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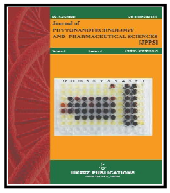

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Evaluation of antibacterial activity of the endophytic fungi isolated from *Bixa orellana* L.

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Abstract

The current study was carried out for evaluation of antibacterial activity of the endophytic fungi isolated from *Bixa orellana* L. plant leaves. *Schizophyllum commune* and *Fusarium incarnatum* were isolated and identified through molecular analysis of the internal transcribed region (ITS) of the ribosomal DNA from *B. orellana*. For large scale crude extraction of different endophytes grown on PDA was carried out and ethyl acetate was used for extraction. Antibacterial activity of ethyl acetate extract of endophyte *Schizophyllum commune* revealed MIC values against *Staphylococcus aureus* (0.16 µg/ml), *Pseudomonas aerugonosa* (2.56 µg/ml), *Streptococcus pyogenus* (5.12 µg/ml), *Proteus mirabilis* (0.08 µg/ml) and *Bacillus subtillis* (0.08 µg/ml), respectively and no effect was found against *Escherichia coli* and *Salmonella typhimurium*.

1. Introduction

Antimicrobial drugs are main pillars to treat any infectious diseases. Due to injudicious and discriminate use of antibacterial drugs, pathogens develop resistance and discovery of newer compound against resistance organism is again big challenge to world. In this scenario, it is worth to explore natural material with new scientific knowledge that can have a broad spectrum action and do not induce resistance in the microbial pathogens (Varia *et al*., 2021). In this situation, endophytic fungi from leaves, fruit or stem had shown great potential sources for new bioactive compounds. Endophytic fungi are microfungi that colonize or live within the healthy plant tissues without producing any apparent symptoms or obvious negative effects to their hosts. They may benefit the host plant by producing bioactive substances to enhance plant growth and competitiveness of the host in nature. In addition, they produce novel antimicrobial secondary metabolites, which are now recognized as novel bioactive compounds (Kaul *et al*., 2012; Kusari *et al*., 2013). Therefore, the present study was undertaken to explore the endophytic mycoflora, isolated from *B. orellana* (annatto), as plants material had shown great potential for obtaining useful and biologically active compounds (Vilar *et al*., 2014) and annatto extracts from leaves, roots and seeds have traditionally been used for treatment of skin, respiratory, liver and urinary ailments. Moreover, there is still limited information available about the endophyte species of *B. orellana* spp.

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2. Materials and Methods

2.1 Isolation of endophytic fungi

Healthy leaves were collected from *B. orellana* from the Navsari district of South Gujarat and processed within less than 48 h prior to the isolation of endophytic fungi. Samples were cleaned under running tap water for 5 min and then air dried. Briefly, leaves were sterilized by immersion in 70% ethanol for 1 min, sodium hypochlorite solution (5% available chlorine) for 5 min and sterile distilled water for 2 min two times. The surface-sterilized leaves and stems were cut into small pieces about 1 x 1 cm^2 using a sterile blade and placed in petri-plates on potato dextrose agar (PDA) medium supplemented with chloramphenicol (50 µg/ml) to inhibit bacterial growth and cultured at 25°C in the dark for 2-3 weeks (Qadri *et al*., 2013).

2.2 Identification of endophytic fungi

Active endophytic fungi were identified by molecular analysis of the internal transcribed region (ITS) of the ribosomal DNA. Isolation of genomic DNA, PCR amplification and sequencing was performed at Scigenom genomic services (Cochin, Kerala). DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) following manufacturer's instruction. Primer pairs ITS1 and ITS4 universal primer were used for the PCR reactions (ABI 3730XL). The purified PCR-DNA products were sequenced by gene sequencer (Ilumina) using the same primers. The sequences were then compared with the Gen Bank database by the BLAST program. Phylogenetic relationships were estimated (Qadri *et al*., 2013).

2.3 Total extraction of culture media and mycella

For large scale crude extraction of different endophytes grown on PDA plates at 25°C for 3-6 days were cut (1 x 1 cm). About 6 to 8 pieces were inoculated into 3 of 250 ml Erlenmeyer flasks containing sterile potato dextrose agar (PDA) medium broth. These flasks

were incubated at 30°C for 21 days.The biomass of endophyte with culture media was extracted for 24 h with ethyl acetate (500 ml) and mycelium was separated by filtering through gauze cloth. The culture filtrate liquid was transferred to separating funnel. Organic and water phases were separated and aqueous layer was extracted 2-3 times with ethyl acetate (200 ml). The ethyl acetate extract was evaporated to dryness using rotary evaporator and weighed to constitute the crude extract (Qadri *et al*., 2013).

2.4 Antibacterial activity

Minimum inhibitory concentrations (MICs) of ethyl acetate crude extract were determined for different organisms like *Staphylococcus aureus* (ATCC25923), *Escherichia coli* (ATCC43888), *Salmonella typhimurium* (ATCC25241), *Pseudomonas aerugonosa* (ATCC 27853), *Streptococcus pyogenus* (ATCC12384), *Proteus mirabilis* (ATCC 25933) and *Bacillus subtillis* (ATCC 19659) by microbroth dilution technique. A test compound was prepared with the known weight of the crude extract, dissolved in dimethyl sulfoxide (DMSO) at an initial concentration of 1.31 mg/ml. The serial two fold dilutions of the crude extracts were made in a concentration which ranged from 0.08 µg/ml to 5.12 µg/ml. All bacterial cultures were prepared to McFarland 0.5 standard (1.5×10^8 cfu/ml) after overnight incubation. Final dispensing inoculum was prepared in a sterile 50 ml Erlenmeyer flask by taking 200 µl Mcfarl and 0.5 standard of respective organism in to 19.8 ml of sterile broth. Test extract (50 µl) was added in all wells except sterility control, vehicle control and growth control wells. Microbial suspension (50 µl) containing bacterial concentration of 1.0×10^6 cfu/ml was added into each well of the 96 well microplate except sterility control wells. Gentamycin (25 µg/ml) and 1% DMSO were used as the positive control and vehicle control, respectively. Whole antibacterial assay was performed in triplicate and was incubated for 16-18 h at 37°C. Next day, INT dye (Iodonitrotetrazolium chloride, Sigma) (1 mg/ml) was prepared in sterile water. After completion of incubation period, 30 µl of INT dye was dispensed in all wells of microtiter plate. Thereafter, plates were incubated for 20-30 min for development of purple colour, which indicated presence of live bacteria. Minimum inhibitory concentration for respective bacterial species was read as absence of colour development (Wiegand *et al*., 2008; Varia *et al*., 2020).

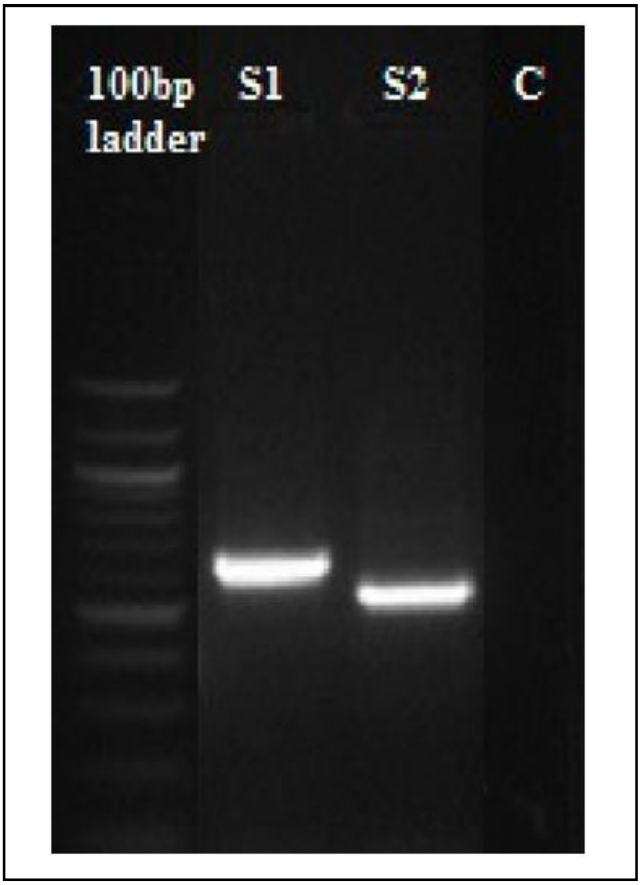

Figure 1: Amplicon photograph of *Schizophyllum commune* and *Fusarium incarnatum*.

>sample.1

TGCGGAAGGATCATTAACGAATCAAACAAGTTCATCTTGTTCTGATCCTGTGCACCTTATGTAGTCCCAAAGC
CTTCACGGGCGGCGGTTGACTACGTTTACCTCACACCTTAAAGTATGTTAACGAATGTAATCATGGTCTTGAC
AGACCCTAAAAAGTTAATACAACTTTCGACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAA
ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTA
TTCCGAGGGGCATGCCTGTTTGAGTGTCATTAAATACCATCAACCCTCTTTTGACTTCGGTCTCGAGAGTGGCT
TGGAAGTGGAGGTCTGCTGGAGCCTAACGGAGCCAGCTCCTCTTAAATGTATTAGCGGATTTCCCTTGCGGGA
TCGCGTCTCCGATGTGATAATTTCTACGTCGTTGACCATCTCGGGGCTGACCTAGTCAGTTTCAATAGGAGTCT
GCTTCCAACCGTCTCTTGACCGAGACTAGCGACTTGTGCGCTAACTTTTGACTTGACCTCAAATCAGGTAGGA
CTACCCGCTGAACTTAAGCATATCAATA

>sample.2

TGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCTATACGTTGCCTCGGCGGATC
AGCCCGCGCCCCGTAAAACGGGACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTA
AAACAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTAT
TCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCAGCTTGGTGTTGGGACTCGCGGTAACCC
GCGTTCCCCAAATCGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAATCATACACCTCGTTACTGGTAAT
CGTCGCGGCCACGCCGTAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTT
AAGCATATCAATA

Figure 2: Sequence of *Schizophyllum commune* (Sample 1) and *Fusarium incarnatum* (Sample 2).

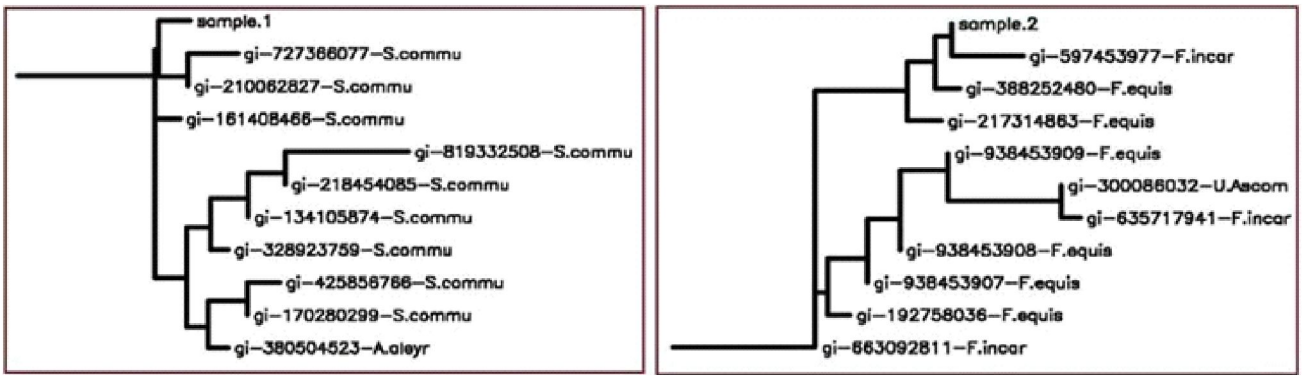

Figure 3: Phylogenetic relationship of (a) *Schizophyllum commune* and (b) *Fusarium incarnatum*.

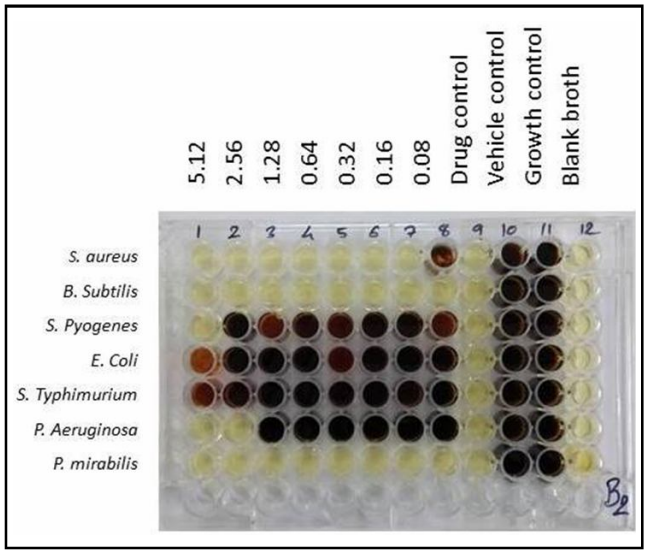

Figure 4: Antibacterial activity of ethyl acetate extract of endophyte *Schizophyllum commune*.

Table 1: Antibacterial activity of ethyl acetate extract of endophyte *Schizophyllum commune*

Sr. No.	Bacteria	MIC value (µg/ml)
1	*Staphylococcus aureus*	0.16
2	*Escherichia coli*	NE
3	*Salmonella typhimurium*	NE
4	*Pseudomonas aerugonosa*	2.56
5	*Streptococcus pyogenus*	5.12
6	*Proteus mirabilis*	0.08
7	*Bacillus subtillis*	0.08

NE: Not effective

3. Results

In this study, endophytic fungi, *i.e.*, *Schizophyllum commune* and *Fusarium incarnatum* were isolated and identified through molecular analysis of the internal transcribed region (ITS) of the ribosomal DNA from *B. orellana*. Amplicon photograph (Figure 1), sequence obtained (Figure 2) and phylogenetic relationship between different fungal endophytes (Figure 3) are depicted. BLAST analysis of sequence showed 99% homology with *Schizophyllum commune* and *Fusarium incarnatum*. Antibacterial activity of ethyl extract of endophyte *Schizophyllum commune* showed less than 5.12 µg/ml against one or more of the test pathogens (Table 1 and Figure 4). Antibacterial activity of ethyl extract of endophyte *Fusarium incarnatum* was not carried out as extract obtained was not sufficient for assay.

4. Discussion

There are more than one million species of endophyte globally, many of which inhabit individual leaves or other parts of the host plant. Endophytic fungi are linked to their metabolic potential to produce a large variety of bioactive molecules that can protect the plant against pathogens. These organisms are known to produce bioactive secondary metabolites such as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols, and lactones, which in turn benefit the host plant by enhancing plant growth and competitiveness of the host in nature (Tan and Zou, 2001; Strobel, 2004). The isolation and identification of endophytic fungi is necessary to rule out biologically active secondary metabolites medicinal properties (Kaul *et al*., 2012; Kusari *et al*., 2013). This was the case in the classic example of taxol, an anticancer agent produced by *Taxus brevifolia* Nutt., and its endophyte *Taxomyces andreanae* (Stierle *et al*., 1993). In the present work, sequences obtained which on alignment with homologous nucleotide sequences revealed 100% similarity with *Schizophyllum commune* and *Fusarium incarnatum*. Similarly, Vijayakumar *et al*. (2021) identified endophyte Fusarium spp. through morphological and molecular investigations from *B. orellana* leaves. However, Kannan *et al*. (2017) reported that endophyte colonization frequency of *B. orellana* in leaf were dominated by *Pestallotiopsis* sp. followed by Phoma spp., Phylosticta spp. and Nigrospora spp. and in stem, it was dominated by sterile forms (40%), followed by hyphomycetes (20%), coelomycetes (20%) and ascomycetes (20%). In addition, Barakat *et al*. (2019) identified *Botryosphaeri amamane*, an endophytic fungus from *B. orellana*. Variation in the colonisation of endophyte in *B. orellana* may be due to the species composition of the endophytic assemblage and frequency of infection according to host species and site characteristic such as elevation, exposure, associated vegetation, tissue type and tissue age (Fisher *et al*., 1994).

Screening of antibacterial activity of ethyl acetate extract of endophyte *Schizophyllum commune* revealed MIC values against *Staphylococcus aureus* (0.16 µg/ml), *Pseudomonas aerugonosa* (2.56 µg/ml), *Streptococcus pyogenus* (5.12 µg/ml), *Proteus mirabilis* (0.08 µg/ml) and *Bacillus subtillis* (0.08 µg/ml) respectively and no effect was found against *Escherichia coli* and *Salmonella typhimurium*. Similarly, the crude extracts of *Schizophyllum commune* isolated form *Vernonia anthelmintica* plant showed moderate growth inhibition against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* by the well difusion method (12,9.5,11 mm), respectively (Rustamova *et al*., 2020). The ethyl acetate extract of *Schizophyllum commune* fungus showed maximum activity in 100 µl concentration against *Micrococcus leuteus*, *Vibrio cholerae* and *Staphylococcus aureus* in well diffusion method (Joel *et al*., 2013). Ethyl acetate extracts studied in the present work possibly contained secondary metabolites such as steroids, terpenes or terpenoids, phthalate esters, phthalate, alkanes, esters, alcohols, sugar, sesquiterpenoids which may be responsible for the antimicrobial activities (Joel *et al*., 2013; Rustamova *et al*., 2020) and justifies the use of the whole plant for various ailments by traditional practitioners.

5. Conclusion

Screening of antibacterial activity of ethyl acetate extract of endophyte *Schizophyllum commune* revealed MIC values against *Staphylococcus aureus* (0.16 µg/ml), *Pseudomonas aerugonosa* (2.56 µg/ml), *Streptococcus pyogenus* (5.12 µg/ml), *Proteus mirabilis* (0.08 µg/ml) and *Bacillus subtillis* (0.08 µg/ml), respectively and no effect was found against *Escherichia coli* and *Salmonella typhimurium*.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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