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EVALUATION OF ANTIMICROBIAL ACTIVITY OF AN ENDOPHYTIC *ASPERGILLUS SP.* ISOLATED FROM *AZADIRACHTA INDICA* AGAINST PLANT AND HUMAN PATHOGENS.

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ABSTACT

Medicinal plants like neem which have been shown to contain bioactive compounds that exhibit multiple health benefits and are therefore, regarded as potential sources of new and improved therapeutic drugs. The present study aims to evaluate the antibacterial and antifungal activity of an endophytic *Aspergillus* sp. isolated from *Azadirachta indica* against ten selected plant and human pathogens. Out of the ten test pathogens, the *Aspergillus* sp.'showed antimicrobial activity against eight pathogens. The endophytic *Aspergillus* sp. isolated from *Azadirachta indica* was found to exhibit the antibacterial activity against Gram-positive bacteria, *Bacillus subtilis*, and Gram-negative bacteria *Escherichia coli* (MTCC 40) and *Pseudomonas fluorescens* (MTCC 1748). The crude extract of *Aspergillus* sp. isolate inhibited the growth of opportunistic fungal pathogens, *Candida albicans* (MTCC 227) and *Candida glabrata* (MTCC 3814) which could not be inhibited by even the standard antifungal drug, Fluconazol. The *Aspergillus* sp. also showed zone of inhibition against phytopathogenic fungi *Fusarium graminearum* (MTCC 2089).

KEY WORDS: Endophytes, bioactive, therapeutic drugs, pathogens.

INTRODUCTION

Endophytes are microorganisms that inhabit living internal tissues of plants without causing any immediate, overt negative effects , i.e., they have asymptomatic colonization in plant [1].

Till date, all the plants that were examined were found to harbor atleast one endophyte in their tissues. This states the richness of endophytic microflora in nature. Although each individual plant host one or more endophytes, not many of them have been studied for their endophytic micro flora or their potential for human use [2].

Azadirachta Indica, commonly known as neem, Nimtree, and Indian Lilac belongs to the family Meliaceae. It is native to India and the Indian subcontinent including Nepal, Pakistan, Bangladesh and Sri Lanka. *A. indica* (leaf, bark and seed oil) is known to contain bioactive compounds and it exhibits wide range of pharmacological activities including; antioxidant, antimalarial, antimutagenic, anticarcinogenic, anti-inflammatory, antihyperglycaemic, antiulcer Omit it anti-diabetic properties, antibacterial and antifungal activity [3]. Neem has been used in traditional medicinal systems since ages and can be regarded as a potent source of therapeutic agents.

This study was designed to explore the antimicrobial efficacy of an endophytic fungi of *Aspergillus* sp. isolated from *Azadirachta indica* on selected human and plant pathogens. The sample from the leaves, bark, stem of *Azadirachta indica* were used for the present study.

MATERIAL AND METHODOLOGY

Collection of plant samples from different areas.

Plant samples were randomly collected from Kurukshetra district of Haryana, India. The sample was cut aseptically with a sterile scalpel and brought to the laboratory in an air tight plastic bag.

Surface sterilization of plant samples

Surface sterilization of plant samples was done to remove epiphytes. Leaf/stem/bark sample was washed with running tap water followed by washing with double distilled water. Sample was cut into 2-3 cm sections in Laminar Air Flow. Then sample fragments were sterilized by immersion in 70% ethanol for 1 minute and sodium hypochlorite solution (4% available chlorine) for 5 minute followed by rinsing with 70% ethanol and then sterilized double distilled water. The sample fragments were allowed to dry in Laminar Air Flow [4].

Isolation of endophytic Fungi

3-4 Segments of each sample were placed on Potato Dextrose Agar (PDA) amended with ciprofloxacin (100 mg/l). The Parafilm-sealed petri dishes were then incubated in a

Biochemical Oxygen Demand (BOD) incubator with humidity for 25 days at 12-h light/dark cycle at 27 ±2°C[5]. The plates were checked on alternate days and hyphal tips of actively growing fungi were then sub-cultured.

Purification and maintenance of fungal isolates

Individual fungal colonies were transferred to fresh media plates for purification and identification purposes and their slants were preserved in refrigerator at 4°C[6].

Fermentation

2-3 discs of fungal isolate were inoculated in a liquid medium, potato dextrose broth (PDB) and incubated for 7-10 days at 25- 27˚C in a BOD incubator. After the incubation, the biomass was filtered (Whatman filter paper No. 1), and the cell-free filtrate was used in screening for antimicrobial activity [7].

Procurement and maintenance of test pathogens

The various human pathogenic microorganisms were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, which included Gram positive bacteria, *Streptococcus mutans* (MTCC 497), *Streptococcus pyogenes* (MTCC 1924), *Bacillus megaterium* (MTCC 428) and *Bacillus subtilis* (MTCC 121); Gram negative bacteria *Escherichia coli* (MTCC 40) and *Pseudomonas fluorescens* (MTCC 1748); fungal pathogens *Candida albicans* (MTCC 227) and *Candida glabrata* (MTCC 3814).Two phytopathogenic fungi *Alternaria solani* (MTCC 10690) and *Fusarium graminearum* (MTCC 2089) were also obtained. The slants of brain heart infusion agar were made to preserve the cultures. All the slants were kept at 4ºC in the refrigerator for further studies.

Assessment of antimicrobial activity by agar well diffusion method

The inoculum of different test pathogens was adjusted according to 0.5 McFarland standard [8]. 200µl of test pathogen was spread aseptically on the surface of Muller Hilton agar plates using sterile cotton Swabs. Wells of about 6.0 mm diameter were punched in the agar plates using a sterile cock borer. 100µl of the crude metabolite was transferred into each well. Plates were then kept in laminar Air flow for 30 minutes for pre-diffusion of metabolite to occur and then incubated at 37˚C for 24 hours. All the experiments were performed in triplicates and after 24 hours, the diameter of resulting zone of inhibition were measured in terms of millimeter (mm.) using a Hi-media zone scale [9,10].

Determination of relative percentage inhibition

The relative percentage inhibition with respect to positive control was calculated by applying the following formula: [11]

Relative percentage inhibition of the test extract = (100(X-Y))/(Z-Y))

Where, X = Total area of zone of inhibition of the test extract

Y = Total area of zone of inhibition of the solvent

Z = Total area of zone of inhibition of the standard drug

Total area of zone of inhibition was calculated according to area = πr^2, where, r = radius of the Zone of inhibition.

RESULTS

In the present study, the endophytic *Aspergillus* sp was screened for its antimicrobial activity against eight human and two plant pathogens. Out of the ten test pathogens, *Aspergillus* sp isolated from the bark of the *Azadirachta indica* showed antagonism against eight of the test pathogens. Neither the test isolate nor the standard antibiotic showed any activity against plant fungal pathogen, *Alternaria solani*. Maximum antimicrobial activity was observed against *candida albicans* (24 mm. in diameter) and minimum activity was observed against *Bacillus subtilis* and *Streptococcus pyogenes* (11 mm. diameter each).

The test isolate showed antibacterial as well as antifungal activity. The standard drug, fluconazol, used in the experiment did not showed any activity against the fungal test pathogens whereas the test isolate showed significant antifungal activity against three of the four test pathogenic fungi. *Candida albicans* showed the zone of 24 mm. (in diameter), *Candida glabrata* displayed a zone of 13 mm. (in diameter) and *Fusarium graminearum* demonstrated inhibition zone of 14 mm. (in diameter). This suggests that the test *Aspergillus* sp. can be exploited for its potential to make an antifungal drug.

The *Aspergillus* sp. was found to be broad spectrum in its activity. The antimicrobial activity was found against three of the four gram positive test pathogens, *Streptococcus mutans* (17 mm. in diameter), *Streptococcus pyogenes* (11 mm. in diameter) and *Bacillus subtilis* (11 mm. in diameter) and against both the gram negative test pathogens, *Escherichia coli* (18 mm. in diameter) and *Pseudomonas fluorescens* (12 mm. in diameter).

The relative inhibition percentage was infinity for *Candida albicans*, *Candida glabrata* and *Fusarium graminearum* whereas it was zero for *Alternaria solani*.

The maximum relative percentage inhibition observed against bacterial pathogens was 20.25% observed against gram negative bacteria, *E. coli* (Table1)

Table 1. The zone of inhibition exhibited by fungal isolates against test pathogens.

Code of the sample	Sample part	Zone of inhibition (in mm.)									
		Test pathogens									
		Bacteria						Fungi			
		Gram-positive				Gram-negative		Opportunistic human pathogens		Plant pathogens	
		Bm	Bs	Sm	Sp	Ec	Pf	Ca	Cg	As	Fg
Aza I 2 B1	Bark	NA	11±0.33	17±0.57	11±0.57	18±0.88	12±0.33	24±0.33	13±0.33	NA	14±0.23
Positive Control		48±0.57	35±0.33	39±0.33	38±0.57	40±0.57	50±0.57	00±00	00±00	00±00	00±00
Relative percentage inhibition		0	5.25	19	8.38	20.25	5.76	∞	∞	0	∞

Values are mean inhibition zone (mm) ± S.D of three replicates

Legend

NA: no antimicrobial activity; negative control: dimethyl sulphoxide; positive control: ciprofloxacin as antibacterial agent (1mg/ml); fluconazol as antifungal agent (1mg/ml); B: Bean; L: Leaf; S: Stem; SM: *Streptococcus mutans* (MTCC 497); SP: *Streptococcus pyogenes* (MTCC 1924); BM: *Bacillus megaterium* (MTCC 428); BS: *Bacillus subtilis* (MTCC 121); EC: *Escherichia coli* (MTCC 40); PS: *Pseudomonas fluorescens* (MTCC 1748); CA: *Candida albicans* (MTCC 227); CG: *Candida glabrata* (MTCC 3814); AS: *Alternaria solani* (MTCC 10690); FG: *Fusarium graminearum* (MTCC 2089).

DISCUSSION

Extensive colonization of host plants with endophytes out-competes pathogenic microorganisms and prevents pathogenic microorganisms from taking hold. Endophytes produce a wide range of compounds which are shown to combat pathogens and even cancers in animals including humans. Molecules that act as antidiabetic, immunomodulatory, herbicidal, plant protective agents and plant growth promoting agents have been discovered as products of endophytes[12].

Since, endophytes exists in virtually all plants, i.e., in a huge number of unique biological niches (higher plants) in different unique environments, they are viewed as a reservoir of unexplored microbes. These factors may govern the novelty and biological activity of the products associated with endophytic microbes. Consequently, there exists a huge opportunity to find novel endophyte/s producing novel metabolites with potential for human use [2].

CONCLUSION

In the present study an *Aspergillus* sp. of endophytic fungi isolated from *Azadirachta indica* showed a great antibacterial activity against seven human pathogenic bacteria. The *aspergillus* spp. showed maximum zone of inhibition against gram negative test pathogen, *E. coli*. Although the zones of inhibition observed with the bacterial test pathogens were much smaller as compared to the standard antibiotic but the results were very encouraging against fungal pathogens. Therefore, there is a need of in depth study of this isolated endophyte for inceasing the activity and volume of bioactive compound. Further studies on safety and efficacy should be performed for the isolated *Aspergillus* sp to be used as therapeutic drug.

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