Antimicrobial and phytochemical properties of endophytic fungi associated with *Aloe vera* L.

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Medicinal plant species are making their way from the margins to the mainstream, with an increasing number of people turning to natural products for treatments and potential health solutions. Aloe vera is a well-known traditional or ancient plant for its antimicrobial and therapeutic properties. Because of its antibacterial properties, it has a long history of use as a treatment for skin disorders in both traditional and modern medicine. The pharmaceutical properties of this plant are attributed to its endophytes. A total of twenty-seven endophytic fungi were isolated from the leaves and roots of *A.vera* L. in various regions of Haridwar district of Uttarakhand state. All fungal isolates were recovered and tested for preliminary antibacterial activity against four human test pathogens namely *Escherichia coli* (MTCC 77), *Pseudomonas aeruginosa* (MTCC 3163), *Staphylococcus aureus* (MTCC 96), and *Bacillus cereus* (MTCC 430). Only four isolated endophytic fungi were found to be effective in inhibiting all test pathogens. The phytochemical analysis of fungal crude extracts revealed the presence of constituents such as alkaloids, flavonoids, phenols, tannins, saponins, steroids, terpenoids, and C-glycosides in these four endophytic fungal isolates. According to this finding, *A.vera* L. endophytic fungi were found to produce antibacterial agents. The data generated has provided the foundation for its use in the pharmaceutical industry in the form of traditional and folk medicine.

Keywords: Endophytic fungi, *Aloe vera* L., Antibacterial activity, Phytochemical analysis

Introduction

Plants are natural sources of useful substances that can aid in the development of new medications. As a result, the antioxidant, antibacterial, wound healing, antiulcer, anti-inflammatory, and analgesic effects of plants have been intensively researched [1]. Cultivating the plants and extracting their metabolite takes time, as a result, exploration of

endophytes especially endophytic fungi gathered from medicinal plants are capable of producing plant-specific chemicals are gaining attention. Interaction between the endophytes and host plant leads to the establishment of symbiotic to mutualistic relationship, which helps to transfer metabolite from endophytes to plants and vice versa. Endophytes are the microorganisms especially bacteria and fungi that live within plant tissues without causing any visible symptoms to the plant [7]. They have evolved to spend all or part of their lives in plant tissues. The symbiotic interaction between fungal endophytes and plants helps both parties; host plants provide essential nutrients to the endophytes. Furthermore, fungal endophytes help host plants by limiting the invasion of plant diseases and/or parasites and boosting host plant resistance and tolerance to important biotic or abiotic stimuli [2]. Endophytes, like the host plant, generate bioactive chemicals such as alkaloids, coumarins, flavonoids, glycosides, lignans, terpenoids, phenylpropanoids, saponins, quinones, and xanthones [6]. These metabolites can defend their hosts against biological agents and environmental circumstances while also producing pharmacologically active molecules [20]. Fungal endophytes, in particular, are promising sources of new physiologically active chemicals with significant medical implications [3,18,6]. Paclitaxel, for example, synthesised by an endophytic Taxomyces andreanae from the Pacific Yew, has motivated the search for endophytes that create the plant-specific metabolite taxol [17]. It has been shown that fungal endophytes have potential to alternate chemical biosynthesis [4,14].

The Liliaceae family includes the succulent plant *Aloe vera* (*Aloe barbadensis* Miller). It features elongated, pointed leaves in a whorl [10]. The name is derived from the Arabic word 'alloeh,' which means 'bitter,' and relates to the bitter taste of the liquid contained within the leaves. It is a medical plant that has antioxidant, anticancer, antiulcer, wound healing, and skin protective characteristics [9,15]. The pharmacologically significant chemicals are located in the parenchymatous tissue of the plant's fleshy parts [15]. In the present study, we carried out the evaluation of the bioactivities of an endophytic fungus isolated from the medicinal plant *A. vera* collected from Haridwar district of Uttarakhand state. Moreover, the results affirm that this endophytic fungus has valuable therapeutic applications.

Materials and methods

Collection of plant samples

Healthy plants of *A.vera* were collected from Haridwar district of Uttarakhand state. Four plants were collected from various locations. Samples were packed in ziplock bags, transported to the laboratory within 30 minutes, kept at 4°C, and processed within 24 hours.

Isolation and identification of endophytic fungi

Prior to surface sterilisation, plants were thoroughly rinsed with running tap water to remove surface particulates, they were then surface sterilised [5], which comprised dipping them in 70% ethyl alcohol for 10 seconds, soaking them in 4% sodium hypochlorite for 30 seconds, and rinsing them twice in sterile distilled water. It was crucial to establish that the fungal isolates from the plant *Aloe vera* L. were actually endophytes rather than epiphytes. No microbial growth was seen on control agar plates after 30 days of culture, showing that all epiphytic microorganisms had been eliminated. The surface sterilized leaf and root segments were placed on potato dextrose agar with 0.5 g/L streptomycin. The plates were incubated at 27±2℃ for 1-2 weeks and monitored daily till hyphal growth. Endophytic fungi isolated were identified by morphological features such as fungal growth topography, colony color, spore generation, growth rate, and colony margin using the lactophenol cotton blue staining method. The isolated endophytic fungi were examined under a 40X microscope and identified using the scotch tape method [11].

Purification and preservation of endophytic fungi

Following incubation, different types of fungal strains were appeared from each sample, individual strains were isolated by placing hyphal tips onto fresh potato dextrose agar medium. This process was repeated several times until a pure endophytic fungal strain with a homogenous colony was obtained. Separately, pure endophytic fungal isolates were grown on potato dextrose agar slants in a 15% (v/v) sterilised glycerol solution. Endophytic fungal strains were observed to develop in both mediums for 3-5 days. Glycerol stock solution and potato dextrose agar slants were stored at -20°C and 4°C, respectively, until needed.

Preliminary antibacterial screening

The agar disc diffusion method was used to test antibacterial activity against test pathogens namely *Escherichia coli* (MTCC 77), *Pseudomonas aeruginosa* (MTCC 3163), *Staphylococcus aureus* (MTCC 96), and *Bacillus cereus* (MTCC 430). In potato dextrose agar medium, cylindrical sections of a well-grown culture of the endophytic fungus strain were cut off. The blocks were placed on petridishes after being deep injected with a predetermined amount of test bacteria grown in nutrient broth medium for bacteria

(10^6cells/ml). After 12 hours of antibacterial agent diffusion, the cultures were cultured for 24 hours at 37°C for bacterial test microorganism development and 48 hours at 28°C for fungus growth [19].

Preparation of crude extract

Agar plugs of endophytic fungus (6mm in diameter) were inoculated into 250 ml flasks containing 100 ml of PDB (potato dextrose broth) to cultivate endophytic fungi. Each flask was incubated at room temperature for 21 days under static conditions. The culture broth and mycelia were separated by filtration after incubation; the filtered broth was then extracted three times with an equal volume of ethyl acetate (EtOAc) [12]. The crude extracts were dried and kept in preparation for antibacterial and phytochemical examination of isolated endophytic fungus.

Determination of antibacterial activity

The antibacterial activity against test pathogens, *Escherichia coli* (MTCC 77), *Pseudomonas aeruginosa* (MTCC 3163), *Staphylococcus aureus* (MTCC 96), and *Bacillus cereus* (MTCC 430) was assessed using the agar well diffusion method. The isolated chemicals from suspected endophytic fungi were dissolved in DMSO and placed into 6mm diameter wells bored into petridishes containing Muller Hinton Agar (MHA) for test bacteria, which had been pre-inoculated with a determined number of test-microorganisms (10^6cells/ml). The cultures were held at 2-8°C for 24 hours to allow antimicrobial metabolite diffusion before being incubated at 352°C for test-microorganism growth. The inhibitory zone was measured in millimetres [19].

Qualitative screening of phytochemicals

The extracts of endophytic fungi of *A.vera* were subjected to qualitative chemical analysis of constituents viz., alkaloids, flavonoids, phenols, saponins, tannins, steroids and cardiac glycosides following Trease and Evans (1983).

Test for alkaloids

Dragendroff's reagent test: The presence of alkaloids were determined by Dragendroff's reagent test. Few ml of filtrate was taken in a test tube and 1-2 ml of Dragendroff's reagent was added. Observe the formation of an orange colour solution.

Test for flavonoids

Alkaline reagent test: 5 ml of dilute ammonia solution was added to the portion of extract, followed by the addition of a few drops of concentrated sulphuric acid. Appearance of yellow color confirmed the presence of flavonoids.

Test for phenols

Few ml of extract was taken in a test tube, little amount of H_2O was added to it, then few drops of $FeCl_3$ was added, green color showed the presence of phenols.

Test for saponins

Froth test: The presence of saponins was determined by a frothing test. The extract was vigorously shaken with distilled water and was allowed to stand for 5 min. Formation of a fairly stable emulsion indicated the presence of saponins.

Test for tannins

Ferric chloride test: few ml of extract was taken in a test tube, few drops of 5% ferric chloride was added to the test tube, colour changed to dark green confirmed the presence of condensed tannins and if the colour changed to bluish black color confirmed the presence of hydrolysable tannins.

Test for steroids

Salkowski test: few ml of extract was taken in a test tube, few drops of concentrated sulphuric acid was added to it, red colour at the lower layer confirmed the presence of steroids, and yellow colour at the lower layer showed the presence of terpenoids.

Test for Cardiac glycosides

Few ml of filtrate was added with 1.5 ml of glacial acetic acid along with a drop of 5% $FeCl_3$ in a test tube, concentrated H_2SO_4 was added along the side of test tube, appearance of blue color indicated the presence of C-glycosides.

Statistical analysis

All the experiments were carried out in triplicate. Data were recorded as the average of three independent readings for each of the experiments. The results were subjected to standard error.

RESULT and DISCUSSION

Isolation and Identification of endophytic fungi

In this study, fungal endophytes from *A. vera* L. were isolated using potato dextrose agar medium and surface sterilisation. A total of 27 endophytic fungi of diverse genus and species were isolated from healthy plant leaves and roots. The scotch tape method and lactophenol cotton blue staining were used to characterise these endophytic fungus morphologically and reproductively (Table 1). *Bipolaris* sp., *Aspergillus* sp., *Penicillium* sp., *Chaetomium* sp., *Cladosporium* sp., and *Fusarium* sp. were identified as the isolates. Six strains lacked reproductive features and were labelled as sterlia mycelia, while nine strains remained undetermined.

Table 1: No. of endophytic fungi isolated

S.NO.	Isolates	Source	Media used	Morphological characteristics	Identified fungal isolates
1.	AVL1	Leaf	PDA/WA/RBA	Velvety, at first white than turning pink, reverse white	Sterlia mycelia
2.	AVL2	Leaf	PDA/WA/RBA	Velvety to woolly, white to grayish brown, conidia are 3-6 celled, fusoid to cylindrical, light to dark brown	*Bipolaris* sp.
3.	AVL3	Leaf	PDA/WA/RBA	Velvety or powdery, at first white than turning greenish to gray with a narrow white border, reverse white to tan	*Aspergillus* sp.
4.	AVL4	Leaf	PDA/WA/RBA	Velvety, white to pink in colour reverse white	_
5.	AVL5	Leaf	PDA/WA/RBA	Velvety to powdery, green, white, slight yellow, reverse white to yellowish	*Penicillium* sp.
6.	AVL6	Leaf	PDA/WA/RBA	Powdery, white to yellow	_
7.	AVL7	Leaf	PDA/WA/RBA	Woolly, white to light pink in colour	Sterlia mycelia

8.	AVR1	Root	PDA/WA/RBA	Powdery, white to green in colour	_
9.	AVR2	Root	PDA/WA/RBA	Cottony, at first white than turning olive green in colour	*Chaetomium* sp.
10.	AVL8	Leaf	PDA/WA/RBA	Powdery, white to light yellow in colour	*Aspergillus* sp.
11.	AVL9	Leaf	PDA/WA/RBA	Powdery, white to yellow	Sterlia mycelia
12.	AVL10	Leaf	PDA/WA/RBA	Velvety to powdery, white than turning gray/ brown with white periphery, conidia brown in colour	*Bipolaris* sp.
13.	AVL11	Leaf	PDA/WA/RBA	Velvety, white to greenish in colour	_
14.	AVL12	Leaf	PDA/WA/RBA	Cottony to powdery, white to yellow than turning brown	_
15.	AVL13	Leaf	PDA/WA/RBA	Woolly, white to yellow	_
16.	AVL14	Leaf	PDA/WA/RBA	Velvety to woolly, white to green colour	_

17.	AVL15	Leaf	PDA/WA/RBA	Woolly, at first white to yellow than turning black, reverse white to yellow	Sterlia mycelia
18.	AVL16	Leaf	PDA/WA/RBA	Usually velvety, cinnamon brown/ brown, reverse white to brown	_
19.	AVR3	Root	PDA/WA/RBA	Texture velvety to powdery, gray green, reverse white to yellow	*Penicillium* sp.
20.	AVR4	Root	PDA/WA/RBA	Velvety to woolly, surface of the colony is initially white of grayish brown	Sterlia mycelia
21.	AVR5	Root	PDA/WA/RBA	Velvety, at first white than turning dark greenish to gray with a narrow white border, reverse white to tan	*Aspergillus* sp.
22.	AVL17	Leaf	PDA/WA/RBA	Velvety, brown colour radial colonies	_
23.	AVL18	Leaf	PDA/WA/RBA	Cottony pink to mauve colour colony	_

24.	AVL19	Leaf	PDA/WA/RBA	Velvety green colour colonies	*Cladosporium* sp.
25.	AVL20	Leaf	PDA/WA/RBA	Velvety white colour colonies	*Penicillium* sp.
26.	AVR6	Root	PDA/WA/RBA	Cottony white colour colony	Sterlia mycelia
27.	AVR7	Root	PDA/WA/RBA	Cottony pink to mauve colour colony	*Fusarium* sp.

Preliminary antibacterial screening

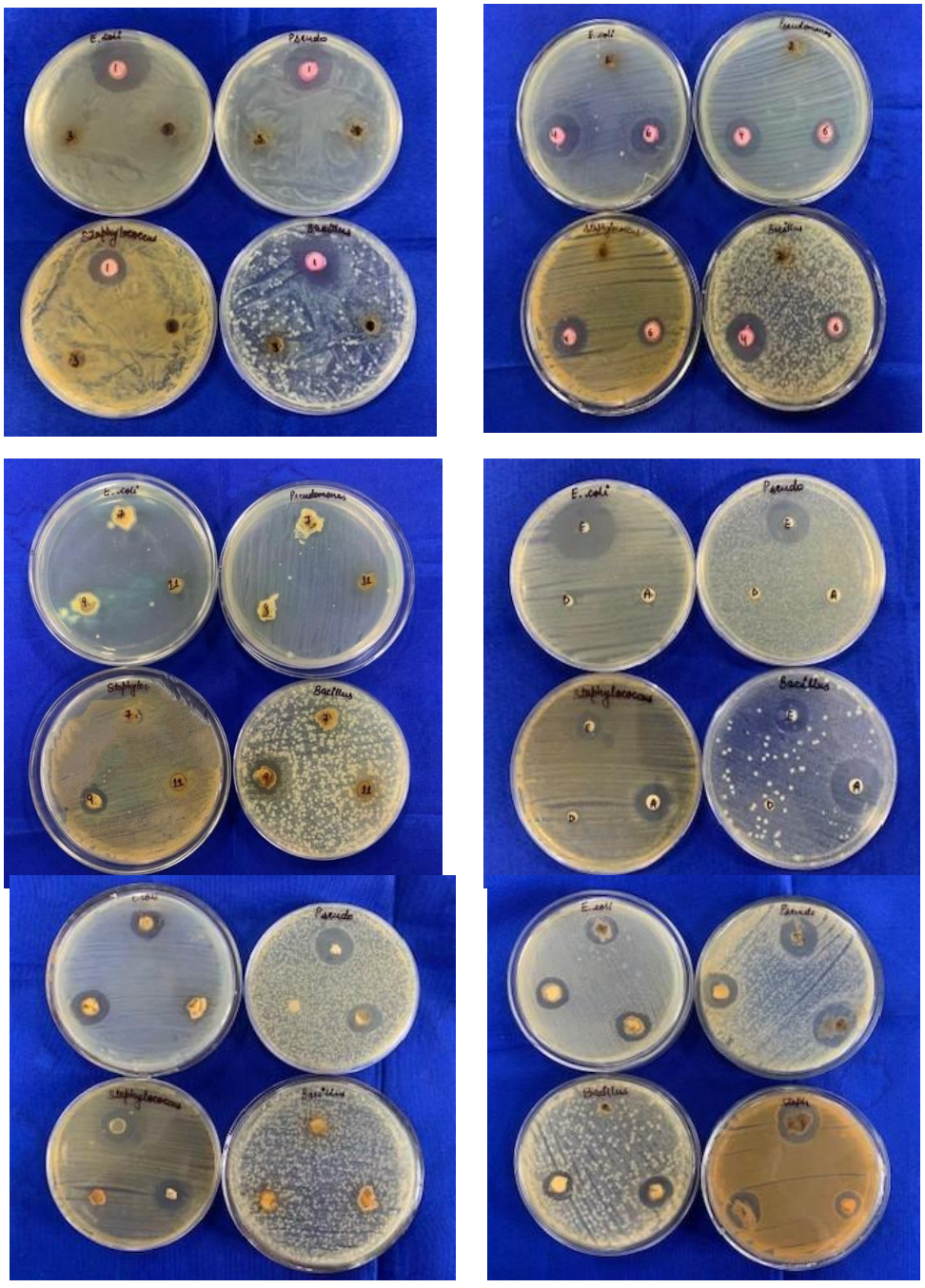
From the leaves and roots of a healthy *A. vera* plant, 27 endophytic fungi were isolated and then exposed to an initial antibacterial screening using the agar disc diffusion method on solid media. Only 19 of the 27 isolates tested showed preliminary inhibition against one or more test pathogens, while the remaining endophytic fungi isolates showed no antibacterial activity against any of the test pathogens.

Table 2: Preliminary antibacterial activity of endophytic fungi isolated from *A.vera* by Agar disc diffusion method

S. NO.	Endophytic fungal Isolate	Zone of inhibition by indicator bacteria (mm)			
		Staphylococcus aureus (MTCC 96)	*Bacillus cereus* (MTCC 430)	*Escherichia coli* (MTCC 77)	*Pseudomonas aeruginosa* (MTCC 3163)
1.	AVL1	11.33	14.66	21	18
2.	AVL4	13	18.66	16.66	16
3.	AVL6	10	10	13	9
4.	AVL9	7	11	-	-
5.	AVL10	6	9	20.33	18
6.	AVL12	10	16	3	5
7.	AVL13	10	-	9	13.33
8.	AVL14	-	-	12	-
9.	AVL15	7	-	-	9
10.	AVL16	12.66	-	9	12
11.	AVL17	9	6	9	10

12.	AVL18	6	9	10	12.66
13.	AVL19	-	11	12.66	10
14.	AVL20	12	-	-	-
15.	AVR3	-	-	-	2
16.	AVR4	11	-	11	10
17.	AVR5	-	3	8.33	-
18.	AVR6	7	-	19	8
19.	AVR7	13.33	9	16	12

*** Values are means of three replicates ; (-) no inhibition; AVL1-AVL20 – Aloe vera leaf, AVR1-AVR7 – Aloe vera root**



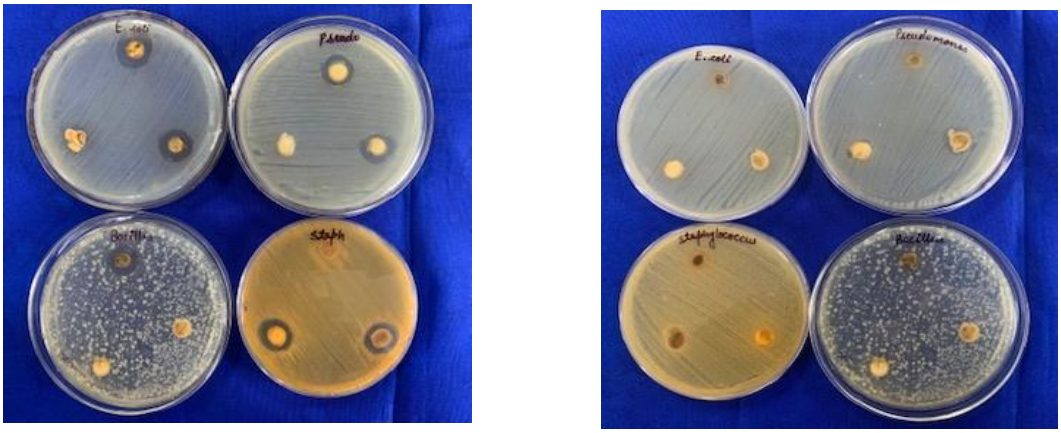


Figure 1: Antibacterial activity of fungal leaf and root isolates of AVL1-AVL20 and AVR1-AVR7 against *Staphylococcus aureus* (MTCC 96), *Bacillus cereus* (MTCC 430), *Escherichia coli* (MTCC 77), *Pseudomonas aeruginosa* (MTCC 3163).

Determination of antibacterial activity

The antibacterial activity was tested against two gram positive and two gram negative bacteria by agar well diffusion method. Isolates AVL1, AVL4, AVL18 and AVR7 shows inhibition against all the test pathogens, which exhibited their broad spectrum antibacterial activity, while .isolates AVL6, AVL10, AVL12 and AVL17 shows inhibition against one or more bacteria (Table 3).

Table 3: Antibacterial activity of endophytic fungi isolated from *A.vera*

S.NO.	Endophytic fungal Isolate	Zone of inhibition by indicator bacteria (mm)			
	Endophytic fungi	*Staphylococcus aureus* (MTCC 96)	*Bacillus cereus* (MTCC 430)	*Escherichia coli* (MTCC 77)	*Pseudomonas Aeruginosa* (MTCC 3163)
1.	AVL1	12±0.0	15±0.30	17±0.30	13±0.30
2.	AVL4	15±0.60	15±0.60	12±0.60	13±0.60
3.	AVL6	12±0.30	-	6±0.0	4±0.30
4.	AVL10	-	-	18±0.70	15±0.0
5.	AVL12	11±0.30	13±0.0	-	-
6.	AVL17	-	-	-	6±0.0
7.	AVL18	10±0.30	8±0.30	13±0.0	12±0.60
8.	AVR7	12±0.0	10±0.33	12±0.30	12±0.30
9.	*Ampicillin	14±0.30	13±0.0	9±0.0	8±0.0
1O.	*DMSO	-	-	-	-

***Ampicillin was used as positive control and DMSO was used as negative control**

*** Values are the mean of three replicates; (±) standard error; (-) No zone of inhibition, (AVL1, AVL4, AVL6, AVL10, AVL12, AVL17, AVL18- *Aloe vera* leaf and AVR7- *Aloe vera* root)**

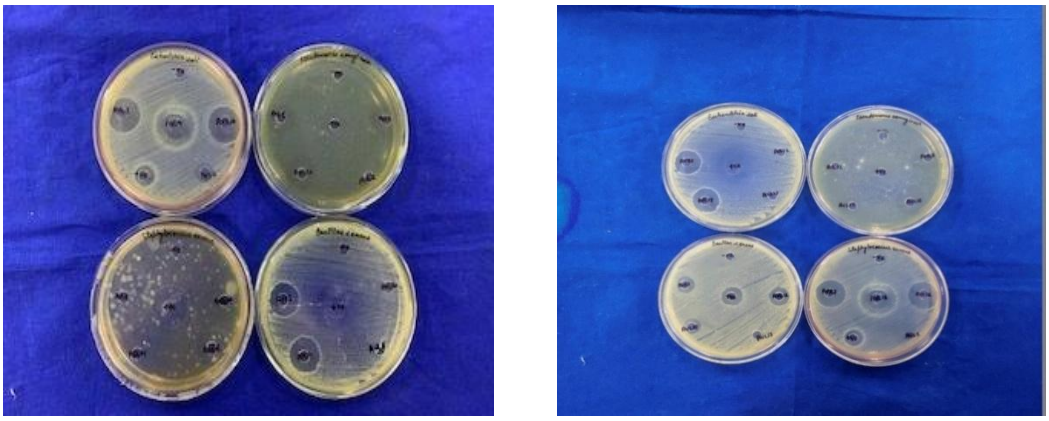


Figure 3: Antibacterial activity of crude extract of endophytic fungi against *Staphylococcus aureus* (MTCC 96), *Bacillus cereus* (MTCC 430), *Escherichia coli* (MTCC 77), *Pseudomonas aeruginosa* (MTCC 3163) of AVL1, AVL4, AVL6, AVL10, AVL12, AVL17, AVL18, AVR7 with positive and negative control respectively.

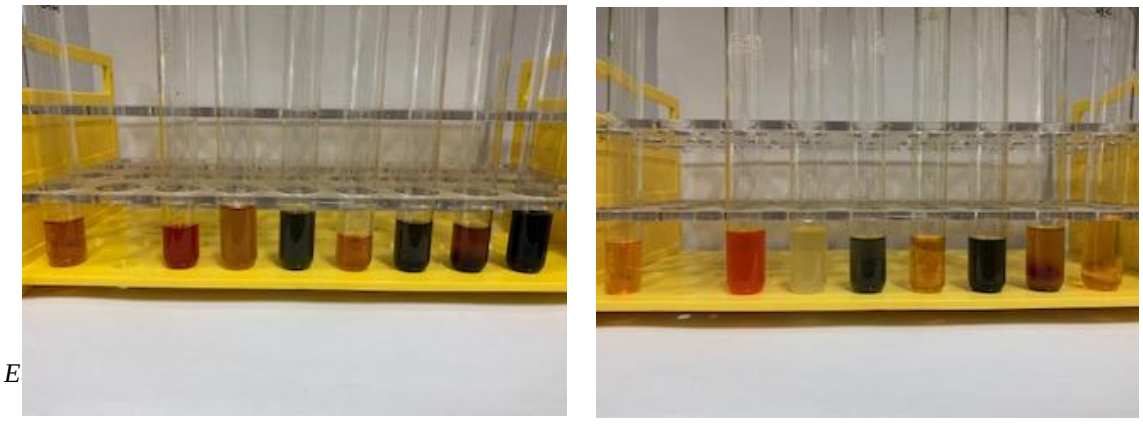
Qualitative screening of phytochemicals

In crude extracts of endophytic fungi isolated from *A.vera* leaves and roots, the presence of different fungal metabolites, phenolic compounds, alkaloids, flavonoids, saponins, tannins, steroids, and cardiac glycosides was identified. (Table 4). It displays the potential of endophytic fungus to offer antibacterial action. Endophytic fungi associated with *A.vera* may be a valuable source of bioactive chemicals for the development of more effective antibacterial medicines with a high therapeutic index value.

Table 4: Phytochemical analysis of endophytic fungi isolated from *A.vera*

S. NO.	Name of the Fungal isolate	Alkaloids	Flavonoids	Phenols	Tannins	Saponins	Steroids/ Terpenoids	C-glycosides
1.	AVL1	+	+	+	+	-	+	+
2.	AVL4	+	+	-	+	-	+	-
3.	AVL18	+	+	+	+	+	+	+
4.	AVR7	+	+	+	+	-	-	-

***(+) indicates the presence, (-) indicates the absence, (AVL1, AVL4, AVL18- *Aloe vera* leaf and AVR7- Aloe vera root)**



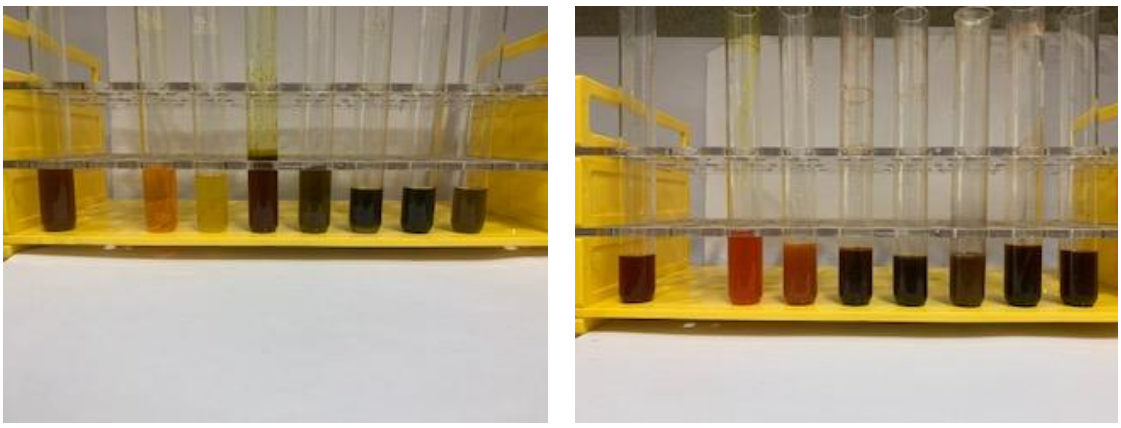


Figure 4: Phytochemical screening to test the presence or absence of Alkaloids, Flavonoids, Phenols, Saponin, Tannins, Steroids and C-glycosides respectively with control of selected endophytic fungi AVL1, AVL4, AVL18 and AVR7.

Discussion

Medicinal herbs have long been utilised to treat a range of ailments in India. Endophytes thrive in the distinct habitat created by medicinal plants. *A.vera* is an antibacterial, antioxidant, anti-diabetic, and anti-inflammatory traditional Indian medicinal herb [13]. Endophytes derived from medicinal plants are particularly essential because they imitate the host metabolic pathway for bioactive metabolite synthesis and protect plants from infections. Resistance to conventional antibiotics is rising, necessitating the development of new antimicrobial medicines. Although *A.vera* has been used to treat a range of diseases in traditional formulations, we identified endophytic fungi from healthy plant leaves and roots. In this work, all of the isolated endophytic fungi from *A.vera* were characterised for antibacterial and phytochemical investigation.

Antimicrobial activity of endophytic fungi was investigated. Crude extracts of AVL1, AVL4, AVL18, and AVR7 revealed an optimistic zone of inhibition against all of the indicator bacteria among all of the identified endophytic fungi. Many earlier studies were congruent with our findings. The phytochemical examination of crude extracts of AVL1, AVL4, AVL18, and AVR7 yielded positive results for alkaloid, flavonoid, phenols, terpenoids, and C-glycoside. Several bioactive chemicals found in our endophytic fungus AVL1, AVL4, AVL18, and AVR7 are similar to numerous previously reported discoveries.

In recent years, endophytic fungi have emerged as a strong source of new chemicals with remarkable biological activity. In our investigation, only four of the 27 isolated endophytic fungi proved positive for broad range antibiotic activity. Because of the presence of bioactive chemicals, endophytic fungi have antibacterial characteristics. Antimicrobial activity studies on endophytic fungi have also showed that specific chemicals, such as alkaloids, phenols, terpenes, and saponins, are responsible for antimicrobials [8].

Additionally, our findings revealed that endophytic fungi of plant *A.vera* have the ability to develop new antimicrobial chemicals, and that the plant's antibacterial nature may be attributable to the impacts of endophytic fungal secondary metabolites.

Conclusion

Because these plants produce natural compounds that are valuable to mankind, fungal endophytes from medicinal plants are being investigated. Endophytic fungi are a poorly understood group of microorganisms that provide a rich and constant source of bioactive and chemically unique substances with medicinal and industrial applications. It was revealed that some plants and endophytes create the same natural chemicals. Endophytic fungi from the *A.vera* plant are a viable source of antibacterial chemicals, according to this study.

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