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POTENTIALS OF ENDOPHYTES OF *Andrographis paniculata* FOR THE PRODUCTION OF PLANT GROWTH PROMOTERS, ENZYMES AND ANTIMICROBIAL COMPOUNDS

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ABSTRACT

In the present study, 9 bacterial and 6 fungal endophytes were isolated from surface sterilized leaf, stem and root samples of the medicinal plant - *Andrographis paniculata* (Kalmegh). The endophytes were screened for plant growth promoting traits (IAA, phosphate solubilization and N_2 fixation), enzymes (cellulase and amylase) and antimicrobial compounds against 3 potent human pathogens- *E. coli*, *Staphylococcus sp*. and *Vibrio sp*. The majority of the isolated endophytes produced the phytohormone - IAA (ranging 2-45µg/ml), and 1 endophyte solubilized phosphate and fixed N_2. All the fungal endophytes possessed cellulase and amylase activity. In the preliminary screening, 4 bacterial and 4 fungal endophytic isolates extract showed antagonistic activity against the 3 potent human pathogens which are known causative agents of urinary tract, skin and gastrointestinal tract infections, respectively. The endophytes of *A. paniculata* exhibiting broad and specific antimicrobial activity make them ideal candidates in medical purposes.

Keywords: *Andrographis paniculata,* Endophytes, Medicinal plant, PGP, Phytohormones

INTRODUCTION

After their discovery by Darnel Germany in 1904 (Freeman, 1904), the endophytes has revolutionized the field of plant microbe interaction. Endophytes are ubiquitous species of bacteria, fungi and actinomycetes (Chanway et al., 1996) that originates in the rhizospheric region and after gaining entry into the root interior by different mechanisms like cell wall hydrolysis, water flow, via wounds and tumour (Hallman, 1997; Siciliano et al., 1998), establish a mutualistic and symbiotic association with the host plant (Quispel et al., 1992). The endophytes are host specific (Dastogeer et al., 2018) and colonize different plant parts such as root, stem, leaves, apical bud etc. (Ulrich et al., 2008) without doing any substantial harm to their host (Kado et al.,

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1992), but provide their host with a ton of benefits in the form of plant nutrients (Chen et al., 1995), phytohormones (Shi et al., 2009), enzymes (Uzma et al., 2016) pest and pathogen resistance and stress tolerance (Yaish et al., 2015) etc.

The endophytes aid in various plant growth promoting (PGP) abilities to the host plant in form of phytohormones such as indole 3 acetic acid, gibberellins, cytokinins (Ali et al., 2017); mineral nutrient assimilation such as N_2 fixation and phosphate solubilization etc. Apart from the plant synthesized phytohormones, the endophyte produce phytohormones like indole 3 acetic acid (IAA) in a L-tryptophan dependent pathway which imparts additive growth promoting abilities to the host (Bal et al., 2013), by enhancing cell elongation, cell division and promoting tissue differentiation etc (Taghavi et al., 2009). Atmospheric nitrogen (N_2), is a limiting nutrient element to plant due to their inability to fix it, hence, the plants have to rely upon the rhizospheric microorganisms and chemical fertilizers for N_2 supply. Recently, the endophytes are emerging as beneficial alternative source of N_2 (Muangthong et al., 2015), because the plant interior with low partial O_2 pressure and sufficient N_2 accessibility, serve as a homely niche for N_2 fixation (James and Olivares, 1998). The endophyte fixed N_2 is required for plant growth and development without any persistent environmental hazards in the form of underground water contamination and greenhouse gas emission, as evident in chemical fertilizers. Phosphorus (P) is an essential primary nutrient element in the form of ATP, sugar and nucleotides in plants (Saber et al., 2005) as well as for secondary metabolic processes like terpenoid biosynthesis, isopentenyl pyrophosphate, precursor of andrographolide (Vickery and Vickery, 1986; Dubay et al., 2003), bioactive compounds of medicinal plant *Salvia miltiorrhiza* (Lu et al., 2013), and *Lens culinaris* (Sarker and Karmoker, 2011). The application of chemical P fertilizers although increased the essential oil content of *Mentha piperita* (Sulandjari et al., 2007) and sunflower (Bahl et al., 2000), but they contain maximum amount of soluble inorganic phosphate, which gets immobilized and unavailable to the plants. Alternatively, rhizobacteria were also used for phosphate mobilization and increased upland crop yields (Goldstein et al., 1986). But nowadays, P solubilization by endophytes is well documented in crop plants like wheat (Jha and Kumar, 2009) and rice (He and Zhu, 1998) via release of protons, chelating agents and organic acids (Hussain et al., 2013) which helps in plant growth and development, pathogen resistance efficiently.

Apart from various primary and secondary metabolites, antioxidants, anticancer agents (Gunatilaka et al., 2006), the endophytic fungi also serves as potential sources of industrially relevant enzymes with valuable roles in biotechnology. They produce hydrolases like pectinase, lipase, proteinase, amylase, laccase, xylanase etc. extracellularly to resist pathogen invasion and nutrient acquisition from the host (Sunitha et al., 2013) which are available to the mankind with industrial and biomedical potentialities (Strobbel et al., 2003).

In a study by Farnsworth et al. (1990), it is observed that an estimation of WHO has clearly reported that approx. 80% population of the developing countries use traditional medicinal plants for primary health care needs, which is also getting in demand in developed countries due to their non-toxicity, abundance and affordable costs. Microorganisms have had promising use since decades as disease control agents. Recently endophytes of medicinal plants are also gaining importance in medical applications such as antibiotics, antivirals, antidiabetic and anticancer agent etc. production (Guo et al., 2010). The endophytes of medicinal plants, *G.mangostana* of Indonesia (Radji et al., 2014), *Catharanthus roseus* (Mukhopadhyay and Adhikari, 2020) etc. are shown to have diverse antimicrobial activity and resisted the growth of potential human pathogens.

Andrographis paniculata (commonly known as Kalmegh) is extensively used in India, China and South East Asia as a household medicine. Studies have reported that this herb has promising results in the treatment of fatal diseases like meningitis, acute hepatitis, influenza, malaria etc (Rao et al., 2014) and is widely used in the prevention of gastrointestinal, respiratory and urinary tract infections (Xu et al., 2006; Zhang et al., 2009). However, very little studies have focused on the abilities of the endophytes of *Andrographis paniculata* to resist against pathogenic infections (Arunachalam et al., 2010; Gusmaini et al., 2013).

Our current study focuses on the isolation of endophytes from medicinal plant - *Andrographis paniculata* and exploring their potential for plant growth promoters, extracellular enzymes and antimicrobial compounds.

MATERIALS AND METHODS

Collection of plant materials

Intact healthy *Andrographis paniculata* plant samples were collected from the local area and taken aseptically to the laboratory.

Surface Sterilization and Isolation of endophytes

From the plant samples, 1g each of stem, root and leaf was taken and thoroughly washed in running tap water and Tween 20 solution by vigorous shaking. This was followed by washing with sterile distilled water, and finally dipped in 70% ethanol for 3-5 minutes. Further, the roots were treated with 1% sodium hypochlorite solution for 2 minutes and rinsed 2-3 times with sterile Milli-Q water following (Sucuia and Cornea, 2019). The sterilized samples were then ground to prepare slurry and various dilutions were prepared with 0.85% sterile saline water. From the prepared dilutions, 0.1 ml was dispensed on Nutrient agar and Czapek Dox agar plates and incubated at 37 °C for 24 h. After the incubation period, the endophyte colonies were selectively isolated and further examined by staining and microscopic analysis.

Screening of bacterial isolates for PGP trait analysis

IAA production

The bacterial isolates were aseptically inoculated into 20 ml of IAA media ($NaNO_3$ 0.2%, K_2HPO_4 0.1%, Na_2HPO_4 0.2%, $MgSO_4$ 0.01%, $CaCO_3$ 0.2%, Glucose 1%) supplemented with 0.5% (v/v) of L- tryptophan and incubated for 10 days at 28°C. After 10 days, the isolates were centrifuged at 3000 rpm for 20 min and the supernatant was analyzed for IAA production following Gordon and Weber, 1951 by using freshly prepared Salkowski reagent and assessed for the development of red colour as positive result. Standard graph was plotted from known concentration of IAA and the amounts of IAA produced by the isolates were measured at 530 nm by spectrophotometeric analysis.

Phosphate Solubilization

The endophytic bacterial isolates were screened for phosphate solubilization by inoculating into Pikovskaya medium (containing dextrose 10gm, Tricalcium phosphate 5gm, $(NH_4)_2SO_4$ 0.5 gm, NaCl 0.2 gm, $MgSO_4$ 0.1 gm, KCl 0.2 gm, $FeSO_4$ 0.002 gm, Yeast Extract 0.5 gm, $MnSO_4$ 0.002 gm, Agar-20 gm, distilled water 1liter) and incubated at 30°C for 7 days (Pikovskaya, 1948). Finally, the media plates were observed for the formation of clear halo around the colony due to the utilization of tricalcium phosphate present in the medium.

Nitrogen Fixation

The bacterial isolates were inoculated into slants of Glucose N_2 free mineral media (containing glucose-10 gm, K_2HPO_4 1gm, $MgSO_4$ 0.20 gm, $CaCO_3$ 1gm, NaCl 0.2 gm, $FeSO_4$ 0.10 gm, Na_2MO_4-0.005 gm, Agar-10 gm, distilled water 1 liter) containing bromothymol blue solution and incubated at 30°C for 7 days and were observed for the appearance of prussian blue colour as indicative of nitrogen fixation by the isolates.

Screening of fungal isolates for Extracellular Enzyme production

Cellulase Activity

The fungal isolates were assessed for cellulase activity by streaking on CMC Agar media (containing K_2HPO_4 1 gm, $MgSO_4$ 0.5 gm, NaCl 0.5 gm, $FeSO_4$ 0.01 gm, $MnSO_4$ 0.01 gm, NH_4NO_3-0.03 gm, CMC 10 gm, Agar-20 gm, distilled water 1 liter) and incubated for 5 days. After fungal growth appearance, the plates were flooded with 0.1% Congo red solution for 15 mins with gentle shaking and then de-stained with 1M NaCl solution for 15 minutes. Appearance of clear zones around fungal colony indicates cellulase activity.

Amylase Activity

The fungal isolates were assessed for amylase activity by inoculating on Starch Agar media (Beef Extract 3 gm, Soluble Starch 10 gm, Agar 20 gm, distilled water 1 liter) and incubated for 24 – 48 h. After incubation, the plates were flooded with 1%

Gram's iodine solution and observed for the appearance of a clear zone of hydrolysis around the fungal growth.

Screening of endophytic bacteria and fungi for production of antimicrobials

The endophytic bacterial and fungal isolates from *Andrographis paniculata* were cultured in 5 ml Nutrient broth and Sabouraud Dextrose broth medium respectively at 32°C for 5 days (allowing them to reach stationary growth phase) in a rotary shaker (150 rpm), followed by centrifugation at 8000 rpm for 8 mins. The culture filtrate was used for the screening of antimicrobial activity by agar-diffusion technique on Luria-Bertani agar media that was previously seeded with test pathogens - *E. coli*, *Staphylococcus* sp. and *Vibrio* sp. Sterile broth was set as control. Formation of any inhibition zone was recorded.

RESULTS AND DISCUSSION

From the surface sterilized *Andrographis paniculata*, a total of 100 endophyte cfu were observed, of which the highest endophytic consortia were isolated from leaf tissue (47cfu), followed by the root (33 cfu) and stem tissue (20 cfu). This signified a diverse number of residing endophytes in *A. paniculata* leaves. Out of 100 endophytic cfu, morphologically distinct 9 bacterial and 6 fungal isolates were considered for further evaluation of their potential roles in the host plant physiological processes (Table 1).

Table 1. Endophytes isolated from *Andrographis paniculata*

Plant specimen	Endophyte Type	Leaf	Stem	Root	Total	Microscopic Characterization
Andrographis paniculata	Bacterial	3	3	3	9	Gram (+) rods, few are gram (-)
	Fungal	3	2	1	6	Fungal hyphae identified to be *Alternaria sp.*

The bacterial endophytic isolates were microscopically characterized as gram positive rods, which are consistent with those found in the *Cassia tora* L. (Kumar et al., 2015) and the fungal endophytic isolate was identified to be *Alternaria sp*. Also, *Aspergillus sp*. is identified as endophytes in *Andrographis paniculata* (Elfita et al., 2015).

In the current study, efforts were made to observe the PGP properties of bacterial endophytes of *Andrographis paniculata* i.e., IAA production, N_2 fixation and phosphate solubilization (Table 2).

Table 2. Plant Growth Promoting (PGP) Traits of Endophytic Bacterial Isolates of *A. paniculata*

Endophyte isolate	IAA Production		Phosphate Solubilization	N_2 fixation
	Ability	Concentration (µg/ml)		
1) APL1-B	-	-	-	-
2) APL2-B	+	2	-	-
3) APL3-B	+	12	-	+
4) APS1-B	-	-	-	-
5) APS2-B	+	31	+	-
6) APS3-B	-	-	-	-
7) APR1-B	+	15	-	-
8) APR2-B	+	3	-	-
9) APR3-B	+	45	-	-

Out of 9 isolates, 6 were found to produce IAA, the main auxin in higher plants. The root isolate, APR3-B was shown to produce maximum IAA (45µg/ml), followed by the stem isolate, APS2-B (31µg/ml) after 10 days incubation with 0.5% L-tryptophan. This signifies their additive roles in promoting plant root and shoot growth, water and mineral uptake etc. (Liu et al., 2010). No IAA detected in isolates in absence of L-tryptophan suggests that the endophytes synthesize IAA in L-tryptophan dependent indole-3 pyruvic acid (IPA) pathway (Lee et al., 2004). This observation may be interpreted as the endophytes may be deficient of the tryptophan biosynthetic pathway and relies upon the host plant for the amino acid L-tryptophan, the extra amount is converted to IAA by the endophyte for efficient plant growth promotion. This signifies a mutually beneficial plant-microbe interaction. Phosphate solubilization by the endophyte, *Achromobacter xylosoxidans* is well documented in wheat (Jha and Kumar, 2009). In our study, 1 stem isolate, APS2-B was shown to solubilize P on Pikovskaya medium, as indicated by a clear halo around the colony is another PGP trait observed in the endophytic isolates. Inorganic P in chemical fertilizers gets immobilized in the soil and is unavailable to the plants. However, P solubilization by rhizospheric microorganisms is well reported (He et al., 2010). The endophytes are also found to be capable of solubilizing the soil P reserve via production of organic acids and extracellular polysaccharides and releasing them into soil (Goldstein et al., 1995). This makes them ideal candidates for the utilization of soil P reserve by the plants thus enhancing the crop yields. The highest P solubilizing endophytes reported seems to be *Streptomyces sp*. (Hamdali et al., 2008), *Bacillus sp*., *Pseudomonas putida* etc (Rajkumar et al., 2006). This solubilized P is further utilized in secondary metabolite biosynthesis like andrographolide in *Andrographis*

paniculata (Vickery and Vickery, 1986). In addition to this, 1 leaf isolate, APL3-B was found to be capable of fixing atmospheric N_2 in glucose N_2 free minimal media, as indicated by prussian blue coloration. This is another PGP trait conferred by the endophytes towars plant growth and development processes. Till date, rhizospheric microorganisms are well known for their capability of atmospheric N_2 fixation (Igiehon et al., 2018), the sole natural source of N_2, which is an important macronutrient of plants. The endophytes also fix atmospheric N_2 by nitrogenase activity which is encoded by the nifH gene (Tonooka et al., 2008) making them ideal for the supply of plant N_2 source. The capability of isolates, APL3-B and APS2-B with diverse PGP traits make them agriculturally potential endophytes.

Beside PGP traits, preliminary screening of *Andrographis paniculata* fungal endophytic isolates for enzyme production (cellulase and amylase) was also carried out. Among 6 isolates, all were positive for the production of extracellular enzymes viz. cellulase and amylase (Table 3), which confers their potential role in plant pathogen resistance. Various plant pathogens attack plant tissues which are outcompeted and inhibited by the residing endophytic fungi with aid of different extracellular lytic enzymes like chitinase, protease, cellulase etc. production (Choi et al., 2005) which breaks down the plant pathogen cell wall constituting chitin, modified cellulose, starch as storage material (de Bashan et al., 2005).

Table 3. Enzyme Production Assessment of Fungal Endophytic Isolates of *A. paniculata*

Endophytic Isolate	Cellulase Activity	Amylase Activity
1) APL1-F	+	+
2) APL2-F	+	+
3) APL3-F	+	+
4) APS1-F	+	+
5) APS2-F	+	+
6) APR1-F	+	+

The bacterial endophytes like *Bacillus* and *Pseudomonas sp*. induce chitinase expression which reduces the disease severity of plant pathogen - *Xanthomonas axonopodis* (Rajendran, 2006). These enzymes producing fungal endophytes can also be used as biotechnological sources for industrially and medically important enzymes (Promputtha et al., 2007).

The antimicrobial potential of *Andrographis paniculata* endophytic extracts were also evaluated against 3 test pathogens - *E.coli*, *Staphylococcus sp*. and *Vibrio sp*.

Out of the 9 bacterial isolates, 4 isolates (APL1-B, APS2-B, APS3-B and APR3-B) were found to inhibit the test pathogens; of which the stem isolate, APS3-B significantly showed broad spectrum antimicrobial activity against *E.coli*, *Staphylococcus sp*. and *Vibrio sp*. 4 out of 6 fungal endophytic isolates namely, APL1-F, APS1-F, APS2-F and APR1-F were also inhibiting the test pathogens, and the latter 3 of them were found to significantly inhibit both *Staphylococcus sp*. and *Vibrio sp*. (Table 4).

Table 4. Antimicrobial Activity of Endophytes of *A. Paniculata*

Isolates	*E. coli*	*Staphylococcus sp.*	*Vibrio sp.*
Bacterial	Zone of Inhibition (in mm)		
APL1-B	15	-	-
APL2-B	-	-	-
APL3-B	-	-	-
APS1-B	-	-	-
APS2-B	-	13	-
APS3-B	16	13	14
APR1-B	-	-	-
APR2-B	-	-	-
APR3-B	-	10	-
Fungal			
APL1-F	-	-	17
APL2-F	-	-	-
APL3-F	-	-	-
APS1-F	-	10	11
APS2-F	-	10	12
APR1-F	-	11	13

E. coli is the leading cause of community acquired urinary tract infection (UTI) among 1/3rd of women population (Minardi et al., 2011). The phytochemicals extracted from *Andrographis paniculata* are capable of inhibiting uropathogenic *E. coli* strains (Sahare and Shinde, 2014) because they contain various essential oils and bioactive compounds with high sensitivity against *E.coli* (Gupta and Shukla, 2017). Contrarily, our study showed that *A. paniculata* leaf and stem endophytes (APL1-B and APS3-B) were strongly combating *E.coli* growth which can be used as an alternative against antibiotics in UTI treatment because the frequent use of antibiotics can cause vaginal and intestinal dysbiosis. Various species of *Staphylococcus* are opportunistic pathogens which causes a wide range of infections from mild skin

lesions (Foster et al., 1996) to severe necrotizing pneumonia, bacteremia, endocarditis etc. (Oliveira, 2018). Studies reported that *A. paniculata* leaf extracts can effectively inhibit *Staphylococcus sp*. at low MIC by oxidative damage via downregulating superoxide dismutase (Hussain and Mustakim, 2017). Our study showed that most of the *A. paniculata* endophytic bacterial (APS2-B, APS3-B, APR3-B) and fungal isolates (APS1-F, APS2-F, APR1-F) have inhibitory activity against *Staphylococcus sp*. which is consistent with results of Arunachalam et al., 2010. *Vibrio sp*., the etiological agent of cholera, gastroenteritis and fulminant sepsis (Morris, 2003) is reported to be potentially inhibited by *A. paniculata* extracts. This is due to the presence of andrographolide, which is reported to have antiquorum sensing potential in *Vibrio harveyi*, thus inhibiting biofilm formation and bacterial virulence (Mary et al., 2017). Our study showed that *A. paniculata* endophytes, mainly fungal endophyte extracts have maximum inhibitory zones against *Vibrio sp*. Our investigation suggested that apart from *A. paniculata* phyto-extracts, the endophytes can also be used for medical purposes as they have broad and specific antimicrobial activity. Currently, endophytes are reported to be used in pharmaceutical purposes for the discovery of diverse human therapeutic agents like antibiotics, antimycotics, anti-carcinogenics etc. (Guo et al., 2008).

CONCLUSION

In conclusion, the endophytes of the medicinal plant -*Andrographis paniculata* are novel and diverse. They exhibit diverse PGP traits that help in the improvement of growth and development of *A. paniculata* and serve as a bioresource of medically important enzymes, which helps the plants in pathogen resistance. They also exhibit potential antagonistic effects against potential human pathogens causing skin, gastrointestinal, and urinary tract infections. Thus, the endophytes are promising sources of bioactive compounds in agricultural, biotechnological and pharmaceutical fields.

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