INTRODUCTION

Endophytic fungi are the ingenious collection of microorganisms which resides within the plant tissues, for entire or part of their life cycle with no apparent symptoms of any infection (Saikkonen et al., 1998). Various fungal endophytes have been recognized to encompass potent anticancer, anti diabetic, insecticidal and immunosuppressive agents (Strobel et al., 1999). The era of discovery of novel therapeutic scaffolds from fungal endophytes was marked with isolation of the anticancer drug, Paclitaxel (Taxol) from endophytic fungi Pestalotiopsis microsora, recovered from Himalayan yew tree, Taxus wallichiana, without resulting any visible injury to the host plant (Strobel et al., 2002). Thus, biodiversity of endophytes is an imperative resource of the chemically diversified bioactive compounds based on their relationship with the plants. Endophytic metabolites produced share the same chemistry as their respective host plant and produce the same compound with more bioactive potential (Strobel et al., 2002; Jia et al., 2016). These are recognized as reasonable untapped and prospective resource of novel natural products for utilization in Pharmaceutical and agro industries. The selection of plant species as a source outlines the screening of fungal endophytes and thereby provides huge spectrum of bioactive metabolites having immense potential as pharmacophores. Rationale of plant selection is based upon their pharmaceutical usage and their ecological niche. Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth and plants growing in these areas are being surveyed for harboring endophytes. Thus, immense potential exists in screening biodiversity of endophytic fungi for exploring novel chemistries possessing enzymatic and anti-bacterial properties (Uzma et al., 2018).

Natural compounds isolated from medicinally important plants are considered to be conventionally leading agents revealing the unexplored chemical diversity incomparable to the principal combinatorial databases (Rajamanikyan et al., 2017). Rauwolfia serpentina, commonly known as sarpagandha and chandrika, is an ethnomedical evergreen shrub of the dogbane or Apocynaceae. It is rich source of indole alkaloids which are available in every part of the plant along with stem and leaves (Yarnell et al., 2004). Reserpine is the most extensively deliberated alkaloid found in R. serpentina which is used in traditional indigenous medicinal preparations in India for centuries as a remedy to various problems, including snake bites, hypertension, uterine stimulant, malaria, abdominal pain, and dysentery (Roy, 2010). Extracts of Rauwolfia serpentina has been shown to encompass potent anti-microbial activity against pathogenic Staphylococcus aureus, Salmonella typhi, Salmonella typhimurium, Verrucaria enterocolitica, Escherichia coli and Candida albicans (Elizabeth, 2017). However, associated side effects including nausea congestion, lethargy, sedation, hypotension, vomiting, abdominal cramping and gastric ulceration limits its use (Lobay, 2015).

Based on these observations, the present work was undertaken to unravel the diversity of fungal endophytes in Rauwolfia serpentina as a potent source of novel fungal endophytes with bioactive potential for pharmaceutical and therapeutic interventions. Rauwolfia serpentina inhabiting the BRT wildlife sanctuary, Karnataka and Neyyer Wildlife Sanctuary, Kerala was collected for the recovery of endophytic fungi and further prospecting for various enzymatic and antibacterial bioactivities.

MATERIALS AND METHODS

Sample Collection and isolation of fungal endophytes

BRT wildlife sanctuary, Karnataka and Neyyer wildlife sanctuary, Kerala were explored for collection of fresh Leaves, stem and bark of Rauwolfia serpentina. The collected plant parts were transported to the laboratory in sealed zip pouches and preserved at 4°C. The samples were subjected to surface sterilization by treating with 0.1% sodium hypochlorite for 3 min followed by 70% ethanol for 45 sec and 30% ethanol for 30 sec under aseptic conditions (Mitchell et al., 2001). The pre-sterilized samples were incised into pieces of 1-2mm in size under sterilized conditions and were cultured on potato dextrose agar (PDA) plates at 28°C for 8-10 days. The inoculated plates were recurrently examined for
the emergence of fungal hyphae from inoculated segments. Appearing fungal hyphae were transferred on PDA plates to attain pure culture. The pure active culture was stored on 10% glycerol-PDA slants (Kapoor and Saxena, 2018).

Screening for extracellular enzyme production

a) Amylase activity assay- The endophytic isolates were examined for the production of extracellular amylase by culturing them on 2% soluble starch-Glucose Yeast Peptone (GYP) agar medium. The plates were incubated at 28°C for 72h followed by flooding with staining solution containing 1% iodine. The zone of clearance appeared around the colony indicated amylase production (Kapoor et al., 2018; Sunitha et al., 2012).

b) Cellulase activity assay- The endophytic fungal isolates were screened for cellulose production on Yeast-Peptone Agar (YPA) medium containing 0.5% carboxymethyl cellulose (CMC). The plates were incubated at 28°C for 72h. Staining was done using 0.2% aqueous Congo red dye followed by destaining with 1M sodium chloride. The zone of clearance appeared around the colony indicated the cellulase activity (Teather and Wood, 2012).

c) Laccase activity assay- The extracellular laccase production by fungal endophytes was screened by inoculating 5 mm mycelial plug on GYP agar medium containing 0.005% α-naphthol. The plates were incubated at 28°C for 24h. Laccase induced oxidation of α-naphthol resulted in the color change of the medium from transparent to blue. Hence formation of blue color around the colony confirms the laccase production (Rao et al., 2019).

Secondary metabolites production

Broth cultures of isolates were produced by following the method of Rodriguez et al. (2000). Briefly, 5 mm mycelial plug of cultured endophytic fungal isolate was inoculated in Tryptone Soya Broth (TSB), Potato Dextrose broth (PDB), Czep-Dox broth (CDB), yeast extract-peptone broth (YPEPDM) medium, PDB:YPEPDB (PY) media, PDB:CDB (PC) media, PDB:TSB (PT) media, PDB:YPEP:TSB:CDB (PYC) media followed by incubation at 28°C with continuous shaking for 10 days. Post incubation period, the fungal mass was separated from the supernatant to obtained cell free filtrates that were further stored at -4°C.

Liquid-liquid Extraction

The culture filtrates of selected fungal endophytes were subjected to extraction with diverse solvent systems viz. Hexane, ethyl acetate, chloroform and acetone. The culture filtrate was extracted with each solvent thrice, organic layers were taken out and dehydrated using sodium sulphate. The organic layer so obtained was evaporated to dryness by using Rotatory evaporator. The obtained crude residue was dissolved in Dimethylsulfoxide (DMSO) and preserved at 4°C for further use.

Maintenance of Test microorganisms

The test organisms included bacterial isolates and were broadly grouped into standard and clinical isolates. The clinical isolates included the following organisms: Staphylococcus aureus (G3), Staphylococcus aureus (G26) procured from Government Medical College, Patiala. The standard isolate was Staphylococcus aureus (NCTC 8571) taken from the Microbial type culture collection (MTCC), IMTECH, Chandigarh. The bacterial cultures were maintained on Muller Hinton Broth (MHB) with 2 % glycerol and stored at 4°C. These cultures were further streaked on Muller Hinton Agar (MHA) plate and incubated at 37°C overnight. A single pure colony was transferred to the MHB and incubated 16–18h at 37°C before being used as test organism. The visual turbidity of the culture was tested against the standard 0.5 McFarland solution.

Kirby-Bauer Antimicrobial Susceptibility Test

A total of 8 different combinations of crude EA and aqueous extract (PDB, CDB, TSB, YEPDB, PY, PT, PC, PYTC media) were investigated for antibacterial activity by KB disc diffusion method. A 5 mm size disc were used having loading capacity of 30μl. Fungal extract (6.4mg/ml) was loaded in sterile discs under laminar conditions and left for drying. The turbidity of test organism was adjusted with 0.5 McFarland solution followed by swabbing of MHA plates. The loaded discs were placed on MHA plates and incubated at 37°C overnight for 18h to 24h. Anti-staphylococcal activity was analyzed by measuring diameter of zone of inhibition in mm (Magaldi et al., 2004). Three independent experiments were performed and the average values of anti-staphylococcal activity were calculated.

Agar well diffusion assay

The agar well diffusion assay was carried out to assess the anti-staphylococcal activity of the crude solvent extracts. Stock solutions of extracts (1 mg/ml) were prepared in 10% DMSO and stored at -4°C till further use. 20μl of test extracts were dispended in 5mm wells prepared using a sterile cork borer on 24h old MH agar plate aseptically. Further, the wells were sealed and kept for 20 minutes for solidification. The turbidity of test organism was adjusted with 0.5 McFarland solution followed by swabbing of MHA plates and incubated at 37°C overnight for 18h to 24h. The anti-staphylococcal activity was determined by measuring the zone of inhibition around the test and control samples (Magaldi et al., 2004; Valgas et al., 2007). Three independent experiments were performed and the average values of anti-staphylococcal activity were calculated.

Molecular identification of potent endophytic fungus

PCR Amplification

The potential fungal endophyte was identified by Molecular tools employing ITS1 and ITS4 primers mediated amplification of ITS1-5.8S-ITS2 region (Felsenstein, 1985; White et al., 1990). Genomic DNA extracted from the endophytic fungal isolates was taken as template along with 0.8 μM primer, 0.2 mM dNTP, 1.5 U Taq polymerase to a final volume of 25 μl. The reaction mixture was subjected to PCR amplification following 39 cycles and a final extension of 5 mins at 72°C (Kapoor and Saxena, 2014). PCR amplicon was purified using Wizard® SV Gel and PCR clean up system kit, Promega, USA following manufacturer’s protocol. The Purified PCR product was sent for direct sequencing to Xcelris Labs, Gujrat.

Sequence assembly and Phylogenetic identification:

Sequencer ver. 5 (www.genecodes.com) was used to edit and verify the attained chromatograms and were submitted in the GenBank under the accession number MH237828. BLAST tool was used to analyze the homology in the consensus sequence. Clustal W in MEGA 5.2 was utilized for obtaining the alignment with the reference taxa (Tamura et al., 2011). The phylogenetic history was determined by the Maximum Parsimony method using the Close-Neighbor-Interchange algorithm (Arnold et al., 2005) in which the tree is formed based on random addition of sequences with 20 replicates. The analysis involved 19 nucleotide sequences. Alignment gaps were taken as missing characters. Bootstrap analysis (1000 bootstrap) was executed to deduce the consensus tree for the demonstration of evolutionary relationship (Kapoor and Saxena, 2014; González-Menéndez et al., 2018).

RESULTS

Isolation of endophytic fungi

Leaves, stem and bark samples of Rauwolfia serpentina were subjected to isolation of fungal endophytes on PDA plates. A total of 21 fungal endophytes were recovered from Rauwolfia serpentina. Each host explant showed disparity in the endophytic fungal colonization (Table 1). The maximum colonization was observed in leaf (42.8 %) followed by bark (33.3 %) and stem (23.8 %). No endophytic fungi were recovered from the stem internal issue of the plant. Isolated endophytes were further sub cultured for preservation of pure colonies.

<table>
<thead>
<tr>
<th>S. NO</th>
<th>Culture Code</th>
<th>Plant part</th>
<th>Place of sample collection</th>
<th>Bioactivities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Amylase</td>
</tr>
<tr>
<td>1.</td>
<td>#1RSLBRT</td>
<td>Leaves</td>
<td>BRT wildlife sanctuary, Karnataka</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>#3RSLBRT</td>
<td>Leaves</td>
<td>BRT wildlife sanctuary, Karnataka</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>#5RSLBRT</td>
<td>Leaves</td>
<td>BRT wildlife sanctuary, Karnataka</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>#7RSLBRT</td>
<td>Leaves</td>
<td>BRT wildlife sanctuary, Karnataka</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>#10RSLBRT</td>
<td>Leaves</td>
<td>BRT wildlife sanctuary, Karnataka</td>
<td>-</td>
</tr>
</tbody>
</table>
Isolated fungal endophytes exhibited potent enzyme production

Isolated fungal endophytes were examined for the production of commercially important enzymes including amylase, cellulose and laccase.

In the preliminary screening of fungal endophytes, four isolates were found to be positive for producing amylase. Out of 4 positive isolates, maximum amylase activity was observed in #15RSBANEY followed by #7RSBANEY, #1RSBANEY and #14RSBANEY. Similarly, three isolates exhibited promising cellulase activity, of which #3RSSTNEY was found to be potent cellulase producer. In laccase assay, four isolates showed positive results. #11 RSLBRT, #12 RSBANEY, #14 RSBANEY and #7 RSBANEY showed blue color around their colony after 4 days of incubation indicating the production of laccase activity showing the ability of fungal isolates to produce de-lignifying enzyme (Figure 1).

Isolated fungal endophytes exhibited potent anti-staphylococcal activity

For evaluation of antimicrobial activity, substrate specificity testing using EA and aqueous extracts of 8 different media combinations using KB disc diffusion method revealed TSB to be the best media for secondary metabolite production. In KB disc diffusion assay, three isolates viz. #16RSLBT, #10RSLBRT and #23RSSTNEY exhibited promising antibacterial activity (Table 2, Figure 2).

Aqueous extract of #10RSLBRT was found to exhibit potent antibacterial action against all test organisms and hence the aqueous extract, was shown to get increased antibacterial activity against pathogenic strains of S. aureus (Figure 3).

Images represent the production of laccase evident from the transition of colour around the colony from transparent to blue. #7RSBANEY, #12RSBANEY and #14RSBANEY exhibited production of laccase enzyme.

Images represent the KB disc diffusion assay based antibacterial activity of ethyl acetate and aqueous extract of #10RSLBRT evident from zone of inhibition. Aqueous extract exhibited maximum anti-bacterial activity against pathogenic strains of S. aureus (D-F) Images represents the KB disc diffusion assay based anti-bacterial activity of ethyl acetate and aqueous extract of #23RSSTNEY evident from zone of inhibition. Ethyl activity extract exhibited anti-bacterial activity against pathogenic strains of S. aureus

Aqueous extract of #10RSLBRT was carried out to find out whether the activity remained constant for the optimum pH needed to exhibit antibacterial activity. A pH gradient ranging from 4 to 7 was prepared and the extracts were subjected to agar well diffusion method for examining the antimicrobial action. It was observed that maximum activity appeared in range of pH 5 to 6 (Figure 3). Since potent activity was shown by aqueous extract, further fractionation with acetone and chloroform for evaluation of antibacterial activity was carried out. The anti-staphylococcal activity of aqueous layer was observed to get increase by two folds after fractionation with maximum activity against S. aureus G3 followed by S. aureus G26 and S. aureus NCTC 6571 (Table 3). These results indicated that fractionation with non polar solvents tends to increase the potency of aqueous layer.

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<table>
<thead>
<tr>
<th>#</th>
<th>Isolate</th>
<th>Media</th>
<th>Production Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>#1RSLBRT</td>
<td>Leaves</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>#15RSLBRT</td>
<td>Leaves</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>#16RSLBRT</td>
<td>Leaves</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>#2RSLBRT</td>
<td>Leaves</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>#1RSBANEY</td>
<td>Bark</td>
<td>++</td>
</tr>
<tr>
<td>11</td>
<td>#7RSBANEY</td>
<td>Bark</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>#1RSBANEY</td>
<td>Bark</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>#12RSBANEY</td>
<td>Bark</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>#14RSBANEY</td>
<td>Bark</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>#15RSBANEY</td>
<td>Bark</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>#22RSBANEY</td>
<td>Bark</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>#2RSSTNEY</td>
<td>Stem</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>#3RSSTNEY</td>
<td>Stem</td>
<td>++</td>
</tr>
<tr>
<td>19</td>
<td>#4RSSTNEY</td>
<td>Stem</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>#15RSSTNEY</td>
<td>Stem</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>#23RSSTNEY</td>
<td>Stem</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: poor activity (+); Good activity (++); Excellent activity(+++); No activity (-)
Enhanced anti-staphylococcal activity by solvent fractionation

Based on above observations, it was hypothesized that antibacterial compound resided in aqueous layer may be a strong non polar compound or increasing the non polarity in extraction may channelize the removal of non polar molecules that may hinder in potent antimicrobial activity. To test the proposed hypothesis, hexane was used for fractionation of culture filtrate of #10RSLBRT followed by extraction with chloroform, Acetone and EA. The agar well gel diffusion assay of obtained extracts revealed that EA and hexane extracts exhibited antibacterial action against all test organisms. Maximum activity was exhibited by EA extract against S. aureus NCTC 6571 with zone size of 11.33 mm (Table 4, Figure 4).

Table 4 represents the anti-staphylococcal activity of ethyl acetate and hexane extracts of #10RSLBRT

<table>
<thead>
<tr>
<th>Culture Code</th>
<th>S. aureus NCTC 6571</th>
<th>Zone size (in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EA</td>
<td>Aqueous</td>
</tr>
<tr>
<td>1. #16RSLBRT</td>
<td>11.33 ± 0.57</td>
<td>8.66 ± 0.57</td>
</tr>
<tr>
<td>2. #10RSLBRT</td>
<td>9.33 ± 0.577</td>
<td>9.33 ± 0.577</td>
</tr>
<tr>
<td>3. #23RSSTNEY</td>
<td>11.33 ± 0.57</td>
<td>9.33 ± 0.577</td>
</tr>
</tbody>
</table>

Mean ± SD 11.33 ± 0.57 7.66 ± 0.57 9.66 ± 0.57 7.0 ± 0 9.66 ± 0.57 8.66 ± 0.57

Figure 4 Ethyl acetate extract of #10RSLBRT exhibited more potent antibacterial activity: To test the possibility of removing the non polar hindering compounds thereby enhancing the antibacterial activity, sequential extraction was done using non polar to polar solvents. (a-c) Images represent the agar well diffusion assay of hexane extracts against S. aureus G3 followed by S. aureus G26 and S. aureus NCTC 6571. No antibacterial action was professed against the test organisms. (d-f) Images represent the antibacterial activity of ethyl extract post sequential extraction evident from the agar well diffusion assay.

The isolated endophytic fungus was clustered in Fusarium incarnatum-equiseti clade by phylogenetic identification.

The BLAST analysis of the sequence data showed close association of the isolate with Fusarium sp. For the speciation of the isolate, Maximum parsimony tree was constructed with consistency index of 0.822917, retention index of 0.910995, and the composite index of 0.815983 (0.749673) for all the sites and parsimony-informative sites (in parentheses). The tree was divided into 4 different clades of respective Fusarium species viz. Fusarium incarnatum-equiseti complex clade, Fusarium oxysporum clade, Fusarium lateritum clade, Fusarium solani clade. The isolate #10RSLBRT was clustered in Fusarium incarnatum-equiseti clade thereby confirming its placement in Fusarium incarnatum-equiseti complex (Figure 5). Aspergillus niger was taken as outgroup to root the tree.

DISCUSSION

Endophyte inhabiting the host plant acts as defense armor against pathogenic (Strobel and Daisy, 2003). Fungal endophytes have been isolated from many plant species including terrestrial plants from different parts like fruits, stems or bark, mangroves and halophytes. Fungal endophytes have been the repository of potent bioactive metabolites with diverse action including anti-cancer, antioxidant, antifungal, antibacterial, antiviral, anti-insecticidal and immunosuppressant (Tan and Zou, 2001; Togheu, 2019). Endophytic fungi are potent source of enzyme producers. Fungal bio-resource is being exploited by researchers in quest of commercially important enzymes like laccase, cellulase, amylase, L-asparaginase etc. For penetrating the host plant and colonising its tissue, endophytic fungi synthesizes different type of enzymes (Jia et al., 2011). Endophytic fungi are commercially viable as producer of starch digesting enzymes because of efficient conversion of numerous starch sources to sugars without prior gelatinization (Marilda et al., 2015; Togheu et al., 2017).

Medical plants have been the potent reservoir of novel chemistries destined for pharmaceutical and therapeutic interventions. Recent trends in mycology have focused in isolating novel endophytic fungi from aboriginal medicinal plants for extraction of potent bioactive agents. Rauwolfia serpentina, a medicinal shrub has been studied as a source of endophytic fungal diversity with a colonisation of 18% (Nath et al., 2017). Also, the isolated endophytes were shown to exhibit
antibacterial activity. In the present study, Rauwolfia serpentina, a medicinal shrub, was taken as a source of exploring endophytic fungal diversity. Endophytic fungi isolated from the plant were maintained as pure culture and were further assessed for the production of anti-bacterial compound. In all 21 isolates were recovered from different plant parts of Rauwolfia serpentina. Since endophytic fungi have been utilised in production of commercially imperative enzymes, we assessed the isolates for the production of enzymes such as amylase, cellulase and laccase. # 11 RSLBRT, # 12 RSBANEY, # 14 RSBANEY and # 7 RSBANEY showed potent amylase production. Laccase production was shown by # 1 RSBANEY, # 7 RSBANEY, # 15 RSBANEY and # 2 RSTSNTEY and # 3 RSTSNTEY. # 1 RSLBRT and # 1 RSBANEY showed cellulase production.

Further, the antibacterial potential of the isolates was evaluated in various media composition to decipher the required substrate for potent production of secondary metabolite. Trypotine soya broth as substrate elicited the production of secondary metabolites which was evident with the presence of antibacterial activity which was evident with the presence of antibacterial enzymes. Since lysis and sequence alignment. Further, the antibacterial potential of the isolates was evaluated in various media composition to decipher the required substrate for potent production of secondary metabolite. Trypotine soya broth as substrate elicited the production of secondary metabolites which was evident with the presence of antibacterial activity. Since lysis and sequence alignment. Further, the antibacterial potential of the isolates was evaluated in various media composition to decipher the required substrate for potent production of secondary metabolite. Trypotine soya broth as substrate elicited the production of secondary metabolites which was evident with the presence of antibacterial activity.


