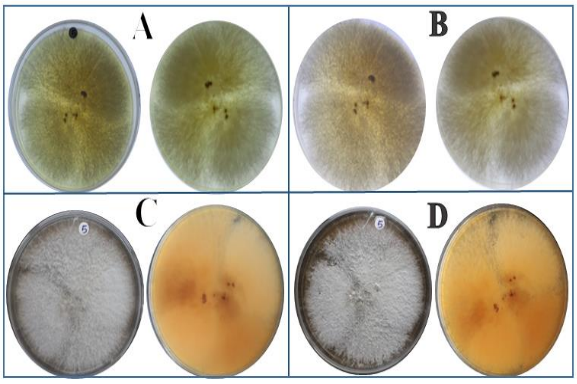

Figure 1. Isolated endophytic fungus Ay05 on PDA media after 2 days (A), 4 days (B), 6 days (C) and after8 days (D) at 28^{o}C

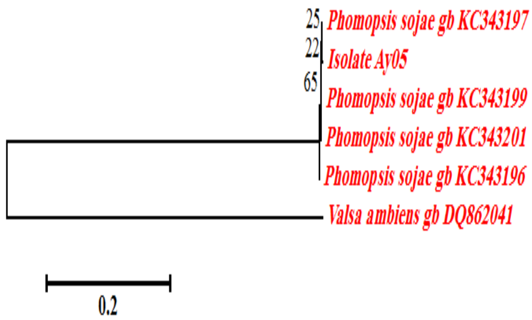

Figure 2: Phylogeny of endophytic fungal isolate from Glycine L. max (soybean). Many patterns of the sequences corresponding to the 18S rRNA gene of the studied isolate were performed followed by neighbor joining clustering. Bootstrap values represented percentages of 1000 replications. Bar represents 0.2 substitutions per nucleotide position.

Antioxidant Activity of The Endophytic Fungi *Phomopsis sojae*

The antioxidant activity using DPPH assay of the different extracts of the isolated *Phomopsis sojae* showed the free radical scavenging ability with different values of IC_{50}. *Phomopsis sojae* aqueous extract exhibited high antioxidant activity with IC_{50} (0.253515953 µg/ml), while the methanol extract of mycelia and organic phase gives lower antioxidant activity (Table 2).

Antitumor Activity of The Endophytic Fungi *Phomopsis sojae*

Screening results of the ethyl acetate extract of P. sojae indicates antitumor activities against tumor cell line MDA-MB231 breast using MTT assay with IC_{50} value 22.9 µg/ml compared with the control and other concentrations from 15 µg/ml to 300 µg/ml.

Table 1: Antimicrobial activity of various extracts of the endophytic fungus *Phomopsis sojae* with 20 mg/ml for organic and mycelia extracts using Tetracycline (30 µg/ml)for bacteria and Nystatin(20 µg/ml)for fungal strain as +ve control , DMSO (10%) as –ve control , showing inhibition zones (mm)by disc diffusion test.(NI= No Inhibition, AQU=Aqueous, ETH=Ethyl Acetate, MET=Methanolic, Tetra= Tetracycline**)**

Pathogens	**DMSO**	***P. sojae* extracts**			**Tetra**
		AQU	ETH	MET	
Streptococcus pyogenes	NI	NI	7	7	10
Staphylococcus aureus	NI	10	9	8	12
Staphylococcus epidermidis	NI	8	8	7	11
Bacillus subtilis	NI	10	8	NI	14
Pseudomonas aeruginosa	NI	NI	NI	NI	12
Escherichia coli	NI	8	7	NI	11
Klebsiella pneumoniae	NI	10	10	12	16
Proteus vulgaris	NI	NI	7	9	13
Enterobacter cloacae.	NI	NI	7	8	15
MRSA	NI	NI	7	8	9
Candida albicans	NI	NI	NI	7	14
Erwinia	NI	7	9	7	10
Shigella	NI	NI	7	NI	13

Discussion

Fungal endophytes have been identified as a source of new secondary metabolites, many of which have significance biological activities [37]. Endophytic fungi enhance the ability to adapt of the host and regulate the growth of host plants by producing phytohormones, like abscisic acid derivatives [38, 39]. Also the bioactive metabolites of fungi are included in the host's chemical defenses, such as taxol and ergot alkaloids [40, 41].

The attention of the scientific community worldwide has been directed towards discovering the active biological fungal metabolites such as new, chemotherapeutic agents, antibiotics and agrochemicals, these active fungal metabolites are realized as potentially powerful, with minor toxicity, and have a low ecological impact [42]. For example, *Pestalotiopsis* that is proved to be strongly forming of over 130 new effectively medicinal metabolites discover [43] , Also *Phomopsis* is an innovative genus with many significant findings involving unique and structurally relevant fungal metabolites that are physiologically active[44].

Table 2: Antioxidant activity of the aqueous extract, ethyl acetate extract of organic phase of filtrate and the methanolic extract of the endophytic fungus *Phomopsis sojae* showing IC_{50} values by DPPH Assay

Extract	IC50 (µg/ml)
Aqueous	0.253515953
Ethyl Acetate	1.786489518
Methanol	2.398213005

The genus Phomopsis has been approved to be a rich source of secondary metabolites, including, for example, phomopsichalasin1 and cytochalasins2, phomopsin A, convolvulanic acids, phytotoxic isobenzofuranones, oblongolide and phomopsolides (phytotoxic sesquiterpene ç-lactones)5,6[45].

In this study, endophytic fungus Ay05 isolated from *Glycine L. max* (soybean) plant was identified as *Phomopsis sojae* via 18S rRNA gene sequencing. The outcomes of our study evaluate the antimicrobial, antitumor and antioxidant activities of *Phomopsis sojae.* Bioactive metabolites produced in vitro by *Phomopsis sojae* have shown inhibitory activities against *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis* which are Gram-positive bacteria and *Klebsiella pneumonia* which is Gram-negative bacteria (**Table 1**). The findings are agreed with those collected from previous studies as the antimicrobial compounds from *Phomopsis* sp. by using the ethyl acetate extract [46, 47]. The fluctuated susceptibility of bacterial species may be due to the morphological and structural characteristics [48].

The results revealed that *Phomopsis sojae* aqueous extract exhibited high antioxidant activity with IC_{50} (0.253µg/ml). Theantana *et al.*,[49] realized that phenolic compounds are the basic power reduction antioxidant compounds and the other secondary metabolites could have an important role as significance free radical scavengers. The high antioxidant activity of *Phomopsis sojae* may be due to the existence of cooperation of phenolic compounds with other metabolites. Phenolic compounds are known to have antioxidant properties [50].

On the other hand, *Phomopsis sojae* extracts by the ethyl acetate shows potential antitumor activity versus tumor cell line MDA-MB231 breast depending on concentrations, with IC50 value (22.9 µg/ml) which considered low value.

Kumaran *et a.,l* [22] tested the endophytic fungus Phomopsis for formation of drug taxol which is anticancer compound, and proposed that *Phomopsis* may be possible alternative source for the compound taxol and could work as a potential genetic-engineered species for increase the generation of taxol. Hemtasin *et al.***,**[51]published that there are modern sesquiterpenes metabolites from ethyl acetate extract of the fungus *Phomopsis* sp.with cytotoxic activity. New oblongolides secondary metabolites from ethyl acetate extract of filtrate of *Phomopsis* sp. culture possess cytotoxic activity.[52]

Conclusion

The present study concludes that the presence of bioactive compound in the extract exhibited antimicrobial, antioxidant and antitumor activity in Phomopsis *sojae*. Furthermore, active crude extracts are pending more work to ease their potential production into new antioxidant, antimicrobial and antitumor agents for pharmaceutical industry in the next day

4. References

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