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Research Article

ISOLATION, CHARACTERIZATION, ANTIMICROBIAL POTENTIAL OF ENDOPHYTIC FUNGUS FROM *ANETHUM GRAVEOLENS* AND ITS *INSILICO* VALIDATION

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ABSTRACT

The research was mainly focused to identify bioactive endophytic fungus which serves as an alternative source of alkaloids produced by their hosts. Fungal diversity interprets significant conservation strategies and its medicinal importance. Although medicinal herbs serve against many ailments, fungal endophytes in today's scenario play a vital role in acting against certain infections but their mode of distribution and pattern of growth are still unexplored. In this research an attempt was made to isolate endophytic fungus from leaves of *Anethum graveolens* and to determine its antimicrobial potential along with molecular characterization studies thereby developing insilico docking interactions for the compounds that was quantitatively analyzed by GC-MS. The results revealed that antimicrobial resistance was significant against methicilin resistant *Staphylococcus aureus*. The molecular identification of the strain confirmed that it belongs to *Colletotrichum sp* which comes under Glomerellaceae family. The bioactive compound in the metabolite was found to be 2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane which exhibited maximum peak. The compound showed best docked pose with least binding energy -117.142 kcal/mol and binding was found to be predominant in active site of MRSA receptor. Thus the outcome revealed significant antimicrobial activity against MRSA which provides a suitable drug target using iGEMDOCK and the metabolite isolated has a potential source on its own.

Keywords: endophyte, bioactive metabolite, *Anethum graveolens*; MRSA receptor.

INTRODUCTION

The ability to synthesize different chemical compounds is a major attribute of Medicinal plants. Biological functions are performed by these plants and they defend themselves against all the microorganisms, insects and herbivorous animals too. About 80% of the world's populations are still dependent on traditional medicines[1]. *Anethum graveolens* which referred to as Dill was chosen for this study. This belongs to celery family Apiaceae. It is believed that the entire plant was found to possess medicinal value and that the dill seeds were used in traditional medicines against anti flatulence, dyspepsia, diuretics and several other ailments. It is used as an antispasmodic agent, anticonvulsant, anti-emetic and anticramp (in children) remedy and it is topically recommended as a wound healer[2].

Endophytic fungi are those that colonize living plant tissue without causing any immediate, over negative effects[3]. The fungal diversity in a host plant is highly influenced by characteristic of its host and growth condition or spatial factor of its host plant[4].

Endophytes are presumably ubiquitous in the plant kingdom and some fungi are even reported to exhibit antimicrobial activity[5]. Endophytic fungi such as *Acremonium sp*. are capable of producing various secondary metabolites[6]. Various different compounds have been isolated from the seeds, leaves and inflorescence of this plant; It is noted that 17 volatile compounds have been identified[7]. The endophytic fungus isolated from ethnomedically important plant *Crescentia cujete* was used to screen their bioactive principles towards various pharmacological studies and applications [8]. Endophytes were isolated from the leaves of *Lannea coromandelica* and produced secondary metabolites by testing its antifungal activity and predicting its mechanism[9].

In this extended research an attempt was made to isolate endophytic fungus from leaves of *Anethum graveolens* and to determine its antimicrobial potential along with molecular characterization studies thereby developing insilico docking interactions for the compounds that were quantitatively analyzed by GC-MS.

MATERIALS AND METHODS

Plant Collection

Fresh young leaves of *Anethum graveolens* L. were collected from Saidapet, Chennai in a sterile condition and used for further purposes. The herbarium specimen was identified and authenticated by Prof P. Jayaraman (Director of Plant Anatomy Research Centre).

Microbial Cultures and chemicals Required

Potato and Dextrose Agar (PDA), Ethyl Acetate, Chloroform, Dimethyl Sulphoxide (DMSO) were used. All chemicals were of the highest purity and of an analytical grade. The strains of *Bacillus subtillis* (MTCC 121), Methicillin resistant *Staphylococcus aureus* (MRSA) (MTCC 84), *Escherichia coli* (MTCC 443), *Klebsiella pneumoniae* (MTCC 530) were acquired from Institute of Microbial Technology, Chandigarh.

Isolation Of Endophytic Fungus

Isolation of leaf fungal-endophytes was carried out by modified technique[10]. The leaves were completely washed in distilled water, soaked in 70% ethanol for 30sec by surface disinfecting it and then dipped in 0.1% mercuric chloride ($HgCl_2$) solution for 60 sec, followed by rinsing it in sterile distilled water. Sterile blotting sheets were used to dry the leaves. The leaves were trimmed using sterile blades after drying. These pieces were placed on Potato Dextrose Agar augmented with antibiotic Cephalosporin (3mg/100mL) and incubated at room temperature. After 3-4 days the fungal hyphae tips were detached and laid on potato dextrose agar medium (PDA: 300g/L 1 diced potato, 20g/L dextrose and 20g/L agar) followed by incubating it for 7 days.

Pure Strain Isolation

The endophyte isolated was checked for its purity and assessed by examination of colony morphology. The final pure cultures were transferred to PDA slants and maintained at 4°C.

Secondary Metabolite Production

Subculturing the pure strain into Potato Dextrose Broth followed by incubating at 37°C for 21 days is carried out for secondary metabolite production. It was then centrifuged at 5000 rpm for 10minutes at 17ºC. Ethyl acetate was added in the ratio of 1:1 to the supernatant for extracting the bioactive metabolites[11].

Qualitative Phytochemical Analysis

Phytochemical screening of the crude extract was carried out using standard procedures to reveal the presence of chemical constituents such as Carbohydrates, Tannins, Saponins, Flavonoids, Alkaloids, Quinones, Glycosides, Cardiac glycosides, Terpenoids, Phenols, Coumarins, Steroids, Phytosteroids, Phlobatannins, Anthraquinones. This was carried out with the standard procedure described in Phytochemical methods[12].

Antimicrobial Activity of the Metabolite

The antimicrobial resistance of the metabolite was determined using Cross streak assay[13]. Gram positive strains (*Staphylococcus aureus, Bacillus subtilis*) and gram negative strains (*Klebsiella pneumoniae*, *E.coli*) were seeded by a single streak followed by swabbing the strain of interest perpendicular to the central streak and kept for incubation. Similarly, the antifungal activity was evaluated by seeding *Aspergillus niger* against the target strain and incubated.

DPPH Free Radical Scavenging Assay

The ability of the samples to annihilate the DPPH radical (1,1-diphenil-2-picrylhydrazyl) was investigated[14]. Stock solution of compound was prepared to the concentration of 10 mg/ml. Different concentration of the metabolite (250, 500, 750, 1000 µg) of sample were added. Methanol was used as blank and the control used was Methanol + DPPH solution. The reaction mixture was incubated for 30min in dark at room temperature; and the absorbance was recorded at 517 nm.

$$\text{Inhibition \%} = \text{Ac-As/Ac} \times 100$$

Where Ac is the absorbance of the control and As is the absorbance of the sample.

Fungal Genomic DNA Isolation

Genomic DNA was isolated for molecular characterization studies[15]. The fungal sample was scraped and homogenized with lysis buffer and centrifuged at 10,000 rpm for 10 minutes. Equal volume of phenol:chloroform:isoamyl alcohol was added to the supernatant and centrifuged at 10,000 rpm for 10minutes. To the aqueous layer containing nucleic acid equal amount of absolute ethanol was added. The pellet contains the precipitated nucleic acid by spinning at 10,000rpm for 15 minutes at 4ºC. 70% ethanol was used to wash the pellet and it is air dried. The pellet was then dissolved in Tris EDTA (TE) buffer and stored at -20ºC. Further the samples were subjected to Agarose gel electrophoresis.

Polymerase Chain Reaction

The amplification was carried out for ITS gene sequence (ITS1-5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4- 5-TCCTCCGCTTATTGATATGC-3') and the reaction mixture contains 11.5µl of MilliQ water, 2µl of Buffer, 2µl of dNTP's, 2µl of forward primer(ITS-1), 2µl of reverse primer(ITS-4), 0.25µl of template DNA and 0.2µl of Taq polymerase. The cycling conditions prevailing in the thermal cycler are as follows: Initial denaturation at 94ºC for 5mins followed by denaturation which occurs at 94ºC for 45sec, annealing occurs at 50ºC for 1min 30sec, continued by extension at 72ºC for 1min and final extension at 72ºC for 5mins. The sequence of reactions is set for about 32 cycles. The amplified DNA sequences are then sequenced using DNA sequence analyzer.

Sequence Analysis

Nucleotide BLAST algorithm was used to check the sequence similarity of the fungal ITS sequences obtained against the essential database maintained by the National Center for Biotechnology Information. Clustal W tool was used to align the BLAST sequences. The neighbor joining trees were generated by MEGA 7.0 software to determine the closely related species[16].

GC-MS Qualitative Analysis

Hewlett-Packard (now Agilent) trade name "Mass Selective Detector" (MSD) was used for analysis of the bioactive metabolite. Experimental conditions were as follows: HP-5 MS Ultra Inert (30 m x 250 µm x 0.25 µm) was used. The flow rate of helium which serves as the carrier gas (mobile phase) was set at 1.0 ml/min. Temperature in the oven was initially set upto 50 °C and after 10 mins it was increased to 240 °C. Samples were injected at a volume of 1 milli litre. Samples which dissolved in ethyl acetate were run fully at a range of 50-6650 m/z.

Insilico Docking

For molecular docking, the structure of the receptor (MRSA) was retrieved from RCSB PDB database and the entire structure was targeted against the ligand molecule. According to the hit value provided in GC-MS result the active ligand compound for docking was selected[17] and the structure of ligand was retrieved from PUBCHEM database. The 3D format of the ligand was obtained by using Discovery visualizer software. iGEMDOCK was used to study the insilico docking interactions which involves the binding of single ligand molecule with MRSA (Methicillin resistant *Staphylococcus aureus*) receptor where the fitness, bonding interactions were estimated and based on which the fitness of the binding against the target was determined. The pharmacological interactions and scoring function based on energy were combined by visualizing the compounds that were screened and ranked for the process[18].

RESULTS AND DISCUSSION

Isolation and Purification of Endophytic Fungi

The pure strain of endophyte was identified as represented in Figure 1 which was successfully isolated from the leaves of *Anethum graveolens.* The isolate was porous in nature with a mat like appearance as incase of *Penicillium sp.*

Secondary Metabolite Production

The endophyte was sub cultured to produce bioactive metabolite which was then extracted using ethyl acetate as shown in Figure 2 that significantly showed the distinction between the aqueous and the organic phase of the extract obtained and it possessed high polarity.

Qualitative Analysis of the Metabolite

The phytochemical analysis of the metabolite is tabulated below in Table 1 by carrying out the standard procedure for detection of the phyto constituents that are present in the metabolite showed that the bioactive compound was completely devoid of all these phytochemicals. The absence of these phytochemicals is acceptable as it is not mandatory that the metabolites from fungus should possess these bioactive compounds in it.

DPPH Free Radical Scavenging Assay

The antioxidant activity showed that when concentration is found to increase the activity of the free radicals is more thereby producing antioxidant effect by scavenging process. The obtained results were almost similar to the scavenging activity of AGLE and AA which exhibited 87.57% and 96.47% extracted from *Anethum graveolens*[19].

Antimicrobial Potential of the Metabolite

The endophytic fungus was checked for antimicrobial activity and it showed that there was maximum inhibition against Gram positive species specifically MRSA (Methicillin Resistant *Staphylococcus aureus*) as shown in Figure 4 and similarly the inhibition in the case of antifungal resistance was not prominent as depicted in Figure 5. The multidrug resistant pathogens had significant impact and so the drugs which possessed inhibitory effects on such micro-organisms become indispensable. Hence, for proper screening of the metabolite Methicillin resistant *S. aureus* was incorporated. It was found to have maximum inhibition against gram positive bacterial species especially *S. aureus*. The inhibition for gram negative species was not significant enough when compared with the strains of gram positive species.

Molecular Characterization Studies

Agarose gel was used to analyze the fungal DNA which produced crisp and high molecular bands. The quality of the DNA was checked using UV Spectrophotometer. For amplification process ITS primers were used wherein a template DNA and a defined annealing temperature is vital to be maintained in correct proportion. Sanger's method of sequencing was used to sequence the purified PCR products in a 3730 DNA sequencing analyzer (ABI) [11].

Sequence Analysis

A nucleotide BLAST for the strain was obtained and it was found to be congeneric to *Colletotrichum sp* with query coverage of 94%. Using Mega 7.0 software the neighbor joining tree was generated which indicated the evolutionary relationship between the various species of *Colletotrichum* as depicted in Figure 6.

GC-MS Analysis

The phytocomponents in the crude ethyl acetate extract of the metabolite were identified based on the retention time and area covered by the peaks on HP-5 MS Ultra Inert (30 m x 250 µm x 0.25 µm) column shown in Figure 7. Mass spectrum was interpreted using the database of National Institute Standard and Technology (NIST), Gaitherburg, Maryland. The GC-MS spectrum confirmed the presence of some compounds at various retention times. The compound 2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane was found to have the maximum peak area as illustrated in Table 3. The bioactive properties of this compound are still under research and it is possible that this compound can possess antioxidant activity in it.

Insilico Docking Studies

The compound with the least binding energy serves as the lead component. In the present study the binding energy of the compound 2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane was found to be -117.142 kcal/mol as illustrated in Table 4 and hence it tends to have highest fitness energy as represented in Figure 8. The structure of the ligand and the receptor is depicted in Figure 9 and Figure 10.

Table 1: Phytochemical Screening of the Metabolite

Sl. No.	Tests Perfomed	Analysis
1	Saponins	-
2	Flavanoids	-
3	Alkaloids	-
4	Glycosides	-
5	Cardiac glycosides	-
6	Coumarins	-
7	Phlobatanins	-
8	Anthraquinones	-

'+' – Presence ' - ' – Absence

Table 2: Annihilation Activity of the Metabolite

Concentration (µg/ml)	% inhibition	
	Test Sample	Positive control
250	78.37	94.35
500	80.945	95.26
750	81.645	96.035
1000	83.02	96.79

Table 3: List of Compounds and Its Corresponding Peak Area Analysed By Gc-Ms Analysis

Peak	RT	Area %	Compound name
1	3.085	100	Azetidin-2-one 3,3-dimethyl-4-(1-aminoethyl)
2	8.338	13.95	2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane
3	11.068	26.31	2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane
4	13.533	28.75	2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane
5	15.741	27.22	2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2-yl]oxy]phenyl]propane

Table 4: Fitness Energy Required for Insilico Docking Process

Compound	Energy	VDW	H Bond	Elec
5m18-Structure3D_CID_568149-1.pdb	-117.142	-100.003	-17.1395	0

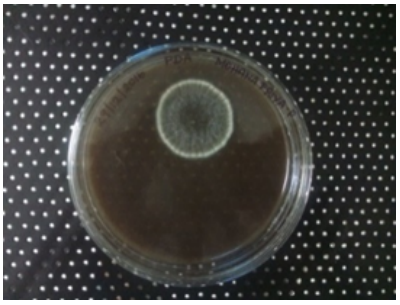

Figure 1: Pure isolate of Endophytic fungus from *A. graveolens*

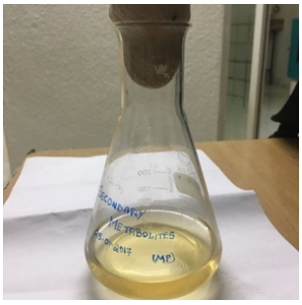

Figure 2: Crude extract of the bioactive metabolite

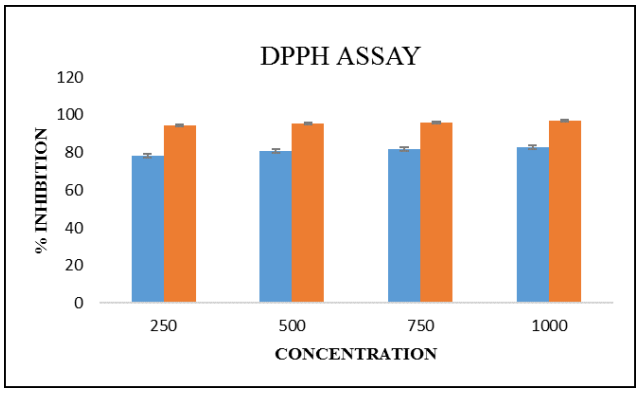

Figure 3: Graph showing the free radical inhibition of the metabolite

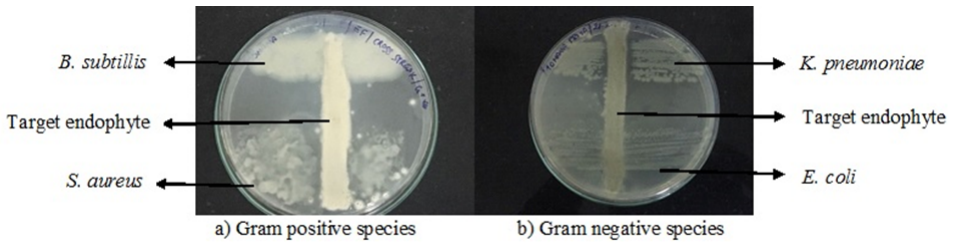

Figure 4: Antibacterial cross streak assay

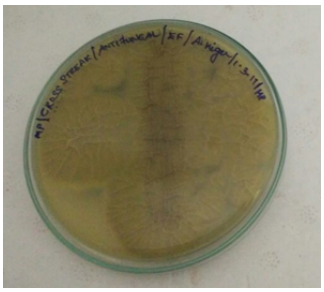

Figure 5: Antifungal cross streak assay of *A. niger*

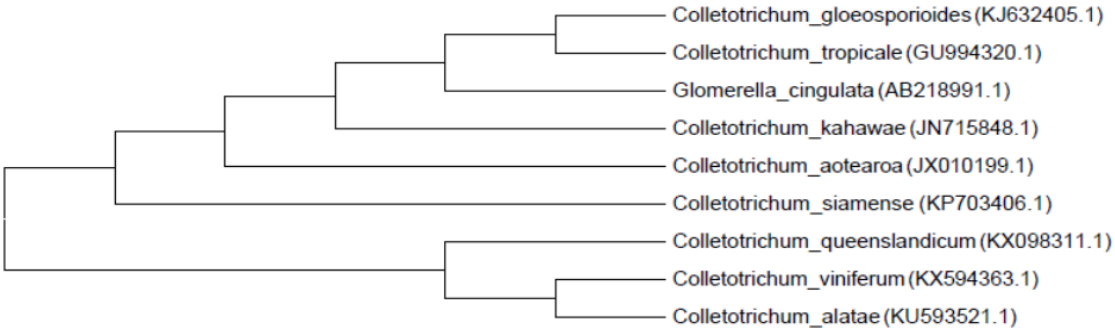

Figure 6: Evolutionary relationship between various species of *Colletotrichum* by MEGA 7.0

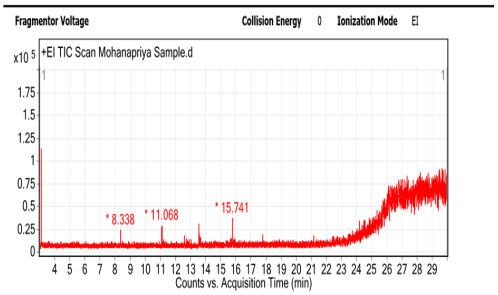

Figure 7: Peaks at various retention time using GC-MS analysis

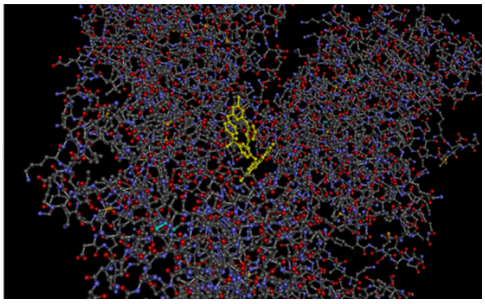

Figure 8: In silico docking interactions of the ligand and the receptor molecule

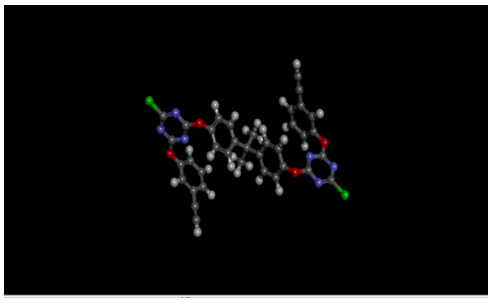

Figure 9: Structure of ligand 2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2 yl]oxy]phenyl]propane

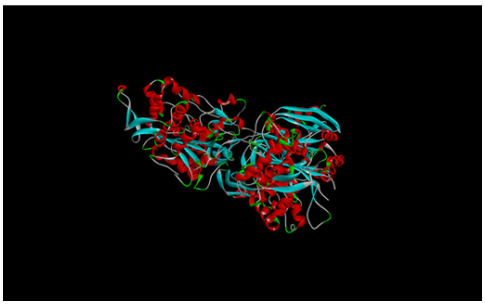

Figure 10: Structure of MRSA receptor molecule

CONCLUSION

Traditionally there are lot of medicinal herbs prevailing in this world. *Anethum graveolens* is one such type where it has a special trait of inbound medicinal value from the endophyte which is found inside the plant. Current research explained the benefits of the endophyte *Colletotrichum sp* by exhibiting antimicrobial resistance for Methicillin resistant *Staphylococcus aureus* which helped the binding of target molecule against the receptor. The interactions inferred that 2,2-Bis[4-[[4-chloro-6-(3-ethynylphenoxy)-1,3,5-triazin-2yl]oxy]phenyl]propane could be the source of activating the MRSA receptor. Thus, physicians can rely on this to check if they can develop suitable drug targets against MRSA infections.

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