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EVALUATION OF ANTIMICROBIAL POTENTIAL OF *ASPERGILLUS IBERICUS* ISOLATED FROM RHIZOSPHERIC SOIL OF *FICUS RELIGIOSA*

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ABSTRACT

Inappropriate and irrational use of antimicrobial medicines has prompted the resistant microbes to emerge, spread and persist. Antimicrobial resistance may be intrinsic or acquired as it can develop through the spontaneous mutation of existing genes or through the transfer of genes from other resistant species or strains. Therefore, antimicrobial resistance has resulted research and development for the discovery of new antibiotics against pathogens at all times. Natural products with industrial applications can be produced from secondary metabolism of living organisms including plants, animals and microorganisms. The present study highlights the bioactivity of rhizosphere soil fungi Aspergillus ibericus from Ficus religiosa against opportunistic pathogens, through the production of secondary metabolite exhibiting antimicrobial activity. Antimicrobial metabolite obtained from A. ibericus was found to be effective against test microbes gram-positive bacteria, Bacillus subtilis, Staphylococcus aureus, Streptococcus mutans and Streptococcus pyogenes, gram-negative bacteria, Pseudomonas aeruginosa and Escherichia coli and yeasts such as Candida albicans and C. tropicalis. The antimicrobial metabolite was thus broad spectrum in nature. The effect of fermentation medium, temperature and pH was also observed on the production of antimicrobial metabolite.

KEY WORDS

Antimicrobial Resistance, Rhizosphere Soil, Oppurtunistic Pathogens, Aspergillus ibericus

INTRODUCTION

Antibiotics are the most important pillar of current medications [1]. The antimicrobial compounds have been found to cure various kind of bacterial and fungal infections, but the discovery of these agents has been tempered by appearance of resistant microbial pathogens. Emergence of drug resistance in them has emphasized the need of research for new compounds [2,3,4]. Antibiotics nowadays are taken for granted to treat bacterial infections. The antibiotics are successfully used for prophylactic or therapeutic purposes regularly in clinical, veterinary and agricultural purposes. However, overconsumption of antibiotics caused an enormous selective pressure on the bacteria to gain resistance or die [5]. The increasing frequency of multi-resistant pathogenic bacteria has compromised the clinical treatment of an emerging infectious diseases. There is an urgent demand for new antimicrobial compounds active against current resistant pathogens and emerging pathogens [6].

Natural drug discovery involves the search for natural sources such as soil, bacteria, mold and plants for new chemical entities (NCE). Natural products provide a vast source of chemically diverse biologically active leads for

therapeutic agents [7]. Most of the antibiotics used all over the world are derived from natural compounds.

Fungal antagonism has been reported against many pathogenic organisms by researchers [8]. Besides the antimicrobial property, fungi isolated from soil are also the sources of other industrially important compounds like enzyme inhibitors, antihelminthics, antitumour agents, insecticides, vitamins, immunosuppressant and immunomodulators [8]. *Penicillium*, *Cephalosporium* and *Aspergillus* species have gained importance as produced commercial antibiotics, penicillin G, V, cephalosporin, griseofulvin, fumagillin, variotin, fusidic acid, siccanin and xanthocillin [9]. Antibacterial, antifungal and antitumour compounds are found to be produced by many *Aspergillus* species isolated from various soils such as asterric acid, asterriquinone, aspulvinone, citrinin, emodin, butyrolactone I, geodin, terrecyclic acid, questrin, itaconate, sulochrin and lovastatin [10]. Many antimicrobial and antitumour quinones have been reported from *Aspergillus* species [11]. In keeping view of the above justifications, for the continuous search of new isolates from rhizosphere soil of medicinal plants, having antimicrobial activity, the present study highlights the bioactivity of rhizosphere soil fungus *Aspergillus ibericus* from *Ficus religiosa* against opportunistic pathogens, through the production of secondary metabolite exhibiting antimicrobial activity. The isolated PIF2 strain (*Aspergillus ibericus*) belongs to Order *Eurotiales*, Class *Eurotiomycetes* and Family *Trichocomaceae*. Serra et al. [12] for the first time isolated a new species of section Nigri, *A. ibericus* from wine grapes and dried vine fruit in Portugal and Spain and reported that strains of *A. ibericus* did not produce detectable ochratoxin A (carcinogenic) in culture media, therefore could be exploited for biotechnological applications.

MATERIALS AND METHODS

Collection of soil samples

Soil samples were collected from rhizosphere of medicinal plant Peepal (*Ficus religiosa*) from Botanical garden, Kurukshetra University, Kurukshetra. by removing 1-1.5 inch of top soil with sterilized spatula.

Isolation of *Aspergillus ibericus* from soil

The serial dilution agar plate method was used for isolation of *Aspergillus ibericus* from soil sample [13,14]. Potato dextrose agar (PDA) (CDH) for fungi was used as isolation medium. One gram of soil (finely pulverized and air dried) was suspended in 9ml sterilized distilled water blank no. 1 and shaken vigorously to obtain uniform suspension. 1ml of suspension was transferred, while in motion, from stock suspension (No. 1) to sterile water blank no. 2 with sterile pipette under aseptic conditions to make 1:100 (10^{-2}) dilution. For fungi, 10^{-2} to 10^{-5} dilutions were used. Approximately 20-25ml cooled (45°C) molten nutrient agar and potato dextrose agar was added to each petri plate. After solidification of agar media, 100µl aliquots of suspension of dilutions were added to labeled and sterilized petri plates and spread with spreader. Inoculated plates were incubated at 30°C for 4-5 days for fungi.

Purification and maintenance of isolates

Fungal colonies appearing on their respective media were transferred to potato dextrose agar plates (one colony on each plate) at 30°C for 4-5 days. The colonies were then transferred on potato dextrose agar slants and incubated at 30°C for 4-5 days for fungi and were maintained at 4°C in a refrigerator for further studies.

Morphological and Molecular identification of fungal isolate

Fungal isolate was identified as *Aspergillus ibericus* based on colony morphology, pigmentation, growth pattern and sporulating structure by lactophenol cotton blue staining by following various manuals and monographs [15,16,17]. Molecular identification was performed by National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune

Screening for antimicrobial activity of soil microbes by agar well diffusion method

The antimicrobial activity of pure fungal isolate *Aspergillus ibericus* was evaluated by using agar well diffusion assay [18] against gram-positive bacteria, *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 87), *Streptococcus mutans* (MTCC 497), *Streptococcus pyogenes* (MTCC 1924); gram-negative bacteria, *Pseudomonas aeruginosa* (MTCC 424), *Escherichia coli* (MTCC 40) and yeasts namely *Candida albicans* (MTCC 227) and *Candida tropicalis* (MTCC 3421) which were procured from CSIR- Institute of Microbial Technology, Chandigarh, India. All test microbes were grown in their specific media, nutrient agar, malt extract agar and brain heart infusion agar. Nutrient agar plates were inoculated with 100µl of standardized inoculum (0.5 McFarland Standard) of each test microbe (in triplicates) and was spread with sterile swabs. Wells were made into agar plates

containing the test microbe inoculum. 200µl volume of extract was poured into a well of inoculated plates. Uninoculated potato dextrose broth (Hi-Media) was used as negative control. Antibiotics ciprofloxacin (antibacterial) and fluconazole (antifungal) were used as positive control. Then plates were left at room temperature for ten minutes allowing the diffusion of extract into agar. After incubation for 24 hours at 37°C, the plates were observed for inhibition zone surrounding the well containing extract. The zone of growth inhibition was measured and expressed in millimeters (mm). The mean and standard deviation of diameter of inhibition zones were calculated.

Effect of fermentation medium on antimicrobial metabolite production

3 disks of fungal isolate were transferred to four fermentation broths like czapek dox broth, potato dextrose broth, sabouraud dextrose broth and tryptone soya broth (Hi-media) and incubated at 30°C for 12-14 days. Wells were made into nutrient agar plates swabbed with test microbes adjusted at.0.5 McFarland Standard. Fungal broths were filtered and 200µl of filtrates was added into well with help of micropipette. Plates were incubated at 37°C for 24 hours and observed for zone of growth inhibition [10].

Effect of incubation temperature on antimicrobial metabolite production

The optimization of incubation temperature for antimicrobial metabolite production was carried out at different temperatures viz. 25°C, 28°C, 30°C, 35°C and 40°C. 100ml of potato dextrose broth was taken in 250ml Erlenmeyer's flasks. These flasks were autoclaved at 121°C for 15 minutes. For each value, three replicates were used. Three disks cut from four days old colony of selected fungus were added as inoculum in each flask. The inoculated flasks were incubated at 25°C, 28°C, 30°C, 35°C and 40°C for 12-14 days under stationary condition. The extracts after filtration with filter paper were evaluated for antimicrobial activity against test microbes by using agar well diffusion method [10].

Effect of pH on antimicrobial metabolite production

The optimization of pH of the fermentation broth for antimicrobial metabolite production was done by carrying out fermentation at different pH values viz. 4, 5, 6, 7, 8, 9, 10 and 11. For each pH value, 100ml of potato dextrose broth (adjusted to desired pH by using 1N NaOH or 0.1 N HCl) was taken in 250ml Erlenmeyer's flasks. These flasks were autoclaved at 121°C for 15 minutes. For each value, three replicates were used. Three disks cut from four days old colony of selected fungus were added as inoculum in each flask. The inoculated flasks were incubated at 30°C for 12-14 days under stationary condition. The extracts after filtration with filter paper were assayed for antimicrobial activity against test microbes by using agar well diffusion method [10].

Statistical analysis

The data obtained from experiments of optimization of media, temperature, pH, carbon source, nitrogen source and their concentration were subjected to analysis of variance (One Way ANOVA) to evaluate the significance of each parameter by estimating p-value and f-value. The level of significance was considered as $p<0.05$ [19].

Determination of minimum inhibitory concentration (MIC) of antimicrobial metabolite

The minimum inhibitory concentration (MIC) is defined as minimum concentration of the antimicrobial compound that is required to inhibit the visible growth of a microorganism after overnight incubation [20]. MIC of crude antimicrobial compound against all the test microbes was determined by two-fold dilution method. In this method, two-fold serial dilution of antimicrobial metabolite was prepared by first reconstituting the metabolite (10mg/ml) in 10% dimethyl sulphoxide (DMSO). The dilutions were made in 10% DMSO to achieve a decreasing concentration range. A 200µl volume of each dilution was introduced into wells (triplicate) in nutrient agar plates already seeded with 100µl of inoculum of the test microbes. All plates were incubated at 37°C for 24 hours and were observed for the inhibition zones to know the minimum concentration of metabolite which is sufficient to inhibit growth of test microbes [14].

RESULTS AND DISCUSSION

In the present study, the fungus *Aspergillus ibericus* was isolated from rhizosphere soil of medicinal plant Ficus religiosa and was studied for its antimicrobial potential against test microbes *B. subtilis, S. aureus, S. mutans, S. pyogenes, P. aeruginosa, E. coli, C. albicans* and *C. tropicalis* by agar well diffusion method, which could cause opportunistic infections in host. Isolated strain of *A. ibericus* (PIF2) seems to be broad spectrum in its mode of action as it inhibited the growth of all test

microbes including gram-positive, gram-negative and yeasts (Table 1) (Fig. 1).

Table 1. Antimicrobial activity of rhizosphere soil fungal isolate PIF2

Fungal isolate	Zone of growth inhibition (mm)							
	Test microorganisms							
	Bacteria						Yeast	
	Gram-positive			Gram-negative				
	Bs	Sa	Sm	Sp	Pa	Ec	Ca	Ct
PIF2	**24.66±0.57**	**25.00±0.00**	**22.66±0.57**	**25.00±0.00**	**21.00±0.00**	**22.33±0.57**	**26.66±0.57**	**15.00±1.00**
Ciprofloxacin	24.00±0.00	NA	25.00±0.00	26.00±0.00	25.00±0.00	23.00±0.00	ND	ND
Fluconazole	ND	ND	ND	ND	ND	ND	NA	NA

Values are mean inhibition zone ± Standard deviation of three replicates

NA: No antimicrobial activity; ND: Not determined; Bs: Bacillus subtilis; Sa: Staphylococcus aureus; Pa: Pseudomonas aeruginosa; Ec: Escherichia coli; Sm: Streptococcus mutans; Sp: Streptococcus pyogenes; Ca: Candida albicans; Ct: Candida tropicalis

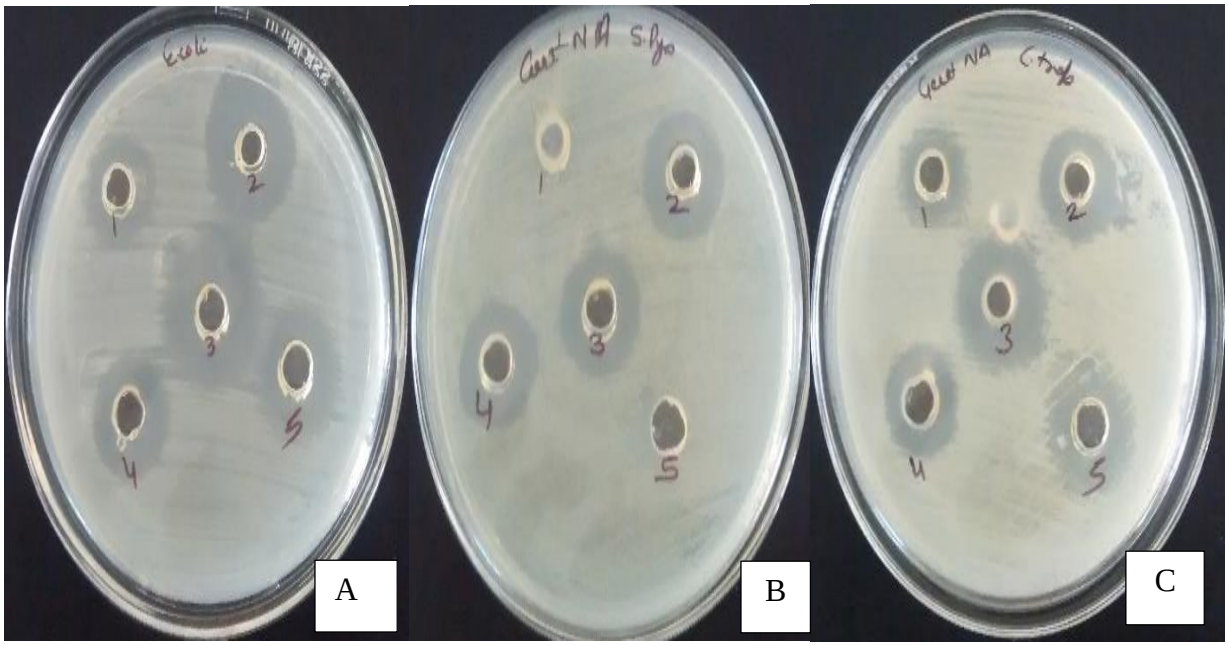

Fig. 1. Antimicrobial activity of Aspergillus ibericus against test microorganisms.

A. ibericus has been reported to bioremediate olive mill wastes by using it as culture media to produce enzymes like lipases [21], cellulase and xylanases [22]. Fungus *A. ibericus* has also been reported to produce aflavinine [23] and naptho-γ-pyrone compounds [24]. The morphological characteristics of *Aspergillus* sp. were identified on the basis of colony colour (black), reverse side (yellow), texture (granular), sporulating structure (biseriate), spores (rough with maturity), conidia diameter (5-5.5µm), conidia head diameter (55-70µm), colonies begin with white hyphae and quickly form jet black conidia (Figure 2). Fungal isolate PIF2 was identified as *Aspergillus* ibericus on the basis of above characteristics and by National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India as it showed 99% sequence similarity to *Aspergillus* ibericus strain A-1618 in BLAST (Basic Local Alignment Search Tool) analysis. Figure 3 represents sequence of ITS- rDNA of PIF2 fungal isolate. Top five results of BLAST analysis are shown in Table 2. Alignment statistics of PIF2 ITS-rDNA sequence (query sequence) with *Aspergillus* ibericus strain A-1618 in BLAST analysis is shown in Figure 4. Therefore, isolated PIF2 strain (*Aspergillus ibericus*) belongs to Order *Eurotiales*, Class *Eurotiomycetes* and Family *Trichocomaceae*. The choice of a good fermentation

medium is as important as the selection of an organism for the success of an industrial fermentation [25]. Antibiotic biosynthesis is a specific property of microorganisms which depends greatly on culture conditions. Therefore, media composition plays a crucial role in the efficiency and economics of the ultimate process [26]. In the present study, fungal discs of *Aspergillus ibericus* were inoculated in four fermentation media such as Czapek Dox Broth (CDB), Potato Dextrose Broth (PDB), Sabouraud Dextrose Broth (SDB) and Tryptone Soya Broth (TSB) to select the best media for the growth of fungus *A. ibericus* and antibiotic production. Maximum antimicrobial activity was observed in PDB as it showed zone of growth inhibition of 25mm against *B. subtilis*, 21mm against *S. aureus*, 25mm against *S. mutans*, 21mm against *S. pyogenes*, 20mm against P. aeruginosa, 19mm against *E. coli*, 21mm against *C. albicans* and 19mm against *C. tropicalis*. Antimicrobial activity was decreased in CDB (12mm to 14mm), SDB (0mm to 21mm) and TSB (0mm to 19mm). Therefore, PDB was found the best medium for antimicrobial metabolite production. One-way ANOVA analysis at 5% significance level shows calculated F value (7.27) greater than F critical value (2.94) and P value (0.000937) less than 0.05, which indicates that null hypothesis (there is no significant difference between the values) is rejected and there is significant difference between values. Table 3 and Figure 5 shows antimicrobial activity of *A. ibericus* in different fermentation media.

(A) (B)

Fig.2. Morphological characterization of fungal isolate PIF2 (*Aspergillus ibericus*); A) Fungal colony on plate and B) Microscopic characteristics.

> PIF2 ITS-rDNA

GTTGGAGTTGTCGGCAGGCGCCGGCCAATCCTACAGAGCATGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGCC
GCCGCTGCCTTTCGGGCCCGTCCCCCCGGAAAGGGGGACGGCGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACG
CTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCA
CATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCATTGTTGAAAGTTTTAACTGATTGAAAACA
ATCGACTCAGACTTCACGATTTCAGACAGTGTTCGTGTTGGTGTCTCCGGCGGGCGCGGGCCCGAGGGGCAGAGATGCCCCC
CCGGCGGCCGACAAGCGGCGGGCCCGCCGAAGCAACAGGGTACAATAGACACGGGTGGGAGGTTGGGCCCAAAGGACCC
GCACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGATTTTTTACTT

Fig. 3. Sequence of ITS-rDNA of PIF2 (*Aspergillus ibericus*) isolate (NFCCI, Pune)

The results in present study are in accordance with various previous research which also found the PDB as best medium for production of fungal metabolites; for the production of cladospolide D antifungal antibiotic from *Cladosporium* sp. [27] and diketopiperazine novel antifungal antibiotic from marine fungus [28]. Vijaykumar et al. [29] described that maximum activity of isolate may be due to the production of bioactive compound in high concentration in that specific growth media.

Physical factors such as incubation temperature influences directly the growth and development of any organism and production phases of secondary metabolism. It affects the physiology and subsequently the synthesis of various metabolites [30]. Low temperature may cease the metabolic activity and high temperature kills the cells of the fungus [31]. Fungal discs of *Aspergillus ibericus* were inoculated in potato dextrose broth and incubated at different temperatures of 25°C, 28°C, 30°C, 35°C and 40°C to select the best incubation temperature for growth of fungus *A. ibericus* and antibiotic production. Fungal discs of *Aspergillus ibericus* were inoculated in potato dextrose broth and incubated at different temperatures of 25°C, 28°C, 30°C, 35°C and 40°C to select the best incubation temperature for growth of fungus *A. ibericus* and antibiotic production. Maximum antimicrobial activity of strain *A. ibericus* was recorded in potato dextrose broth which was incubated at 30°C as when broth was filtered and assayed against test microbes (21mm to 24mm). The diameter of zone of growth inhibition was 24mm against *B. subtilis*, 23mm against *S. aureus*, 21mm against *S. mutans*, 22mm against *S. pyogenes*, 22mm against *P. aeruginosa*, 24mm against *E. coli,* 22mm against *C. albicans* and 22mm against *C. tropicalis*. The antimicrobial activity was decreased at 25°C (17mm to 20mm), at 28°C (19mm to 23mm), at 35°C (18mm to 22mm) and at 40°C (0mm to 20mm). Antimicrobial metabolite production was increased from 25°C to 30°C then decreased from 30°C to 40°C. Therefore, 30°C was the optimized temperature for antimicrobial metabolite production. One-way ANOVA analysis at 5% significance level shows calculated F value (4.17) greater than F critical value (2.64) and P value (0.007201) less than 0.05, which indicates that null hypothesis (there is no significant difference between the values) is rejected and there is significant difference between values. Antimicrobial activity of fungus *A. ibericus* at different incubation temperatures is shown in Table 4 and Figure 6. The result of present study was in accordance with study of Bhattacharyya and Jha [8], where the *Aspergillus* strain TSF 146 showed a narrow range of incubation temperatures for effective growth and antibiotic production. The increase of the incubation temperatures from 25 to 30°C enhanced the growth of the cells and production of bioactive metabolite.

Table 2. Blast analysis of sequence of PIF2 (*Aspergillus ibericus*) isolate (NFCCI, Pune)

Gene Bank Accession No.	Description	Max score	Query cover	Query coverage	E value	Identity (%)
AY656627.1	*Aspergillus ibericus* strain A-1618	980	980	100%	0.0	99%
AY656622.1	*Aspergillus ibericus* strain A-1082	980	980	100%	0.0	99%
NR_119514.1	*Aspergillus ibericus* IMI 391429	975	975	100%	0.0	99%
EU440775.1	*Aspergillus carbonarius* strain MPVCT 340	966	966	100%	0.0	99%
NR_111094.1	*Aspergillus carbonarius* CBS 111.26	966	966	99%	0.0	99%

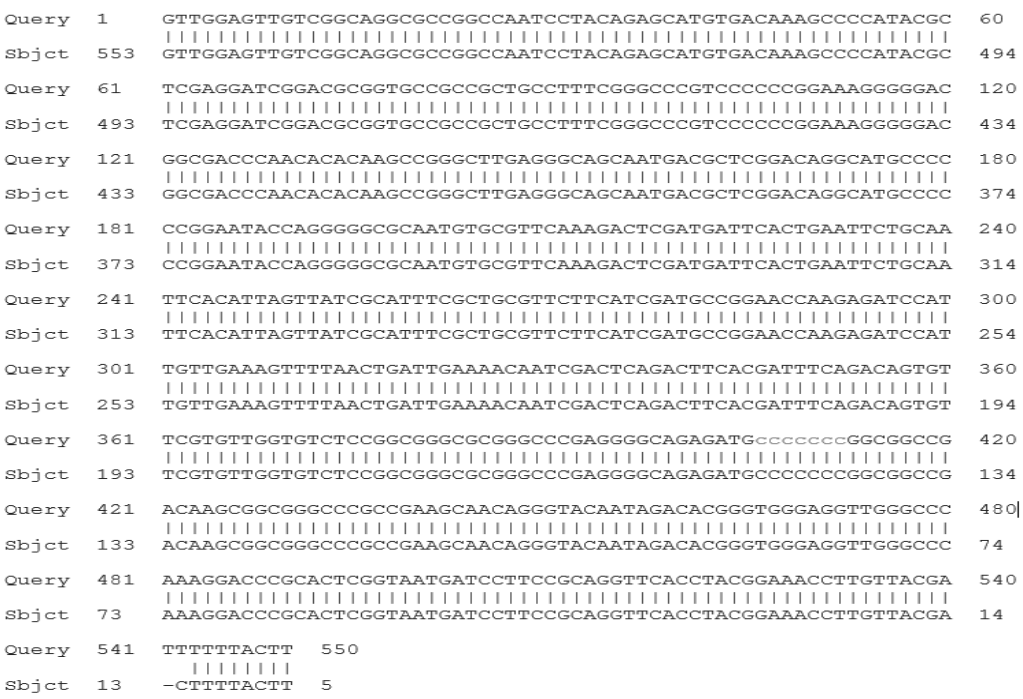

Fig. 4. Blast analysis of sequence of PIF2 (*Aspergillus ibericus*) isolate (NFCCI, Pune)

Table 3. Optimization of fermenation medium and antimicrobial activity

Media	Zone of growth inhibition (mm)							
	Test microorganisms							
	Bacteria						Yeast	
	Gram-positive				Gram-negative			
	Bs	Sa	Sm	Sp	Pa	Ec	Ca	Ct
Czapek Dox Broth	12.33±0.57	14.00±0.00	14.66±0.57	12.00±0.00	13.66±0.57	12.33±0.57	14.33±0.57	14.00±0.00
Potato Dextrose Broth	**25.66±0.57**	**21.33±0.57**	**25.33±0.57**	**22.00±1.00**	**20.66±0.57**	**19.33±0.57**	**22.00±1.00**	**20.00±1.00**
Sabouraud Dextrose Broth	20.00±0.00	17.66±0.57	21.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Tryptone Soya Broth	0.00±0.00	12.66±0.57	19.33±0.57	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Values are mean inhibition zone ± Standard deviation of three replicates

Bs: *Bacillus subtilis*; Sa*: Staphylococcus aureus*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichia coli*; Ca: *Candida albicans*; Ct: *Candida tropicalis*

The pH of the culture medium is one of the most important environmental factors, because it exerts a significant effect on the activity of several enzymes that catalyze metabolic reactions, on the intermediate products, their dissociation and solubility as well as on complex physiological phenomena such as membrane permeability (effect on either ion uptake or loss to the nutrient medium) and cell morphology (conidia formation). The pH level of the growth medium affects many cellular processes such as regulation and biosynthesis of secondary metabolites with synthesis falling rapidly either side of an optimal level [9,25,30].

The fermentation medium, potato dextrose broth was adjusted at different pH values of 4, 5, 6, 7, 8, 9, 10 and 11 to select the best pH for growth of *A. ibericus* and antibiotic production. The antimicrobial activity of

culture filtrate of strain *A. ibericus* against test microbes was found to be maximum at pH 7 as diameter of zone of growth inhibition was ranging from 18mm to 30mm. The diameter of zones of growth inhibition were 18mm against *B. subtilis*, 24mm against *S. aureus*, 24mm against *S. mutans*, 30mm against *S. pyogenes*, 21mm against *P. aeruginosa*, 25mm against *E. coli*, 28mm against *C. albicans* and 21mm against *C. tropicalis*. The diameter of zone of growth inhibition was found to reduce at pH 4 (0mm to 20mm), at pH 5 (16mm to 24mm), at pH 6 (16mm to 27mm), remained constant at pH7 and pH 8 (18mm to 30mm) and decreased at pH 9 (18mm to 26mm) and showed no activity at pH 10 and pH 11. The antimicrobial activity was increased from pH 4 to pH 7, then remained constant at pH 8 and decreased at pH 9 and finally became zero at pH 10 and 11. Therefore, neutral pH 7 was found to be the optimized pH of media for antimicrobial metabolite production. One-way ANOVA analysis at 5% significance level shows calculated F value (40.45) greater than F critical value (2.29) and P value (2.48E-17) less than 0.05, which indicates that null hypothesis (there is no significant difference between the values) is rejected and there is significant difference between values. Table 5 and Figure 7 shows antimicrobial activity of fungus *A. ibericus* at different pH. Compaore et al. [32] Optimized the pH of medium for the strain *Aspergillus fumigatus* for antimicrobial metabolite production and found that isolate preferred pH 7 (neutral) rather than acidic or alkaline.

MIC is defined as the lowest concentration of an antimicrobial agent which, under defined in vitro conditions, prevents the appearance of visible growth of a microorganism within a defined period of time. MIC value can be influenced by many factors such as period of incubation, type of culture medium, dissolving solvent, nature of test microorganisms and the size of inoculums. Therefore, standardization is important to carry out the test. In the present study, MIC of antimicrobial metabolite obtained from *A. ibericus* was found significant as 19.5µg/ml against *P. aeruginosa, E. coli, S. pyogenes, C. albicans, C. tropicalis*, moderate as 625µg /ml against *S. aureus, S. mutans* and weak as 1.25mg/ml against *B. subtilis* and also compared to selected antibiotics fluconazole (showed no activity against any test microbe at any concentration) and streptomycin (120µg for *P. aeruginosa*, 5µg for *S. mutans, S. pyogenes, E. coli*, 0.1µg for *S. aureus* and *B. subtilis* (Table 6).

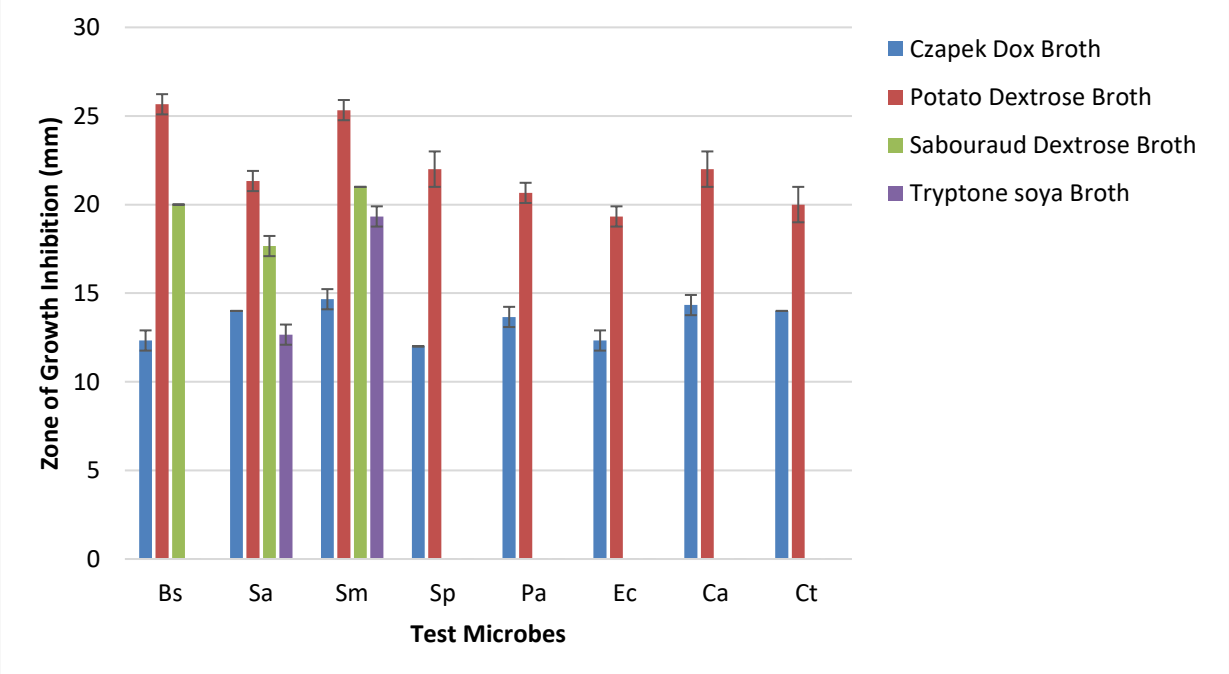

Fig. 5 Optimization of fermentation medium and antimicrobial activity

Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichia coli*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Ca: *Candida albicans*; Ct: *Candida tropicalis*. When statistically analyzed at significance level 0.05 by One Way ANOVA, proved to be significantly different.

Table 4 Optimization of incubation temperature and antimicrobial activity

Temperature (°C)	Zone of growth inhibition (mm) Test microorganisms							
	Bacteria						Yeast	
	Gram-positive				Gram-negative			
	Bs	Sa	Sm	Sp	Pa	Ec	Ca	Ct
25°C	19.66±0.57	19.66±0.57	18.33±0.57	0.00±0.00	19.00±0.00	19.33±0.57	0.00±0.00	16.66±0.57
28°C	22.33±0.57	20.33±0.57	18.66±0.57	19.66±0.57	19.66±0.57	22.66±0.57	20.00±0.00	19.33±0.57
30°C	**24.33±0.57**	**23.33±0.57**	**21.33±0.57**	**22.66±0.57**	**22.00±0.00**	**24.66±0.57**	**22.66±0.57**	**22.33±0.57**
35°C	21.66±0.57	19.66±0.57	19.33±0.57	19.00±1.00	19.33±0.57	19.66±0.57	19.00±0.00	18.00±0.00
40°C	18.00±1.00	18.00±1.00	18.66±0.57	0.00±0.00	17.66±0.57	18.66±0.57	0.00±0.00	19.66±0.57

Values are mean inhibition zone ± Standard deviation of three replicates

Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichia coli*; Ca: *Candida albicans*; Ct: *Candida tropicalis*

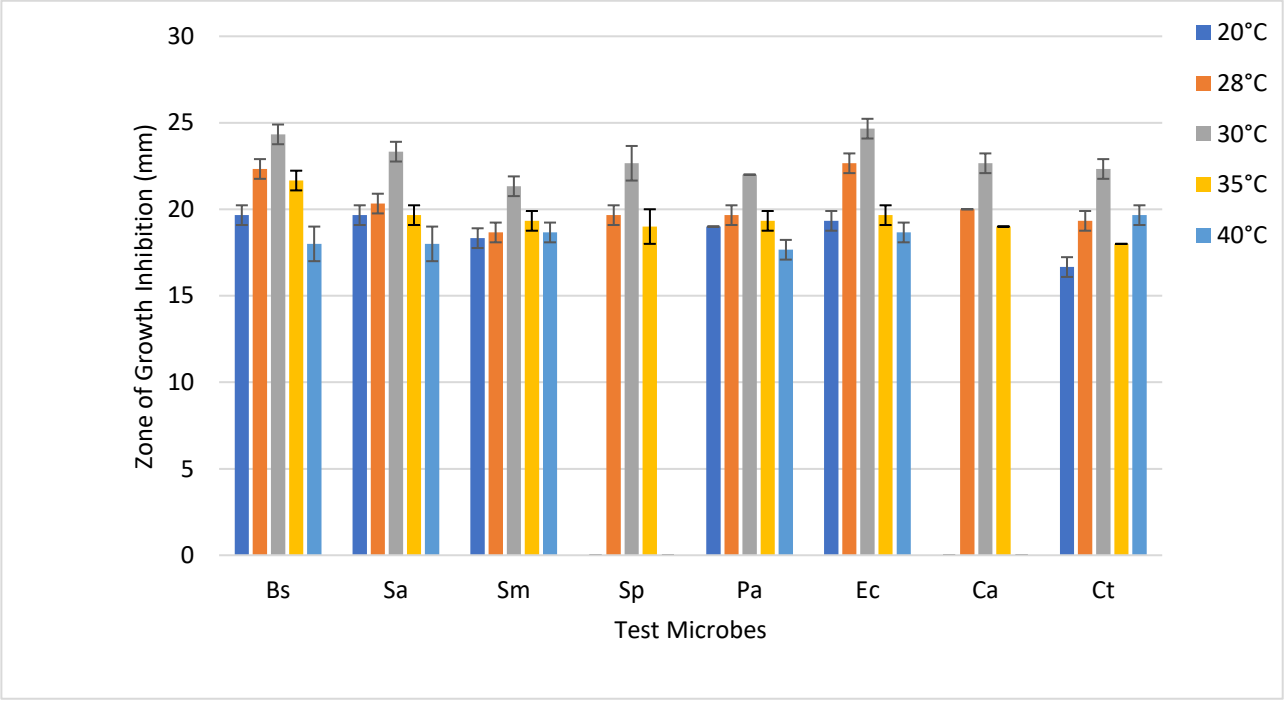

Fig. 6. Optimization of incubation temperature and antimicrobial activity

Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichia coli*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Ca: *Candida albicans*; Ct: *Candida tropicalis*. When statistically analyzed at significance level 0.05 by One Way ANOVA, proved to be significantly different.

Table 5. Optimization of pH and antimicrobial activity

pH	Zone of growth inhibition (mm) Test microorganisms							
	Bacteria						Yeast	
	Gram-positive				Gram-negative			
	Bs	Sa	Sm	Sp	Pa	Ec	Ca	Ct
4	0.00±0.00	17.66±0.57	15.66±0.57	17.66±0.57	16.66±0.57	20.00±0.00	17.66±0.57	17.00±0.00
5	15.66±0.57	21.66±0.57	19.00±0.00	22.33±0.57	23.66±0.57	23.66±0.57	22.00±0.00	19.66±0.57
6	16.00±0.00	23.66±0.57	20.66±0.57	24.66±0.57	25.66±0.57	26.66±0.57	25.66±0.57	20.66±0.57
7	**18.00±0.00**	**24.33±0.57**	**23.66±0.57**	**30.00±0.00**	**21.00±0.00**	**25.00±0.00**	**28.00±0.00**	**21.33±0.57**
8	17.33±0.57	23.00±1.00	25.00±0.00	29.66±0.57	20.66±0.57	24.33±0.57	27.66±0.57	19.66±0.57
9	17.33±0.57	22.66±0.57	19.66±0.57	23.66±0.57	20.33±0.57	19.66±0.57	26.00±0.00	18.00±0.00
10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
11	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Values are mean inhibition zone ± Standard deviation of three replicates

Bs: *Bacillus subtilis*; Sa*: Staphylococcus aureus*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichia coli*; Ca: *Candida albicans*; Ct: *Candida tropicali*

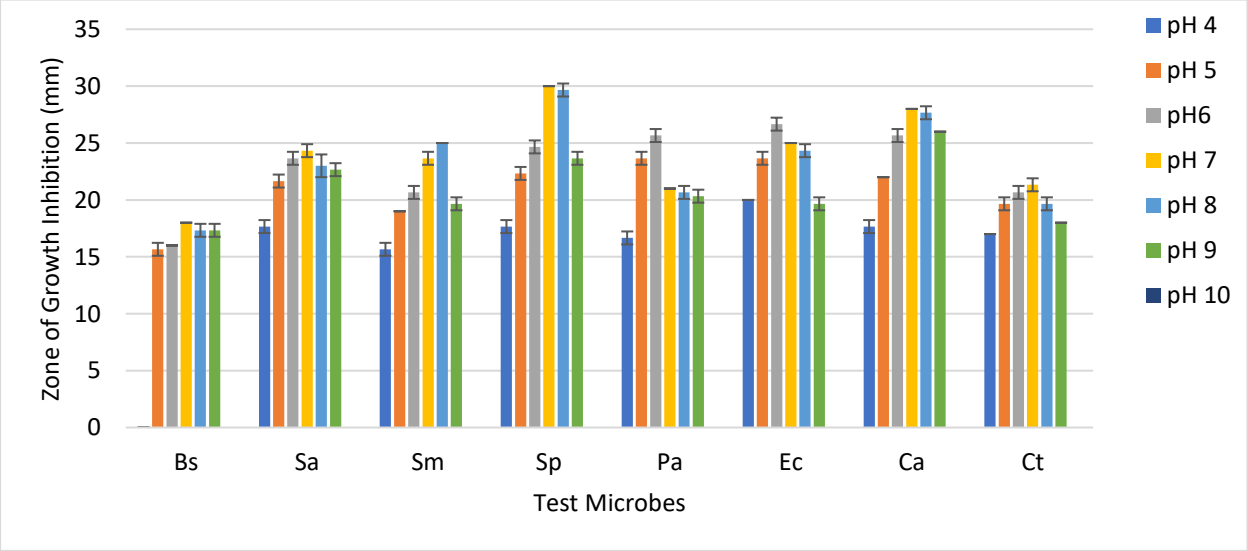

Fig. 7. Optimization of pH and antimicrobial activity

Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Pa: *Pseudomona aeruginosa*; Ec: *Escherichia coli*; Sm: *Streptococcus mutans*; Sp: *Streptococcus pyogenes*; Ca: *Candida albicans*; Ct: *Candida tropicalis*. When statistically analyzed at significance level 0.05 by One Way ANOVA, proved to be significantly different.

Table 6 Determination of minimum inhibitory concentration (MIC) of antimicrobial compound

Conc.	Zone of growth inhibition (mm) Test microorganisms							
	Bacteria						Yeast	
	Gram-positive			Gram-negative				
	Bs	Sa	Sm	Sp	Pa	Ec	Ca	Ct
10 mg/ml	21.33±0.57	25.33±0.57	24.00±0.00	28.33±0.57	30.00±0.00	25.66±0.57	30.66±0.57	20.33±0.57
5 mg/ml	20.66±0.57	21.00±0.00	20.66±0.57	25.66±0.57	22.66±0.57	22.33±0.57	28.66±0.57	19.66±0.57
2.5 mg/ml	15.00±0.00	18.00±0.00	17.00±0.00	22.00±0.00	21.33±0.57	19.00±0.00	24.00±0.00	17.0±0.00
1.25 mg/ml	12.66±0.57	14.33±0.57	15.00±0.00	19.33±0.57	19.00±0.00	17.00±0.00	22.33±0.57	16.00±0.00
0.625 mg/ml	-	12.66±0.57	13.33±0.57	17.33±0.57	17.00±0.00	16.00±0.00	19.00±0.00	15.00±0.00
0.312 mg/ml	-	-	-	16.00±0.00	17.66±0.57	16.66±0.57	18.33±0.57	14.66±0.57
0.156 mg/ml	-	-	-	14.00±0.00	16.33±0.57	15.33±0.57	18.66±0.57	13.33±0.57
0.078 mg/ml	-	-	-	13.33±0.57	15.00±0.00	14.66±0.57	15.00±0.00	13.00±0.00
0.039 mg/ml	-	-	-	13.33±0.57	15.33±0.57	13.66±0.57	15.00±0.00	12.66±0.57
0.0195 mg/ml	-	-	-	12.66±0.57	12.66±0.57	12.00±0.00	14.66±0.57	12.00±0.00
10% DMSO	-	-	-	-	-	-	-	-

Values are mean ± S.D. of triplicates

Bs: ***Bacillus subtilis*****; Sa:** ***Staphylococcus aureus*****; Sm:** ***Streptococcus mutans*****; Sp:** ***Streptococcus pyogenes*****; Pa:** ***Pseudomonas aeruginosa*****; Ec:** ***Escherichia coli*****; Ca:** ***Candida albicans*****; Ct:** ***Candida tropicalis***

CONCLUSION

It may be concluded that fungus *Aspergillus ibericus* isolated from rhizosphere soil of medicinal plant *Ficus religiosa* is a promising source of antimicrobial metabolite. The research work shows rhizospheric soil of medicinal plants is a rich source of clinically important microorganisms. It may also be suggested that further research is needed to determine the cytotoxicity and in vivo efficacy against opportunistic pathogens before it is used for commercialization purpose. Improvement of strain by gene manipulation is recommended to produce new strains with increased antibiotic production capacities.

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