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ANTIMICROBIAL AND IMMUNOMODULATORY POTENTIAL OF ENDOPHYTIC FUNGUS *FUSARIUM SOLANI* ISOLATED FROM *WITHANIA SOMNIFERA*

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ABSTRACT

Recent research indicate that pharmaceutical compounds produced by medicinal plants can also be produced by their inhabitant endophytes and this paves way to a new methodology of science utilizing endophytes as a source of bioactive compounds. This study aimed to add more information on endophyte driven biodiversity and bioactive potential of *Withania*. About 29 endophytic fungal isolates belonging to 16 different species were isolated from *Withania* root segments. Preliminary screening for antibacterial agents against common pathogenic bacteria led to isolation six endophytic fungi viz, *F.semitectum, F.avenaceum, Fusarium sp, F.oxysporum, Aspergillus sp* and *F. redolens*. The minimum inhibitory concentration of the extracts ranged in concentrations 21-78µg/ml. Endophytic isolate *Fusarium solani* also possessed immunomodulatory role and bioactive compounds in its the ethyl acetate extract were analysed by GC-MS. Based on its antibacterial and immunomodulatory potential, *Fusarium solani* was selected, characterized genotypically by 18S rDNA typing and the sequence was deposited in NCBI database under accession number KJ193849. The current study also describes *F.solani* as a suitable source for biocompound extraction due to its antibacterial and immunomodulatory role. Works on the mycotic biodiversity of endophytes of *Withania* and their potential to produce novel bioactive compounds are meager and this work becomes relevant.

KEYWORDS: *F solani, Withania, endophyte, GC-MS, Immunomodulation.*

INTRODUCTION

Natural bioactive compounds from animals, plants and microorganisms, have been a rich source of pharmaceutical agents from time immemorial (Newman and Cragg 2007). Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases. Historically many compounds have been isolated from the natural environment, particularly plants. It is the most successful source of drugs ever (Harvey et al. 1982).The World Health Organization estimates that 80% of the people in developing countries of the world rely on traditional medicine for their primary health care, and about 85% of traditional medicine involves the use of plant extracts (Verma and Singh 2008). This means that about 3.5 to 4 billion people in the world rely on plants as sources of drugs.

Virtually all plants harbor numerous endophytic bacteria and fungi that reside within the plant tissues, without causing any harm and establishing a mutualistic association (Hallmann et al. 1997). Plants benefit extensively by harboring these endophytic microbes, as they promote plant growth (Compant et al. 2005) and confer enhanced resistance to various pathogens (Clay and Schardl 2002) by producing antibiotics (Ezra et al. 2004). It has been suggested that the presence of a mutualistic endophyte acts as a "biological trigger" to activate the stress response system more rapidly and strongly than non-mutualistic plants (Redman et al. 2002).Recent reports state that endophytes can produce the same rare and important bioactive compounds as their host plants (Strobel et al. 2004). This would not only reduce the need to harvest slow growing and possibly rare plants but also preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a valued product may be easier and more economical to produce effectively reducing its market price (Stinson et al. 2003).Extraction from natural sources (such as plants/animals) presents some disadvantages such as dependency on seasonal, climatic and political features and possible ecological problems involved with the extraction, thus calling for innovative approaches to obtain such compounds (Bicas et al. 2009).

Withania somnifera (Ashwagandha) is one of the most valued plants in Ayurveda and is commonly used in Indian traditional health care systems. It comes under the Family *Solanaceae* and grows as erect evergreen shrubs distributed throughout the drier parts of India. The plant has traditionally been used to promote youthful vigor, endurance, strength, health, nurturing the time elements of the body and increasing the production of vital fluids, muscle fat, blood, lymph, semen and cells (Williamson 2002). But works on the biodiversity

of endophytes of *Withania* and endophyte derived novel compounds are few. Thus study was done to add more information on endophyte biodiversity and its bioactive potential, taking *Withania* as the source plant. On the emergence of new diseases, development of drug resistant pathogenic microorganisms and appearance of life threatening viruses are major challenges in human therapy, harnessing of novel better effective antimicrobial and immunomodulatory compounds from endophytes could be a very promising approach.

MATERIALS AND METHODS

2.1.Collection of Plant Material

Healthy *W. somnifera* plant samples were collected from botanical garden of Ayurvedic college Puthiyakavu at Ernakulam district and were carefully uprooted for the isolation of endophytes from the roots (the part which is effectively used for the preparation of Balarishta).The plant material was brought to the laboratory in sterile bags and processed within a few hours after sampling. Fresh plant materials were used for isolation to reduce the chance of contamination.

2.2.Isolation and Identification of Fungal Endophytes

Surface sterilized blot dried root samples were placed on Potato Dextrose Agar medium, amended with Chloramphenicol 0.4mg per 100 ml and were incubated at 26^{o}C for 3 weeks in an incubator. Fungal colonies were transferred to fresh Potato Dextrose Agar or Malt Extract Agar plates without antibiotics and identified using standard monographs. Taxonomic identification of the isolated endophytic filamentous fungi was done through observation of macroscopic and microscopic characters (G.S.de Hoog 2000).

2.3.Screening for Antibacterial Activity

Primary screening of antimicrobial potential of endophytic fungal isolates was done by agar well diffusion method against bacterial pathogens (using 5 test organisms- *B.Subtilis, P.aeruginosa, S.aureus, E.coli* and *K.pneuminiae*), turbidity equal to 0.5 Mc Farland (1×10^{8} CFU/ml) were spread with sterile cotton swab on Muller Hinton Agar. Fungal culture extract, concentration 150µg/ml was added (100µl) in to wells (6mm) formed by Cork borer on the MHA agar. The plates were incubated in suitable temperature for 24 – 48h. The zone of inhibition was measured and recorded.

Secondary level screening for antibacterial activity using different extracts of endophytes (petroleum ether, ethyl acetate, and methanol) was further done by agar disc diffusion method

as described above. Standard antibiotic, streptomycin (30µg/ml) was used as a positive control. The loaded discs were placed on petridish containing sterile MHA medium seeded with test organism. All test plates were incubated at 37^{o}C for 24 hours. The inhibition zone was observed, measured and recorded. The minimum inhibitory concentrations of the best extracts were calculated by microtitre plate-based antibacterial assay using resauzarin method (Sarker et al. 2007).

2.4.Immunomodulatory Activity of Endophytes

Immunomodulatory role of compounds were assessed in vitro using phagocytosis as a screening tool (Wang et al. 2010).This assay involves the spectrophotometric measurement of congo red-stained yeast cells which have been phagocytized by macrophages. Blood was used for isolation of leucocytes by density gradient centrifugation using Histopaque. Mononuclear cells and platelets are collected on top of the Histopaque layer because they have a lower density, in contrast red blood cells (RBC) and granulocytes have a higher density thus get collected at the bottom of the gradient. Platelets were then separated by subsequent washing or by centrifugation. The viability of the cells was checked by trypan blue dye exclusion method.

2.5.GC-MS Analysis of the Biomodulatory Compound

The GC-MS analysis of ethylacetate extracts of WEF 7 was done. Instrument used was a GC varian CP 6800 and MS model Saturn 2200. The interpretation on mass spectrum GC-MS performed based on the database of National Institute Standard and Technology having more than 62000 patterns. The spectrum of the unknown compound was compared with the spectrum of the known compound in the NIST library.

2.6.Molecular Identification of Selected Fungal Isolate

Identification of selected fungal cultures was done using D1/D2 region of Large Subunit by 28S rDNA typing. Fungal DNA was isolated using Sigma DNA isolation kit. The D1/D2 region was amplified by PCR from fungal genomic DNA using universal primers (DF- 5'-CCCGCTGAACTTAAGC-3',DR -5'-GGTCCGTG TTTCAAGACGG-3'). The PCR product of 1.5 kb was purified using illustra GFX PCR DNA and gel band purification kit (GE Healthcare). The purified amplicon was sequenced using big dye terminator v3.2 cycle sequencing chemistry for ABI Bioprism (Applied Biosystems). The sequence was analyzed using the BLAST (www.ncbi.nlm.nih.gov) search algorithm and aligned to their nearest neighbors. The sequence was deposited in the NCBI GenBank database.

RESULTS

3.1.Isolation and Identification of Endophytes

In our investigation a total of 29 fungi belonging to sixteen different genera were isolated from forty five root segments of *Withania somnifera* and *Fusarium sp* wasfound to be the most predominant fungal species in both macro and micro identification procedures. The fungal isolates included *Aspergillus sp, A.niger, A.restructus, Fusarium acutatum, Fusarium armeniacum, Fusarium avenacium, Fusarium begoniae, Fusarium dimerum, Fusarium oxysporam, Fusarium redolens, Fusarium sambusinum, Fusarium semitectum, Fusarium solani, Fusarium udum, Fusarium verticilloides and Mucor sp.* Out of sixteen endophytic fungal species only one of them came under the phylum Zygomycota and all others belonged to Ascomycota. Fig 1 depicts the frequency of endophytic fungi isolated from *Withania* roots as a part of our work. The colonization frequency of endophytic fungi from root of *W.somnifera* was 35% .

3.2.Biological Activities of Fungal Endophytes

a) Antibacterial Activity of Endophytes

Out of endophytes checked, six of fungal isolates (*F.semitectum, F.avenaceum, Fusariumsp, F.oxysporum, Aspergillus sp, F.redolens*) showed antagonism towards bacterial pathogens (table 1). Ethyl acetate extract of *Fusarium* sp (WEF-7) & *Aspergillus* sp (WEF-9) showed maximum (27mm and 25 mm) activity against *S.aureus, E.coli & B.subtilis* when compared to other solvent system. Therefore, the suitable solvent system for further studies was selected as ethyl acetate.

Minimum inhibitory concentration of ethyl extract of two fungal extract (i.e.WEF-7-*Fusarium sp* and *WEF-9-Aspergillus sp*) are shown in table 2.The MIC values of endophyte extracts are shown in µg/ml concentration against 4 indicator strains. The inhibitory effects on gram positive and gram negative bacterial growth were relatively low, concentration of MIC values ranging in 21-78µg /ml.

b) Immunomodulatory Activity of Endophytes

Among the sixteen fungal isolates, only six showed immunomodulatory potential. WEF-7 *Fusarium* sp showed maximum phagocytic activity than others by significantly increasing the selected immune parameter i.e phagocytic activity (Fig 2). Among the various endophytic fungal isolates, *F.solani* (WEF7) and *Aspergillus* sp (WEF 9) showed more phagocytic activity**.**

In GC-MS analysis (table 4), out of the nine compounds obtained from ethyl acetate extract of *Fusarium solani (WEF7),*only 3 compounds were reported to be pharmaceutically important and also isolated from other endophytic fungi. The compounds were identified as Phenol,2,4-bis(1,1-dimethyl ethyl), Benzene propanoic acid,3,5-bis(1,1-dimethyl) and Carnegine using software analysis.

3.3.Molecular Characterization of WEF -7

Endophytic fungi WEF7 showed maximum antibacterial activity, immunomodulatory activity and which produced pharmaceutically important volatile compounds on GC MS analysis was selected and subjected to molecular analysis. D1/D2 region of the fungal isolates was amplified from the isolated genomic DNA using the universal primer and analyzed using bioinformatics tool BLAST of NCBI. The result showed that the organism WEF-7 had close (99%) similarity to *Fusarium solani.* The sequences generated in this study were deposited in the NCBI gen bank and accession KJ193849.

DISCUSSION

Endophytic fungi represent an important and quantifiable component of fungal biodiversity and are known to affect plant community diversity and structure (Krings et al. 2007).*Alternaria* and *Fusarium* species were reported as the dominant taxa obtained from *Withania somnifera, Artemisia annua, Platanus orientalis* and *Rauwolfia serpentina*, as compared to the other host plants (Qadri et al. 2010). Khan *et al* reported colonization frequency of endophytic fungi from *W.somnifera roots as* 14.15%. Present study also satisfies the mycotic diversity and dominace of fusarium sp. aThe maturity of the roots, seasonal changes, other factors like soil conditions, climate and the dynamics of soil microflora may also influence the colonization of endophytes in the plant tissue.

Endophytes colonizing medicinal plants could produce same bioactive natural products or derivatives that are more bioactive than those of their respective hosts (Strobel et al. 2004).To prove the effect of such association, the isolated endophytes were grown and the fermentation broth were extracted with different solvents and screened to evaluate the biological potential. Estimation of Minimum Inhibitory Concentration of ethyl acetate extracts of selected isolates (WEF7 & WEF 9) was done by the resazurin method. This microtitre-plate method is simple, sensitive, rapid, robust and reliable, and could be used successfully to assess antibacterial properties of natural products (McNicholl et al. 2007). Resazurin is an oxidation–reduction indicator used for the evaluation of cell growth, particularly in various cytotoxicity assays. It

is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. Resorufin is further reduced to hydroresorufin (uncoloured and nonfluorescent). Any colour changes from purple to pink or colourless were recorded as positive**.** The lowest concentration at which colour change occurred was taken as the MIC value.Immunomodulators are substances that regulate or modify the function of the immune system. They may act as immunosuppressant by inhibiting the immune response or as immunostimulants by stimulating the immune response. This suggested that the endophyte extracts contained some immunostimulating bioactive compounds.

Previous reports indicate that a decoction prepared from the root of *W.somnifera* consisting of equimolar concentrations of Sitoindosides VII-X and Withaferin A, induced immunomodulant activities (Martinez-Luis et al. 2012).Other withanolides, including their glycosylated products are reported to have immunomodulatory activities (Zhao et al. 2002).Thus immunomodulatory role of *Withania* plant is well established, but the immunomodulatory role of its inhabitant endophytes is novel. Endophytes provide an abundant reservoir of bioactive metabolites for medicinal exploitation, and an increasing number of novel compounds are being isolated from endophytic fungi. Until 2003 approximately 4,000 secondary metabolites with biological activity had been described from fungi (Lane G.A. 2000), most of these metabolites are produced by so called “creative fungi”.

Thus it should be emphasized that, the immunomodulatory properties of *W somnifera* is enhanced or is associated with the endophytic microbial diversity. This finding adds more values, supports and strengthens the objective of the present study and remains a new perspective for future endeavors regarding plant bacterial association. Based on the observations, the ethyl acetate extracts of the fungal species WEF-7 was analysed to understand the components and its chemical nature.

The compound Phenol,2,4-bis(1,1-dimethyl ethyl), is a precursor to many complex compounds and is widely used as antioxidants, light protection agents or UV stabilizers and chemical intermediates for the synthesis of other chemical intermediates. Phenol,2,4-bis(1,1-dimethyl ethyl has free radical scavenging activity (produced from root endophyte, *Frankia brunchorst* from *Casuarina equisetifolia)* (Santhi et al. 2012); anticancer activity (from ethanolic extract of *Solanum torvum)* (Panigrahi et al. 2014), antioxidant activity (from root extract of Plumbago zeylanica) (Ajayi et al. 2011), antitumor potential **(**from *Passiflora*

incarnate) (Sujana et al. 2012), antifungal activity (from medicinal plant *Memecylon umbellatum)* (Rangel-Sanchez et al. 2013). All these reports are stating the fact that the antimicrobial activity of the fungal isolate WEf-7 is a characteristic attribute of possessing this volatile compound Phenol,2,4-bis(1,1-dimethyl ethyl).

Benzene propanoic acid,3,5-bis(1,1-dimethyl),is an isoquinoline alkaloid which is widely used as an anticancer and anti metastatic agent. Benzene propanoic acid,3,5-bis(1,1-dimethyl), has been isolated from Woodfordia fruticosa leaves (Grover 2013) possessing antifungal and antioxidant activities. We know Withanaloid, the effective and proven biologically active component of *Withania* is also an alkaloid then it is clear that other effective alkaloids are also possessed by the plant as a metabolic byproduct of the endophytic fungus WEF-7.

The third and another important compound Carnegine, also called as hydrocinnamic acid since it is prepared by oxygenation of cinnamic acid, a medicinally important product obtained from the cinnamon oil and a synthetic indicative of pharmaceuticals. It is also used as an antioxidant, antimicrobial, flavoring agent and also as a fixative agent in medicine. Carnegine was previously isolated from *Nandina domestica* (Iwasa et al. 2008).

When we correlate the results obtained, it is clear that the medicinal property of the plant, *Withania somnifera* tested is strongly affected by the endophytic fungi *Fusarium solani* isolated from it. These compounds could be considered as strong candidates for the development of effective drugs with broad spectrum activity.

Although many medicinal plants and their secondary metabolites have been screened for antimicrobial and immunomodulatory activities, this is the first report on such biological activities of fungal endophyte *Fusarium solani* isolated from *Withania somnifera*. The present investigation has also opened avenues for identification, characterization of potential plant endophytes as an alternative source for novel metabolites which can be used in biomedicines for therapeutic utility. Such products, if well tolerated by the patient, may be developed into alternative co adjuvants in the treatment of disorders caused by an exaggerated or unwanted immune responses. The result shows the importance of endophytic fungi for producing bioactive compounds. We expect that the combined knowledge of microbial diversity and the ability to target specific biosynthetic genes along with bioprocess

techniques will provide the necessary tools to utilize the endophytic fungus, *Fusarium solani* to produce novel, potent pharmaceuticals on an economically viable scale.

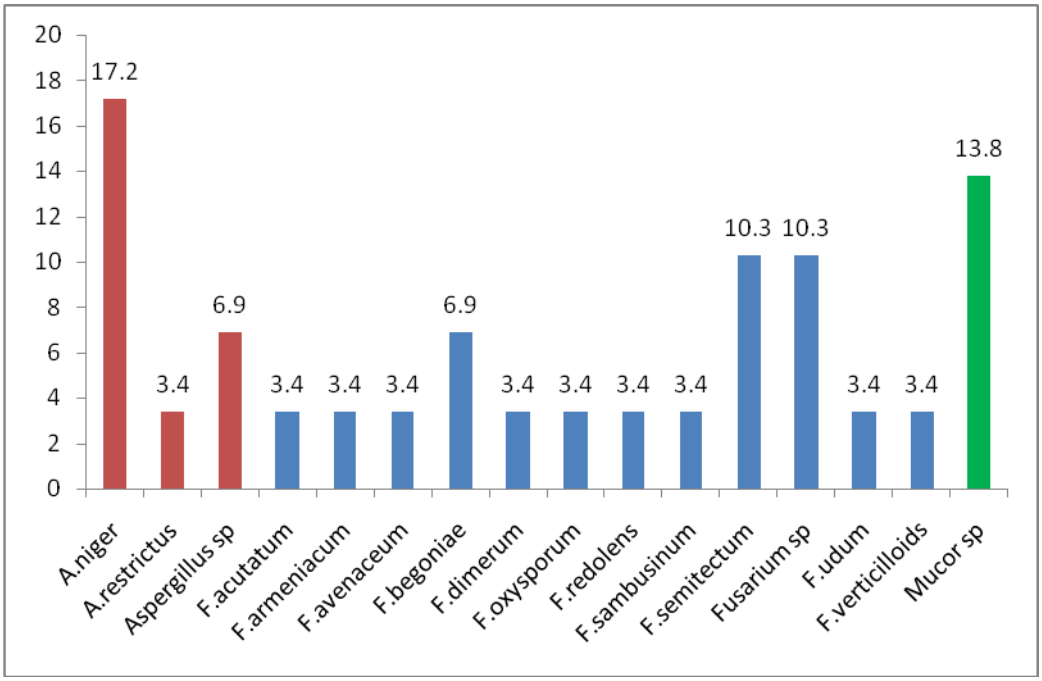

Fig 1. Frequency of endophytic fungal isolates from roots of *Withania somnifera*

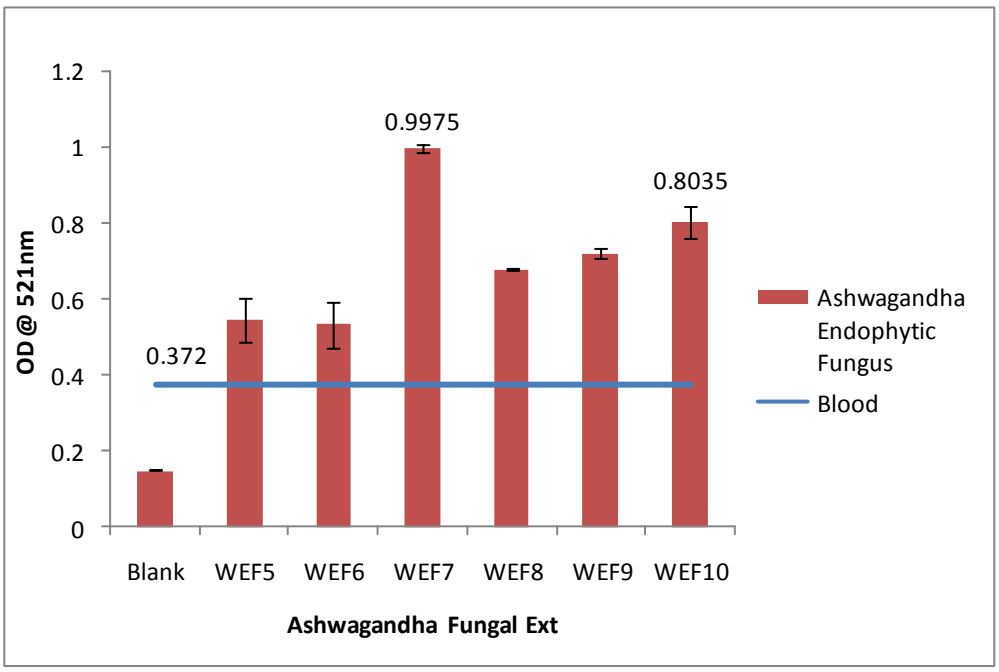

Fig2. Phagocytic activity of endophytic fungal isolates from roots of *W.somnifera*

Table 1.Antibacterial activity of endophytes against common pathogenic bacteria

Sl no	Endophyte		Zone of inhibition in (mm)				
			B.subtilis	***S.aureus***	***E.coli***	***K.pneumoniae***	***P. aeruginosa***
1	WEF-5	*F.semitectum*	19±0.33	35±0.56	29±0.20	20±0.35	-
2	WEF-6	*F.avenaceum*	23 ±0.41	31±0.35	24±0.23	22±0.36	-
3	WEF-7	*F. solani*	21±0.25	35±0.48	22±0.01	22±0.36	-
4	WEF-8	*F.oxysporum*	22±0.27	32±0.39	30±0.28	20±0.42	-
5	WEF-9	*Aspergillus sp*	20 ±0.39	39±0.27	22±0.28	21 ±0.32	-
6	WEF10	*F.redolens*	20±0.46	22±0.27	24±0.44	20 ±0.23	-

Table 2. Minimum inhibitory concentration of Ethyl acetate extract of selected endophytic fungi by Microtitre plate Method.

Ethyl acetate extract	***S.aureus***	***B.subtilis***	***E.coli***	***K.pneumoniae***
	MIC (µg/ml)	**MIC (µg/ml)**	**MIC (µg/ml)**	**MIC (µg/ml)**
WEF-7	21±0.3	48±0.19	42±0.23	72±0.33
WEF-9	24±0.27	51±0.36	45±0.29	78±0.21

Table 3. Compounds identified in Ethyl acetate extract of WEF-7 as per GC-MS analysis

No	RT	Name of compound	Peak area
1	12.438	2-Myristynoyl pantethine	10308
2	14.461	Phenol,2,4-bis(1,1-dimethyl ethyl)-	543126
3	26.757	Phenol,4-(1-methyl-1-phenol	2.043
4	28.539	Benzene propanoic acid,3,5-bis(1,1-dimethyl)	11068
5	29.163	Morphinan-4,5-epoxy-3,6-di-ol,6-[7-nitr]	209659
6	29.934	Carnegine	15195
7	32.819	1-Ethylsulfanylmethyl-2,8,9-trioxa-5-aza	97503
8	33.746	Phenol,4,4'-(1-methyl-ethylidene)bis-	3.856
9	35.036	10-Acetoxy-2-hydroxy-1,2,6a,6b,9,9,12a-h	12576

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