



RESEARCH ARTICLE

Study of antibacterial, anti-proliferative and pro-apoptotic potential of the cell extracts of endophytic fungi and bacteria isolated from *Pajanelia longifolia* (Willd.) K. Schuman

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ABSTRACT

Endophytes contribute to the synthesis of significant metabolites in symbiotic association with their host plants. On considering the medicinal importance of the prominent tree species *Pajanelia longifolia* (Willd.) K. Schuman, the study was conducted to isolate and identify the endophytic bacteria and fungi for their bioactivity. The isolation of endophytic bacteria and fungi were performed by surface sterilisation of the stem and leaf samples of *P. longifolia*. The obtained bacterial and fungal endophytic isolates were maintained in nutrient agar and Potato Dextrose Agar (PDA) media and were examined for colony morphology and microscopic appearances with varied biochemical characterisations. Furthermore, both the fungal and bacterial isolates were subjected to solvent extractions to evaluate antibacterial activity. Also, anti-proliferative effects due to apoptotic induction by the endophytic fungal extracts were checked against proliferative yeast cells. Moreover, endophytic bacteria belonging to *Enterococcaceae* had shown antibacterial activity against *Salmonella* species. In the present study, fungal species belonging to *Cladosporium* predominantly found to inhabit as endophytic fungi in the plant samples. Also, this particular fungus among other selected endophytic fungi attributed to causing effective anti-proliferative activity. The endophytic bacteria belonging to *Enterococcus* and *Micrococcus* genera showed significant antimicrobial activity against *Salmonella typhimurium* (ATCC 23564).

Introduction

Plant-Microbe interaction is known to have crucial biological activities for both the species participating in the association. Endophytic microbes play a vital role in stabilising physiological and biochemical status within the host attributing to the defensive role against invading plant pathogens (1). The role of endophytes and their associations with the medicinal plant contributes significantly to the medicinal properties of the particular plant (2, 3). Endophytic microbes have also been isolated from diverse medicinal plants and have showed significant contributions to the treatment for many ailments by metabolite synthesis and the host biosynthesis (4, 5). According to the literature, the endophytic microbes protect their host from infectious agents and against any adverse conditions by secreting bioactive secondary metabolites (6, 7).

Since time immemorial medicinal plants have played an important role in the treatment of various diseases. *Pajanelia longifolia* (Willd.) K. Schuman, a

deciduous tree belonging to the family of Bignoniaceae, is considered as one of the important plant species among Western Ghat diversity in Karnataka due to its antibacterial and antioxidant properties. The pharmacological importance of this tree is reported in the treatment of skin disorders, the bark extract is used for treating *eczema* by local tribes in certain regions of Dakshina Kannada district in Karnataka. The scientific interventions have reported antioxidant, hepatoprotective and cytotoxic activity of the plant (8, 9). Many important metabolites from the *P. longifolia*, have been isolated and studied over a long time for medicinal properties such as antibacterial, antifungal, anti-tumour, immunosuppressant, anti-parasitic and antiviral, antioxidant and anti-inflammatory (10, 11).

The present study focus on isolation of endophytic bacteria and fungi from leaf and stem of *P. longifolia*. The isolated endophytes were examined for the colony morphology, microscopic staining and biochemical characterisations were also determined.

The Intracellular metabolites were further extracted to check their pharmacological properties such as antimicrobial, anti-proliferative and pro-apoptotic activities. The Isolates fungal strains were subjected to apoptosis induction as a measure of cell death due to cytotoxicity was analysed in the mutagen transformed cell model after treating with intracellular extracts from endophytic fungi. Furthermore the isolated bacterial endophytic strains showed prominent antibacterial activity against *Salmonella typhimurium* (ATCC 23564).

Materials and Methods

Plant collection

Pajanelia longifolia was collected from Honnahanakodu region of Karnataka (12°37'02.1"N 75°51'36.6"E). The healthy mature leaves and stem samples were collected and transferred to the laboratory in sterile bags.

Isolation of Endophytes from *Pajanelia longifolia*

The collected plant parts (as shown in Fig. 1) were washed, dried and cut into small segments measuring about 1 cm. The leaf and stem segments were initially surface sterilised using sterile distilled water, subsequently rinsed with 70% ethanol and 4% sodium hypochlorite. Successive washing were carried out thrice using sterilised distilled water and dried aseptically. Surface sterilised segments were placed on water agar media containing chloramphenicol (0.1 mg), Nutrient agar media and (Potato Dextrose Agar) PDA media. Each plate was placed with ten segments in equidistance, followed by setting standard bacterial and fungal growth conditions.

Identification of Endophytic Bacteria using Biochemical Characterisation

The colony morphology and staining characteristics were observed for each of the Endophytic isolates and documented. The isolates were subjected for

biochemical characterisations for IMViC tests, carbohydrate utilisation test, hydrogen sulphide test, catalase test and gelatin hydrolysis tests (12, 13).

Identification of Endophytic Fungi

Endophytic fungal cultures were examined for their characteristics such as growth pattern, size, the colouration of the mycelium and other colony morphological features. The morphological observation of the fungal isolates were observed by lactophenol cotton blue staining for spore arrangement and seen under Phase contrast Microscope (Lawrence and Mayo LM-52-3501) (14, 15).

The isolation rate (IR) was calculated using the formula: $IR = (Ni/Nt)$, Ni: the number of segments yielding the fungal isolates, Nt: the total number of segments incubated. The colonisation rate (%) was calculated using the formula: $CR = (Ni/Nt) \times 100$ (16).

Intracellular extraction from endophytic bacteria

To obtain bacterial biomass, each of the bacterial isolates was grown by inoculating onto Nutrient broth pH 7.0 and incubated at 37°C for 48 hours in a shaker incubator (ROTEK Incubator shaker ROSI-2). The bacterial biomass was collected and their intracellular fractions were extracted using phosphate buffer pH 7.2 by crushing the biomass in mortar pestle. The buffer extracted fractions were further centrifuged at 10000 rpm (Remi CRP 30 Plus VCEF-6571/450 LAL) and the culture-free extract was obtained. Protein content was estimated by Lowry's method using BSA as standard (17) where as the Carbohydrate was estimated by DNS method using glucose as standard (18).

Biomass extraction of endophytic fungal isolates

Endophytic fungal isolates were cultured in PDB medium for ten days at room temperature on a rotary shaker Remi (Model no. RIS-768) (19, 20). After incubation period the broth was filtered using Whatmann filter paper No. 1 the fungal biomass was then collected and homogenized using mortar and



Fig. 1. Leaf and stem of *Pajanelia longifolia* plant.

pestle in aseptic conditions in biosafety cabinet (ESCO AC2-4EB class II bio-safety cabinet) by adding equal amount of 0.1M phosphate buffer pH 7.2. The intracellular fractions were centrifuged using Remi CRP 30 Plus VCEF-6571/450 LAL at 4000 rpm for 10 min and the supernatant was collected. The culture free extract was obtained for further analysis.

Antibacterial activity of the intracellular extract of endophytic bacterial and fungal isolates

The obtained endophytic bacterial and fungal extracts were tested for growth inhibition against *Escherichia coli* (ATCC 8739), *Salmonella typhimurium* (ATCC 23564), *Klebsiella pneumoniae* (ATCC 9621), *Proteus vulgaris* (ATCC 13315) as performed by well diffusion method (21, 22).

Anti-proliferative activity of the endophytic fungal extract

For checking anti-proliferative activity, the intracellular fungal extracts were examined for anti-proliferative activity; were treated upon proliferative yeast cells. *Saccharomyces cerevisiae* were maintained as seed culture in Sabouraud's dextrose broth (SDB) medium and was incubated at 37 °C for 24 hrs. A standard volume of yeast cells with appropriate cell number from seed culture was transferred to SDB (Glucose 40g/L; Peptone 10g/L) containing phosphate buffer and incubated at 37 °C. The yeast cells were transformed into highly dividing cells by subjecting Ultra Violet irradiation mutagenesis and subsequent growth analysis at an interval of 2 hrs using spectrophotometer (Labman LMSP-UV1900) at wavelength of 670 nm (23).

Determination of Pro-apoptotic activity of the fungal extract

The UV-induced proliferative yeast cell system was treated with Endophytic fungal extract at a concentration of 0.01 mg/ml. The rates of cell divisions were analysed in control and treated groups. The proliferative yeast cells after treating with or without Endophytic fungal fractions were

stained with trypan blue dye. Cell viability counts were performed for the different time interval of incubation. The treated and untreated, proliferative yeast cells were subjected to staining with Giemsa to analyse morphological changes of a cell undergoing death (24). The cells were then fixed on to slide using methanol and glacial acetic acid solvent mixture; the slides were immersed in Giemsa staining solution for 30–40 min, subsequently, washed with distilled water, air-dried and observed under 40X objective using a phase contrast microscope (Lawrence and Mayo LM-52-3501) (25, 26).

Results

Isolation and Identification of endophytic bacteria using Biochemical Characterisation

A total of six endophytic bacterial isolates was isolated from *P. longifolia*, which have showed different colony characteristics as shown in Table 1. Furthermore, the isolated bacterial isolates were subjected to biochemical characterisation as shown in Table 2 with reference to identification mentioned in Bergey's Manual of Determinative Bacteriology, the endophytic bacterial isolates were placed under their taxonomical groups (27).

The endophytic bacteria PL 2(5), PS 1(8), and PS 2(6) were identified as gram-negative cocci and showed positive for Methyl red and positive for glucose, sucrose and lactose fermentation, these isolates were determined belonging to the genus *Enterococcus*. The gram-positive endophytic cocci, PL 1(3) showed positive Methyl red reaction, catalase test positive and all three carbohydrate utilization tests viz. Glucose, Lactose and Sucrose. The endophytic bacteria were identified as *Micrococcus* sp.

Morphological Identification of endophytic Fungi

Eleven fungal isolates have been isolated as shown in Fig. 2 and identified based on their colony and

Table 1. Morphological and staining characterisation of endophytic bacteria

Isolates	Colony characters							Staining methods			
	Shape	Colour	Puncti form	Raised	Convex	Flat	Globose	Septate formation	Simple	Wet mount	Gram staining
PL 2 (5)	Irregular	Cream	+	+	-	-	-	-	Cocci	Cocci	Gram negative
PL 1(3)	Irregular	Cream	-	+	-	-	+	+	Cocci	Cocci	Gram Positive
PS 1 (8)	Entire	Cream	-	+	-	-	-	-	Cocci	Cocci	Gram negative
PS 2 (5)	Entire	Whitish cream	-	+	+	+	+	+	Cocci	Cocci	Gram negative
PS 2 (6)	Irregular	Cream	+	-	-	+	+	-	Cocci	Cocci	Gram negative
PS 2 (8)	Entire	Whitish cream	-	-	-	-	-	-	Cocci	Cocci	Gram negative

Table 2. Biochemical properties of endophytic bacteria

Isolates	IMViC test			Carbohydrate			H ₂ S	Gelatin hydrolysis test	Catalase test
	Indole	MR-VP	Citrate utilisation	D	S	L			
PL 2 (5)	-	+/-	-	+	+	+	-	-	+
PL 1 (3)	-	+/-	-	+	+	+	+	-	+
PS 1 (8)	-	+/-	-	+	+	+	-	-	+
PS 2 (5)	-	+/-	-	-	+	+	+	-	-
PS 2 (6)	-	+/-	-	+	+	+	+	-	+
PS 2 (8)	-	+/-	-	-	+	+	-	-	+

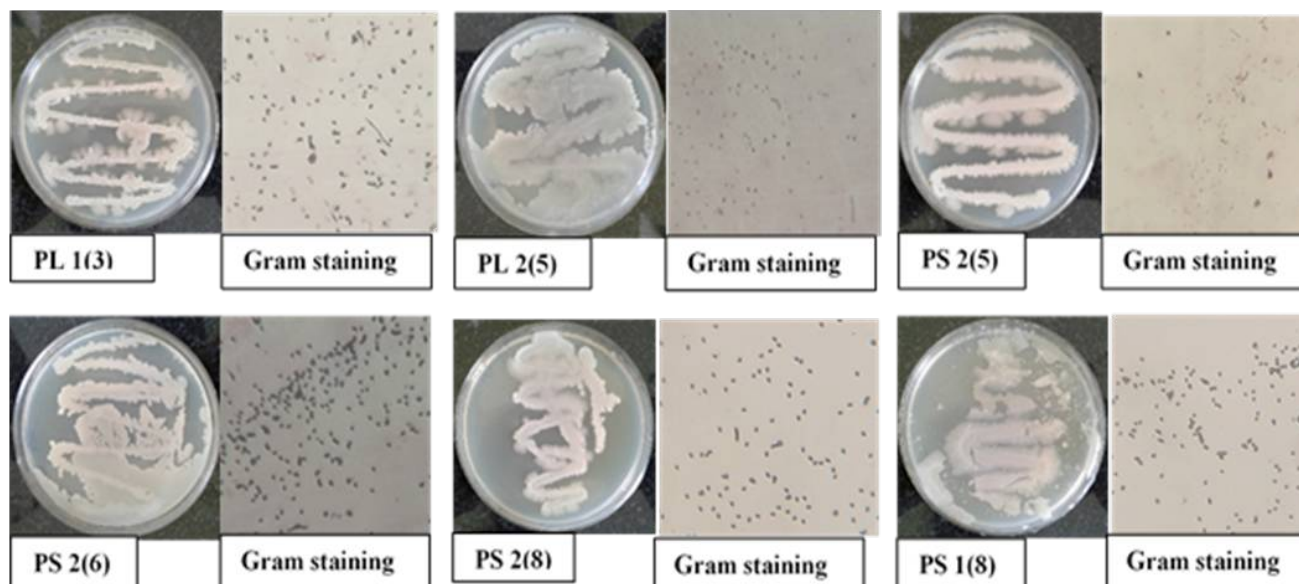


Fig. 2. Morphological and Microscopic view of bacterial endophytes.

microscopic observation with reference to fungi identification manual (28). For every number of segments placed on a growth medium, the isolation and colonisation rates were determined and has been depicted in Table 3.

The spore structure of the fungi identified as *Alternaria* sp. showed club-shaped septate singly formed spore, as shown in Fig. 4A. As shown in Fig. 4B, the conidia are arranged on phialides on the conidiophores and thus shows characteristics of the genus *Verticillium*. *Cladosporium* sp. and were identified from both the stem and leaf samples with simple conidia and dark spores (Fig. 4C, D, E and G). Fig. 4F identified as *Curvularia* sp. with concentric colony growth pattern producing curved spores with two to three transverse divisions. Other Endophytic morphospecies were identified from the plant.

Table 3. Isolation rate and colonisation rate of endophytic fungi

Plant part	Leaf	Stem
Number of segments incubated	100	100
Number of segments yielding endophytic fungi	4	7
Number of isolates	4	7
Isolation rate	0.04	0.07
Colonization rate (%)	4	7

Antibacterial activity of the intracellular extract of Endophytic bacteria

Intracellular extract from each of the endophytic bacterial isolates were tested for their antibacterial activity using well diffusion method allowing diffusion of the bacterial metabolite within the pre-inoculated plates of *E. coli*, *S. typhimurium*, *K. pneumoniae* and *P. vulgaris*. The plates were then observed for the zone of inhibitions. The isolated Endophytic bacterial metabolites showed no zone of inhibition *E. coli*, *S. typhimurium*, *K. pneumoniae* and *P. vulgaris*. As shown in Fig. 3, the intracellular extractions from *Enterococcus* sp. PS 1(8) showed significant inhibitions against *Salmonella* sp. Also, the intracellular extractions from PL 2(5) and *Enterococcus* PS 2(6) *Micrococcus* sp. PL 1(3) endophytic bacteria showed inhibitory activity against *Salmonella* sp.

Antibacterial activity of the intracellular extract of Endophytic Fungal extract

The species belonging to *Cladosporium* sp. was predominantly present in *P. longifolia* and have showed inhibitory effect against *P. vulgaris* and *K. pneumoniae* as illustrated in Table 4. Moreover, *Curvularia* sp. extract showed antibacterial activity against all the selected test pathogens viz., *P. vulgaris*, *E. coli*, *K. pneumoniae* and *S. typhimurium*. The species of *Verticillium* extract inhibited the growth of *S. typhimurium* and *E. coli*.

Anti-proliferative activity of the intracellular extract of endophytic fungal

Highly proliferative yeast cell (UV treated) when incubated along with intracellular extract of endophytic fungus, *Cladosporium* sp. and *Fusarium* sp. induced cytotoxic anti-proliferative effect out of eleven isolates as shown in Fig. 5.

Due to the influence of fungal extract the morphological change occurred in the cell has been noted down at different interval of time depicting the initiation of the cell death within the highly proliferative yeast cell system. Furthermore, the cell counting was carried by the trypan blue dye exclusion method and it was clear that after 6 to 8 hr incubation, the extracts induced a cytotoxic effect against the proliferative yeast cells.

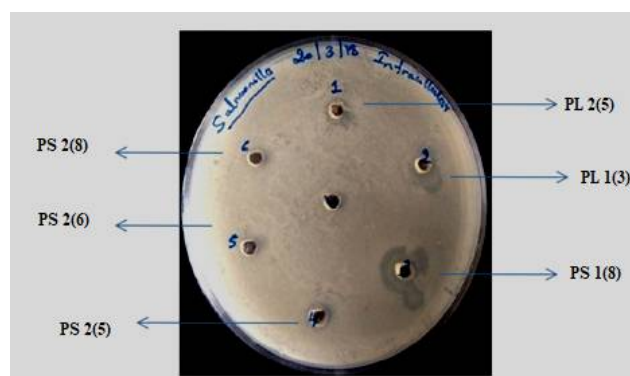


Fig. 3. Intracellular metabolites from endophytic bacteria showing growth inhibition against *Salmonella typhimurium* (ATCC 23564).

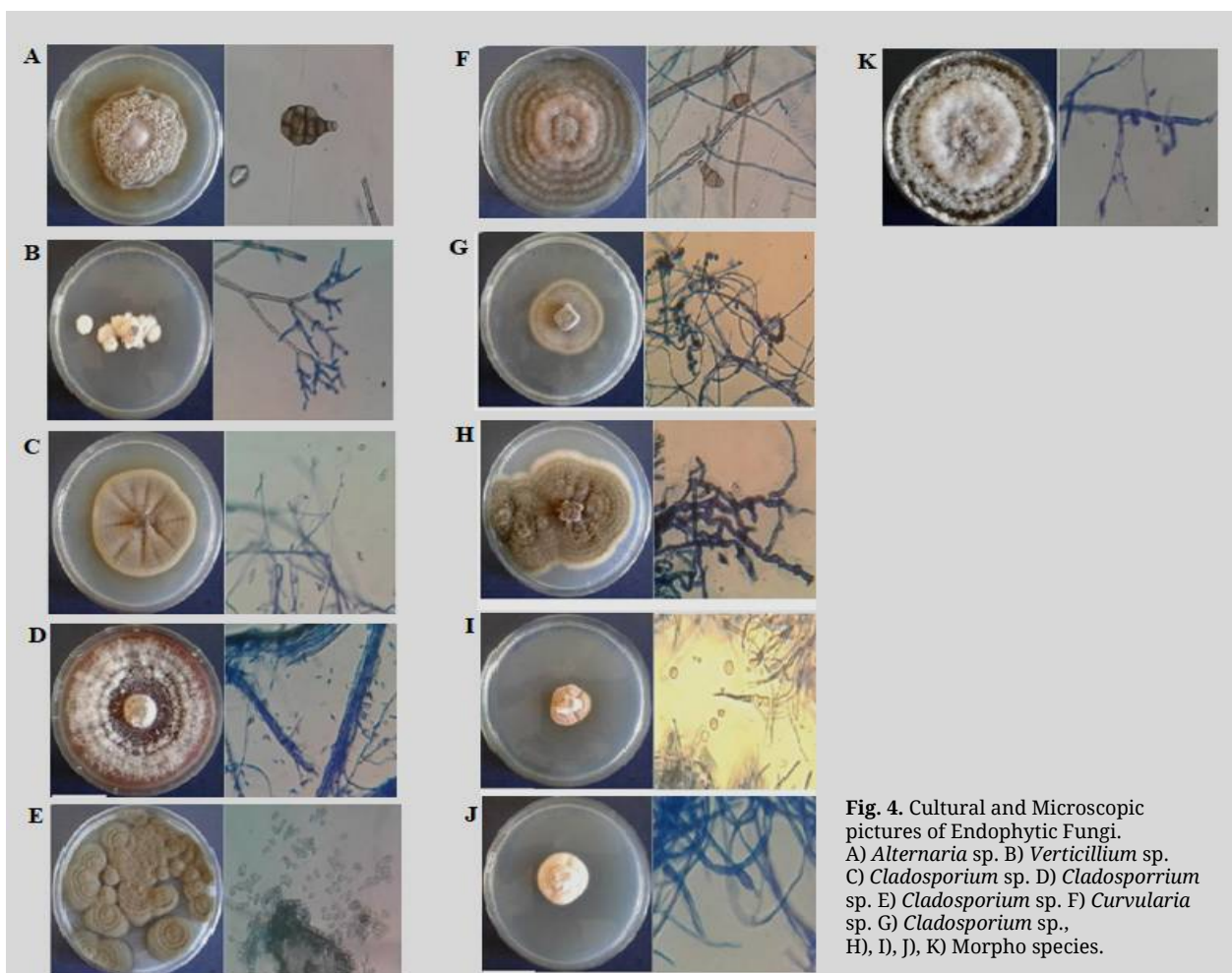


Fig. 4. Cultural and Microscopic pictures of Endophytic Fungi. A) *Alternaria* sp. B) *Verticillium* sp. C) *Cladosporium* sp. D) *Cladosporium* sp. E) *Cladosporium* sp. F) *Curvularia* sp. G) *Cladosporium* sp., H), I), J), K) Morpho species.

Pro-apoptotic effect of intracellular endophytic fungal extract

The intracellular fungal extract of *Cladosporium* sp. and *Fusarium* sp. showed pro-apoptotic effect (Fig. 6). The fungal extract treated yeast cells were stained with Giemsa stain the Yeast cell showed the characteristic morphology of cells undergoing cell death. The characteristic features of chromatin condensation, membrane blebbing and apoptotic bodies are the changes that occur during programmed cell death when treated with the fungal extract.

These results of anti-proliferative and Pro-apoptotic induction activity from *P. longifolia* fungal endophytes provide substantial information for their

detailed analysis of various cancer cell lines and their possible mechanism involved in pharmacological and therapeutic applications.

Discussion

In the present research we have successfully isolated different bacterial as well as fungal species from *P. longifolia*. The intracellular extract of the isolated endophytic strains showed inhibitory activity against pathogenic bacterial strains and have further induced apoptosis in UV mutated yeast cells. The endophytic bacteria belonging to *Enterococci* and *Micrococci* species were isolated from the *P. longifolia* plant that showed effective antibacterial activity against the *K. pneumoniae*, *S.*

Table 4. Antimicrobial activity of the endophytic fungal extracts against bacterial pathogen

Endophytic fungal isolates	Antibacterial Activity			
	<i>E. coli</i>	<i>Proteus</i> sp.	<i>Salmonella</i> sp.	<i>Klebsiella</i> sp.
<i>Alternaria</i> sp.	-	+	-	-
<i>Cladosporium</i> sp.	-	+	-	+
<i>Cladosporium</i> sp.	-	+	-	-
<i>Cladosporium</i> sp.	-	+	-	-
<i>Curvularia</i> sp.	+	+	+	+
<i>Fusarium</i> sp.	-	-	-	+
<i>Verticillium</i> sp.	+	-	+	-
Morpho species 1	-	+	-	+
Morpho species 2	-	+	-	+
Morpho species 3	+	-	+	-
Morpho species 4	+	+	+	+

*(-) Negative, (+) Positive

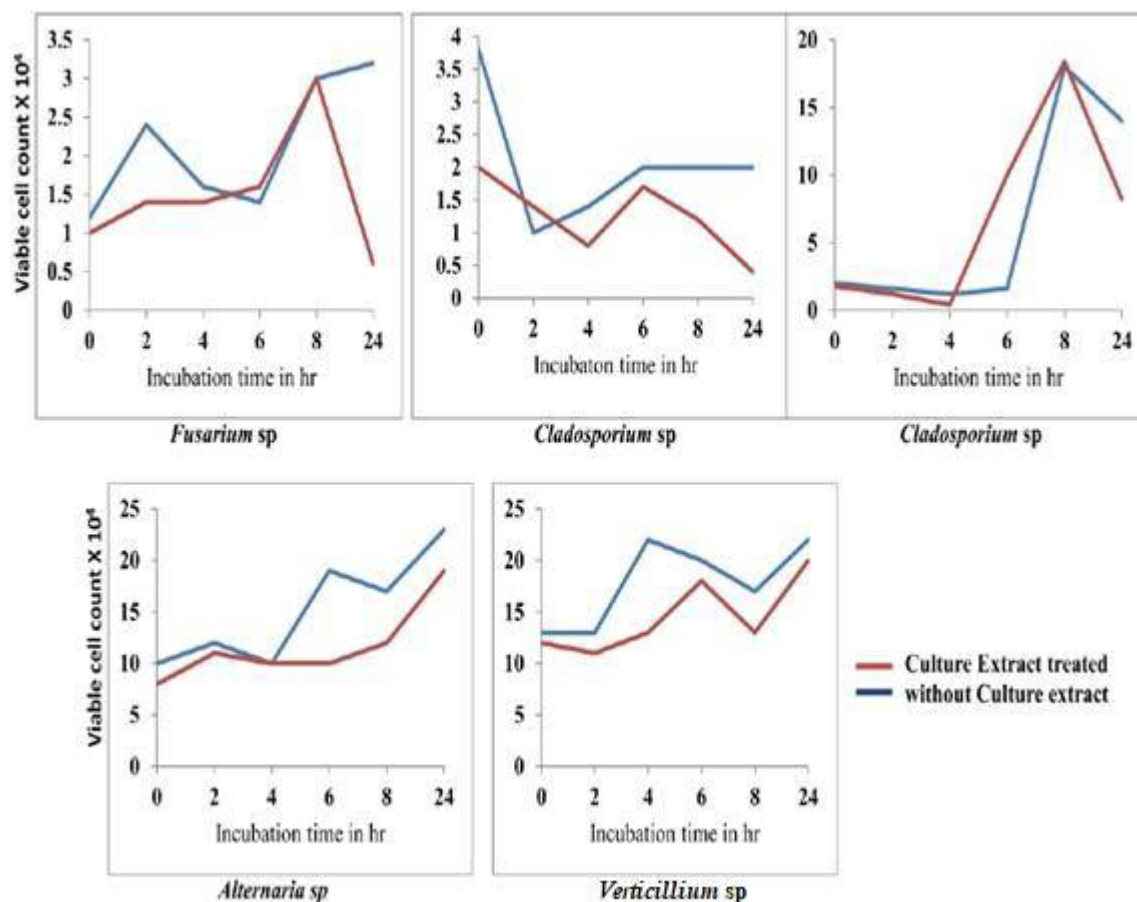


Fig. 5. Anti-proliferative effect of the endophytic fungal extracts on UV induced proliferating yeast cell line.

typhimurium and *E. coli* bacterial pathogens. This was achieved by performing well diffusion method. Similar reports have showed antibacterial activity against *S. typhi*, *S. flexneri*, *S. typhi* and *S. marcescens*, *Klebsiella sp.*, *E. coli*, *Staphylococcus aureus* and *Streptococcus sp.* by solvent extracts of leaves of *P. longifolia* (29, 30). The present study substantiate, significant metabolite contribution from the endophytic bacterial and fungal strain may play a major role thus complementing along with the plant extracts for pharmacological efficacy (31). Furthermore, the endophytic fungi belonging to the Ascomycota division such as *Alternaria sp.*, *Fusarium sp.*, *Cladosporium sp.*, *Verticillium sp.*, *Curvularia sp.* and morpho species were identified from the plant. The genera, *Cladosporium* was predominantly isolated compared to other fungal species thus illustrating that it is one of the prominent fungal endophytes present in the plant (32). *Curvularia sp.* have exhibited antibacterial activity against *S. typhimurium*, *Klebsiella pneumoniae* and *E. coli*, *Proteus vulgaris*.

Also, the intracellular extract obtained from each of the fungal isolates were analysed for their ability to induce apoptotic cell cytotoxicity in the UV mutated Yeast cell lines. Evidently, the endophytic fungi *Cladosporium sp.* and *Fusarium sp.* could demonstrate anti-proliferative activity and was able to induce apoptotic cell death. According to literature, fungal extracts are well capable of inducing apoptosis in tumor cells,

staining the cells for morphological observations treated with the intracellular extracts in the treatment groups showed apoptotic morphological features such as membrane blebbing, apoptotic bodies and cell disintegrations (33). The above morphology was not observed in the control groups that were not incorporated with any of the intracellular endophytic fungal extracts. Thus, the present work gives future insight information of the medicinal properties of endophytic bacterial as well as fungal strain whose medicinal properties could further be explored in near future.

Conclusion

The present study revealed that *Pajanelia longifolia* (Willd.) K. Schuman harboured a number of endophytic fungi and bacteria. Furthermore, intracellular extractions from both bacterial as well as fungal endophytes exhibited antibacterial activity. The isolated endophytic fungal species belonging to *Cladosporium* and their occurrences in the plant samples attributed to effective anti-proliferative and pro-apoptotic activity in the UV induced yeast cell model. The endophytic bacteria belonging to *Enterococcus* and *Micrococcus* genera showed significant antimicrobial activity against *Salmonella typhimurium* (ATCC 23564). These unexplored endophytes from *P. longifolia*, thus offer a valuable source for identifying intracellular metabolites for pharmacological and therapeutic applications in near future.

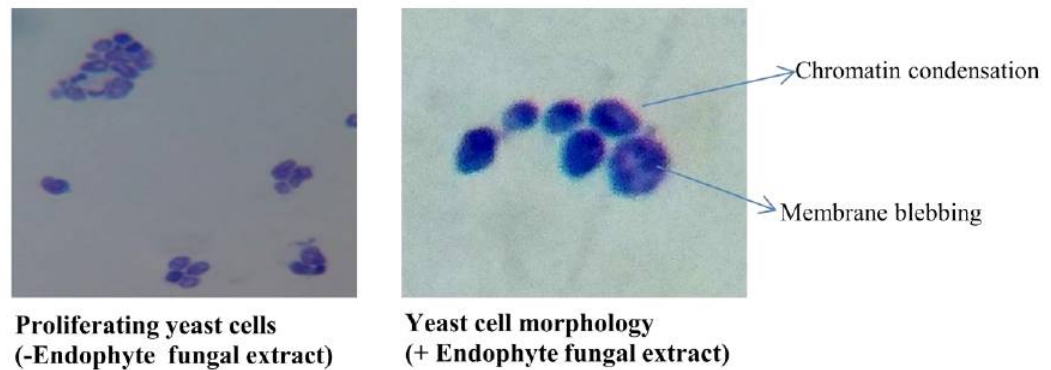


Fig. 6. Chromatin condensation and membrane blebbing in yeast cell line.

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Authors' contributions

GA carried out all the experiments including collection of the plant sample. SD drafted the research article. YK assisted in performing anti-proliferative activity and IKM hypothesized the concept of the research work and guided in writing the manuscript.

Conflict of interests

The authors declare that there is no conflict of interest.

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