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Antibacterial Activity of Endophytic Actinomycetes from *Terminalia arjuna*

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ABSTRACT
Endophytic actinomycetes from medicinal plants produce a wide diversity of secondary metabolites (SM). Endophytic actinomycetes isolated from surface-sterilized living aerial parts of medicinal plant Terminalia arjuna. A simplified method for selective recovery of actinomycetes from internal plant tissues is described. Successful recovery was achieved on the Starch casein agar medium. One isolate was obtained from root of Terminalia arjuna which belonging to actinomycetes genus identified through morphological characteristics, microscopic examination and biochemical test results. Additionally, SG medium used for production of secondary metabolites and 36°C considered as an optimum temperature for culture condition. Conditions were analyzed for active metabolites and the antibacterial activity was observed from metabolites produced with SG medium at 36°C. The antibacterial activity was performed on Gram positive bacteria (Bacillus subtilis, Staphylococcus aureus) and Gram negative bacteria (Escherichia coli, Pseudomonas aeruginosa). Crude B-1 showed highest antibacterial activity against bacterial pathogens.
Keywords*: actinomycetes, endophytes, Terminalia arjuna, secondary metabolites.*

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INTRODUCTION

Endophytes are that microorganism which lives internal tissues or parts of plants without causing any negative effects to plants. In 1809 the German botanist Johann Heinrich Friedrich Link has first described endophytes. Endophytes are also known as endosymbiont often are a bacterium or a fungus. Some endophytes are able to enhance the growth of host, nutrient acquisition and improve the tolerance ability of plant against abiotic stresses such that drought and also helps to decrease biotic stresses by the extent of resistancy of plant against insects, pathogens and herbivores. Transmission of endophytes in plants may through directly from parent to offspring or via among individual plants. Endophytes can enter plant tissues via their stomata, damaged secondary roots zones, aerial portions like stems, flowers, cotyledons, and germinating radicals [1]. Endophytes may help host plants by preventing other pathogenic or parasitic organisms from colonizing internal parts of the plant. Endophytes colonize inside plants and get nutrition from the plant. In return, endophytes increase the health of the plant by producing various bioactive metabolites belonging to the antibiotic, plant growth promoter, plant growth inhibitor, and enzymes. Endophytes are natural resources of novel bioactive secondary metabolites such as steroids, alkaloids, tannins, saponins, quinones and terpenoids which show highly anti-microbial, anti-cancer, anti-insect, and many more properties. The intimate interaction between actinomycetes and plants are commensalistic symbioses such as secreting herbicidal compounds [2], fixing atmospheric nitrogen [2] or protecting roots against fungal infection [3]. Actinomycetes are gram-positive, non-motile, non-spore forming and non-capsulated filamentous bacteria. Actinomycetes are also known as 'ray fungus' because of the morphological structure is similar with fungi and cellular and microscopic characteristics are similar with bacteria. Actinomycetes include in a diverse group of heterotrophic prokaryotes which forms hyphae at

some stage of their growth [4], hence referred to as filamentous prokaryotes. Earlier they were recognized as the exotic group of microorganisms with similar characteristics of both bacteria and fungi. After, determination of their morphological structure and chemical composition confirmed their prokaryotic nature. By using new taxonomic techniques, has led to improvements in the identification and classification of actinomycetes genera and species. Due to their ability to produce secondary metabolites and enzymes, ubiquitous nature, the difference in the morphological structure and overall importance to man, nowadays actinomycetes is widely studied group as compared to another group of bacteria. Approximately 23,000 bioactive secondary metabolites are produced by micro-organisms, out of those secondary metabolites around 10,000 are produced by actinomycetes [5]. Approximately 7,600 compounds are produced by *Streptomyces* sp. [5]. Actinomycetes can play a role as symbionts in the intestinal tract of animals that feed on the organic substances present in the soil. There are so many researches were done on endophytic actinomycetes from different plants. A number of endophytic actinomycetes inhabit tissues of a wide variety of plants. The complicated associations of endophytic actinomycetes with host plants still remain poorly understood. Many actinomycetes have been isolated from the internal tissues of many plants [7]. These actinomycetes have the capacity to survive internal plant tissues as 'endophytes' and that do not harm to the plant host [8,9]. Most of the endophytic actinomycetes inhabit in internal tissues of non-symptomatic plants without causing any effects to host [10]. Various reports indicated that endophytic actinomycetes existed in different tissue types within a broad range of plants. A total of 14 endophytic actinomycetes were isolated from 300 plant stem samples from the upper Amazonian rainforest in Peru [11]. A novel endophytic actinomycetes *Actinomadura syzygii* species was isolated from the roots of *Syzygium cumini* L.skeels [12]. A total of 246 strains of endophytic actinomycetes isolated from different plant tissues belonging to *Streptomyces* (97 strains) *Microbispora* (57 strains) *Micromonospora* (18 strains) *Actinomodura* (4 strains) and *Nocardia* (23 strains) [13]. From the roots of a healthy wheat plant, 38 endophytic actinomycetes were isolated and identified as belonging to the genera *Micromonospora*, *Streptomyces*, *Microbispora*, and *Nocardia* [14]. The endophytic actinomycetes, especially *Streptomyces* species were most common isolates recovered in high number from roots, leaves and less number from stems [15]. Several endophytic actinomycetes play a role as a growth promoter to host plants [16]. Most of the studies regarding endophytic actinomycetes were focused on agricultural and horticultural plant species [17]. The endophytic actinomycetes from rice [18], maize [19], wheat [3] and tomato [20] have been reported. Moreover, studies of actinomycetes have mainly focused on the production of novel secondary metabolites and antibiotics. Few reports suggest that actinomycetes are potent sources of antimicrobial, antidiabetic and antioxidant compounds [21,22]. Endophytic actinomycetes becoming interested are for researchers because of some actinomycetes have the ability to produce bioactive compounds which are inhibiting several pathogenic fungi and bacteria. A study found differences in the types of actinomycetes and bioactive compounds between endophytic and soil actinomycetes [23].

MATERIAL AND METHODS

Sample Collection

Samples of the root, stem and leaf tissues were collected from the selected healthy plant of *Terminalia arjuna* from Botanical garden of the Life Science Department, Gujarat University, Ahmedabad (Gujarat). All the samples were brought to the laboratory in polythene bags and stored at 4°C until further processing.

Isolation of endophytic actinomycetes from the medicinal plant

The leaf, root and stem samples were washed in running tap water to remove soil particles and cut into small fragments of 3-4 cm. Plant tissues were then sterilized by sequential immersion in Tween 20 for 1 min and sodium hypochlorite solution for 15 to 20 min and then washed with three-time dipping in sterile distilled water to remove surface sterilizing agents. After this, the samples were washed in mercuric chloride solution for 2 to 5 min. Sterile fragmented tissues of plants were deposited on the surface of starch casein agar medium (SCA) and the plates were incubated at 28°C for 30 days and were examined daily for the presence of colonies. In order to suppress fungal growth, cycloheximide (50 µg/ml) was added to the medium. A single colony of isolated actinomycetes was grown on various media that marked depending on colonial morphology. For this purpose, four different culture media were used to identify macromorphological characteristics: ISP2- Agar Yeast-Malt extract; ISP4- Agar Starch and Inorganic salts; ISP5- Glycerol Asparagine Agar [24] and Actinomycetes Isolation Agar (AIA) medium. Pure culture was prepared by transferring colonies on ISP-4, ISP-2 and AIA medium by direct streaking method. Incubate all pure streaked plates at 28°C for 21 days. Purified isolates were transferred to freshly prepared SCA slants and kept at 4°C. The isolates were maintained on SCA slants at 4°C. Sub-culturing was done after every 30 days.

Identification of endophytic actinomycetes isolates

Actinomycetes were identified on the basis of cultural and morphological characteristics following directions of International *Streptomyces* Project (ISP) [24]. Morphological identification of the isolate was done by Gram staining. For further identification, the isolate was observed under light microscope for detection of filamentous structure, hyphae and spore formation. Starch hydrolysis, nitrate reduction, gelatin liquefaction, casein hydrolysis were tested following standard procedures for enzymatic activity [25]. Morphological and cultural characteristics of the isolated strain were determined on starch casein agar and ISP 4 agar according to the standard methods [24].

Fermentation and extraction of secondary metabolites

Production of secondary metabolites

Production of secondary metabolites was produced by the fermentation process.

Preparation of inoculums for the fermentation process

SG medium (100 ml) was prepared [26] and autoclaved. Then, under aseptic conditions, a loopful of purified growth was added to the SG medium (which contained glucose 20 g, yeast extract 5 g, soytone 10 g, $CoCl_2 \bullet 6H_2O$ 1 mg, $CaCO_3$ 2 g, D/W 1000 ml, pH 7.2). This broth was incubated at 36°C on shaking at 180 rpm for 3 days. After 3 days of incubation, inoculums for fermentation were ready for use.

Fermentation process

A 250 ml Erlenmeyer flask was used to prepare 100 ml of SG medium (Contained glucose 2 g, yeast extract 0.5 g, soytone 1 g, Cocl2•6H2O 0.1 mg, CaCO3 0.2 g, D/W 100 ml, pH 7.2) which was then autoclave. After that, 1 ml of the prepared inoculum was added in the fermentation medium and incubated at 36°C on shaking at 180 rpm for 10 days. The pH of the fermentation medium was set at 7.2 ± 0.2.

Extraction of secondary metabolite

After the incubation of fermented broth, the medium containing active secondary metabolites and biomass was filtered-off on Whatmann 4 filters. After filtration, the water fraction of the fermented medium was extracted with ethyl acetate (3 × 100 mL). The combined organics were evaporated in a vacuum rotary evaporator at 40°C. After evaporation, active secondary metabolites were diluted in 2 ml of methanol. This crude sample was ready to use for an antibacterial test.

Determination of antibacterial activity

The antibacterial activity of secondary metabolites extracted from endophytic actinomycetes was screened against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtillis*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aerugenosa*) using the agar well diffusion method. Test organisms were spread on nutrient agar plates. Then wells were bored on the agar plate by sterile cup borer and three different concentrations of bacterial crude extract were filled in each well respectively in 50 µl, 100 µl, 150 µl. Antibacterial activities were detected after incubation of 24 Hr. at 37°C. The presence of a clear zone on the plate was used as an indicator of the bioactive nature of the strain. As a positive control, streptomycin was used and methanol was used as a negative control. After incubation, a clear zone was measured by Hi-media Antibiotic zone scale [27].

Antioxidant assay

The antioxidant activity of different bacterial crude extracts was evaluated using the DPPH assay, a widely used method for measuring free radical scavenging capacity [28]. 0.1 mM of DPPH solution was prepared by dissolving 4 mg of DPPH in 100 ml of ethanol. To calculate the % radical scavenging activity of the extract, take different volumes of crude extract (2-20 µl) and makeup 40 µl with DMSO. Add 2.96 ml DPPH solution and incubate in dark condition at room temperature for 20 min. Measure the absorbance of the mixture at 517 nm and take 3 ml of DPPH solution as blank (control). Calculate the % radical scavenging activity of the extract using the following formula:

% Radical scavenging activity = (Abs control−Abs sample/ Abs control) x 100

Where, Abs control is the absorbance of DPPH radical + methanol. Abs sample is the absorbance of DPPH radical + extract.

RESULT

Endophytes are microorganisms that live inside plants without causing any harm to the host plant. Endophytic actinomycetes are a type of endophyte that can be isolated from various medicinal plants. These endophytic actinomycetes have a wide range of biological activities and represent an immense reservoir of novel metabolites. In the present study, 1 endophytic actinomycetes isolated from the root of *Terminalia arjuna*. Isolation efforts resulted in a low number of actinomycetes due to heavy growth of fungus on plates. The Isolated strain was purified on starch casein agar and ISP-4 medium. The slants were maintained at 4˚C. The isolated A1 culture appears to be chalky or powdery in form with smooth surfaced

spores. While the aerial mass colour of the A1 was found to be white with the reverse side pigment like brown which was dispersed all over the plate. The morphological and microscopic characteristic of A1 culture is summarized in Table 1 and figure 1. The isolate A1 was used in the production and extraction of secondary metabolites. Secondary metabolites were extracted with ethyl acetate solvents. Approximately 2 ml of crude extract of endophytic actinomycetes was obtained by using Rotary Vacuum Evaporator. 2 ml of ethyl acetate extract (figure 2) was subjected to determination of antibacterial activity. Crude extract B1 from endophytic actinomycetes showed promising result by exhibiting maximum antibacterial activity against bacterial pathogen by agar well diffusion method which has been reported in the table and figure. The ethyl acetate crude extract has higher antibacterial activity against *E. coli*, *P. aeruginosa*, and *B. subtilis* than Streptomycin at the tested concentrations. It also has higher activity against *S. aureus* at 100 µl and 150 µl but not at 50 µl. Crude B-1 shows maximum zone of inhibition against *Staphylococcus aureus.* The antioxidant activity of B-1 crude extract showed increases with increasing concentration. The highest percentage of DPPH scavenging activity was observed at a concentration of 2 µl with an absorbance of 0.071 and a percentage of RSA of 69.1%. The result of antioxidant activity with DPPH scavenging in percentage described in table.

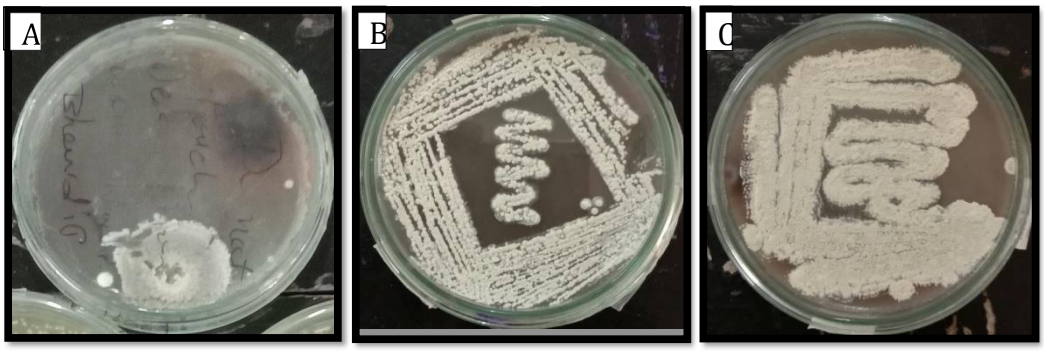

Fig. 1: Isolation and Purification of endophytic actinomycetes

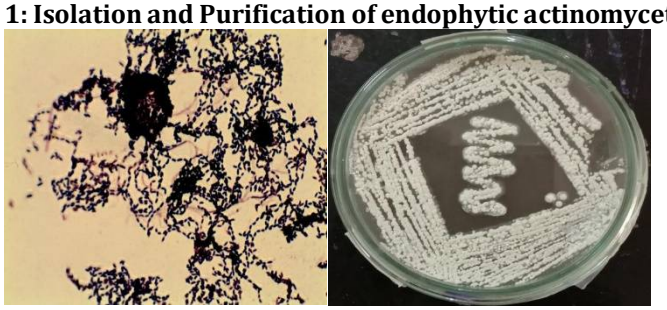

Figure 2: Microscopic observation of endophytic actinomycetes

Figure 3: Endophytic actinomycetes in liquid fermentation medium

Figure 4: Crude extract ethyl acetate solvent

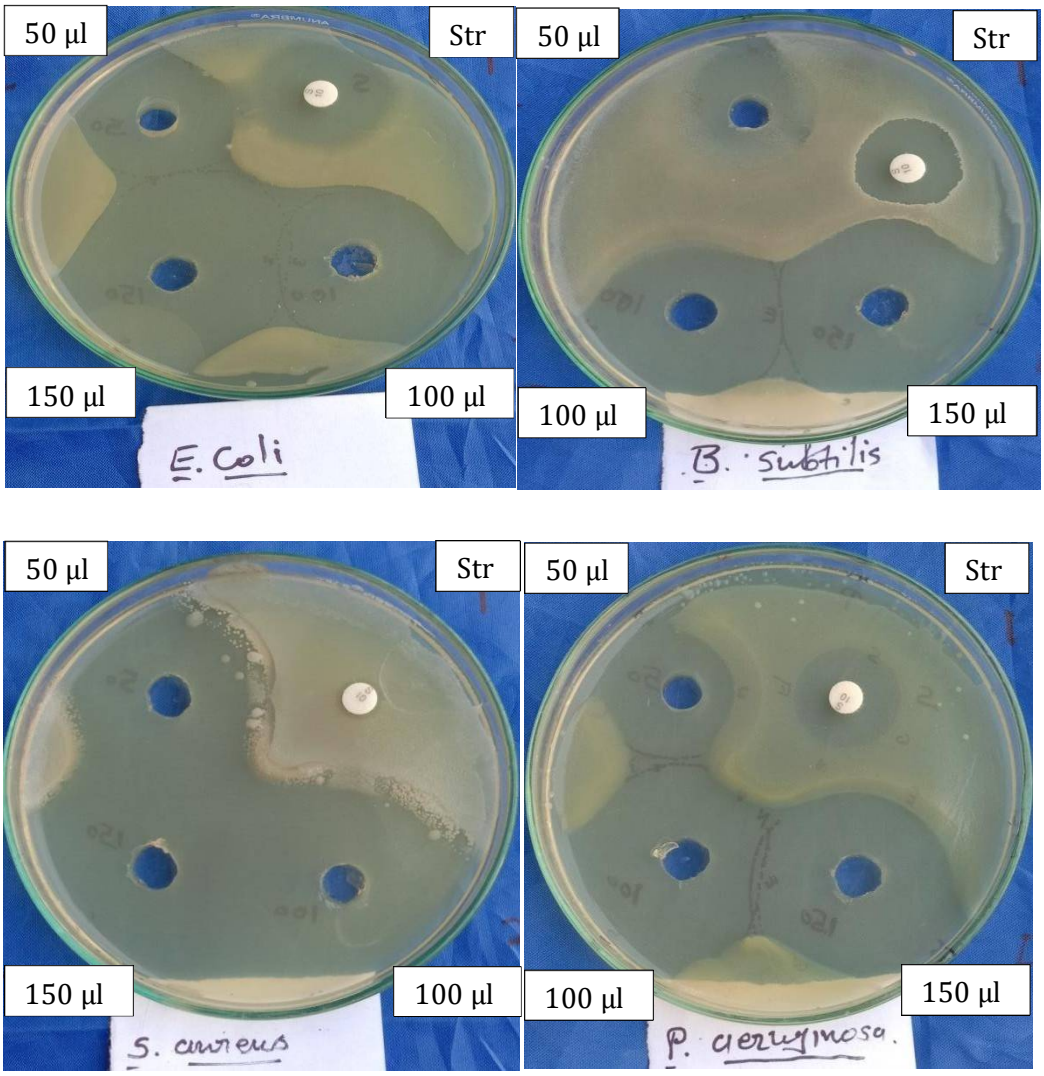

Figure 5: Antibacterial activity of extracted crude (B-1)

Table 1: Morphological characteristics

Size	Big
Shape	Rod
Arrangement	Single, Cluster
Gram reaction	Gram-positive

Table 2: Cultural characteristics

Size	Intermediate
Shape	Irregular
Elevation	Pulvinate
Consistency	Powdery
Surface	Smooth
Margin	Filamentous
Appearance	Chalky
Odor	Earthy

Table 3: Antibacterial activity against crude extract B-1 (Zone of inhibition in mm)

Test Organism	**Crude Extract**			**Ethyl acetate (100 µl)**	**Streptomycin**
	50 µl	**100 µl**	**150 µl**		
E.coli	25	31	35	-	20
P.aeruginosa	21	32	34	-	11
S.aureus	33	36	40	-	-
B.subtilis	24	34	37	-	15

Table 4: Biochemical characteristics of A-1

No.	**Test**		**Interpretation**	**Results**
1	Carbohydrate utilization	Sucrose	Pink color produce due to acid production and/or gasformation observe in Durham's vial	+
		Dextrose		+
		Fructose		+
		Lactose		+
		Manitol		+
		Mannose		+
		Xylose		+
2	M-R		Development of stable red color	-
3	V-P		Development of stable red color within 15 minutes	+
4	Citrate utilization		Development of deep blue color	-
5	Indole production		Observe pink color ring	-
6	H_2S Production		Blacking of filter paper strip	-
7	Nitrate reduction		Development of red color	-
8	Starch hydrolysis		Clear zone surround growth	+
9	Lipid hydrolysis		Formation of clear zone of solubilisation of CaCO	+
10	Gelatine hydrolysis		Check liquefaction of gelatin	-
11	Casein hydrolysis		Clear zone surround growth after adding Lugol's Iodine	+
12	Dehydrogenase		Disappeared of blue color	-
13	Catalase		Production of gas bubbles	+
14	TSI		Shows the presence of acid/gas/H2S	Alkaline slant acidic butt

Table 5: DPPH assay of crude extract B-1

Crude extract (µl)	Abs	%RSA
0.2	0.127	44.7%
0.4	0.115	50%
0.6	0.109	52.6%
0.8	0.102	55.6%
1.0	0.095	58.6%
1.2	0.092	60.0%
1.4	0.091	60.4%
1.6	0.089	61.3%
1.8	0.081	64.7%
2.0	0.071	69.1%

DISCUSSION

Endophytes, especially endophytic actinomycetes isolated from various medicinal plants, represent an immense reservoir of novel metabolites with a wide range of biological activities. Research showed that endophytes, especially endophytic actinomycetes have the ability to produce a diverse range of bioactive compounds which can inhibit the growth of pathogens. Antibacterial compounds are extracted from the bacterial culture to solve the problem of bacteria becoming multi-drug resistant due to several processes including mutation, quorum sensing and excessive use of antibiotics. Endophytic actinomycetes were isolated from stem, leaf, and root of *Rhizophora apiculuta* and *Avicennia marina*. From those samples, a total of 6 endophytic actinomycetes were isolated. Morphological characters, microscopic characteristic, and biochemical characteristics were studied. Also, the effect of salt tolerance, the effect of pH, effect of temperature, assimilation of different carbon sources, nitrogen sources and enzyme activity were studied. Out of that 6 isolates, 1 endophytic actinomycete isolate (MAR1) shows good activity to compare all isolates. Based on the morphology and above characteristics, MAR1 is identified as *Streptomyces* sp. and species may be *Streptomyces coelicolor* [29]. The study isolated 10 endophytic actinomycetes from the medicinal plant *Vochysia divergens* located in the Pantanal, Brazil. Different culture media (SG and R5A) and temperature (28 and 36ºC) were selected for the best culture conditions to produce bioactive metabolites. The LGMB491 extract showed the highest activity against methicillin-resistant *Staphylococcus aureus* with a MIC of 0.04 mg/ml. Bioactive compounds extracted from LGMB491 were 1- acetyl-β-carboline, indole-3-carbaldehyde, 3-(hydroxyacetyl)-indole, brevianamide F, and cyclo-(L-Pro-L- Phe) [30]. The study isolated a total of 46 endophytic actinomycetes from leaf, stem and root samples of 15 tea plants from China. Through 16s rRNA sequence analysis, isolates were classified into 13 genera including *Streptomyces*, *Nocardia*, *Kribbella*, *Actinomadura*, *Kytococcus*, *Leifsonia*, *Microbacterium*, *Micromonospora*, *Mycobacterium*, *Mobilicoccus*, *Nocardiopsis*, *Piscicoccus*, and *Pseudonocardia*. *Streptomyces* was the most prevalent genus whereas *Piscicoccus* and *Mobilicoccus* were rare genera that were first time reported as plant endophytes. Antimicrobial assays against a set of bacterial and fungal pathogens showed that endophytic actinomycetes associated with tea plants have a highly potent source for antimicrobial metabolites production [31]. The study isolated 117 endophytic actinomycetes from *Combretum latifolium* Blume, the Western Ghats of southern India. Streptomyces genera (35%) was the most prevalent isolated strains, followed by *Nocardiopsis*(17%) and *Micromonospora* (13%). Antimicrobial activity was carried out by disc diffusion method. *Streptomyces* spp. strains showed significant antimicrobial activity. Strain CLA-66 and CLA-68 of *Nocardiopsis* spp. inhibited bacterial and fungal pathogens [32]. Endophytic actinomycetes were isolated from the root, leaf, and stem of *Azadirachta indica*, *Ocimum sanctum* and *Phyllanthus amarus*. The isolated strains were tested for antimicrobial activity against bacterial pathogens (*Staphylococcus aureus, Streptococcus pyogens, Klebsiella pneumonia, Pseudomonas aeruginosa*) and the fungi *Rhizopus*. Isolated strains of endophytic actinomycetes which showed efficient antibacterial activity were subjected to study the effect of mutation by physical and chemical method. Mutated endophytic actinomycetes showed higher production of antibiotic than non-mutated endophytic actinomycetes [33]. The study isolated 76 endophytic actinomycetes from six selective medicinal plants of Gibbon wild Life Sanctuary, Assam, India. The isolates were more frequently obtained from roots. Through 16s rRNA sequence analysis identified 16 genera, including rare genera of *Verrucosispora*, *Isoptericola*, and *Kytococcus* which were not previously reported as endophytes. The most prevalent isolated genus was *Streptomyces* spp [34]. 68 chitinolytic endophytic actinomycetes were isolated from various medicinal

plants and 12 isolates were screened for their plant growth promoting abilities and antimicrobial activity against *Sclerotium rolfsii* in chickpea. These isolates were reported for their ability to protect chickpea against *Sclerotium rolfsii*. Through 16s DNA profiling isolates were identified as *Streptomyces diastaticus*, *Streptomyces fradiae*, *Streptomyces olivochromogenes*, *Streptomyces collinus*, *Streptomyces ossamyceticus*, and *Streptomyces griseus* [35]. A number of endophytic actinomycetes inhabit tissues of a wide variety of plants. The complicated associations of endophytic actinomycetes with host plants still remain poorly understood. Some of them are undoubtedly beneficial to the host plant life: the endophytic presence of some actinomycetes may play important role in plant development and health because of their role in nutrient assimilation and in secondary metabolite production. Endophytes are a poorly investigated group of microorganisms that represent a source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial areas. As endophytic microorganisms, particularly actinomycetes, have evolved with the greatest genomic and metabolic diversity, efforts should be directed towards exploring such actinomycetes as a source of novel secondary metabolites. The exploitation of endophytic actinomycetes as a source for novel secondary metabolites is in its infancy. Two-thirds of today's antibiotics are produced on the industrial scale come from actinomycetes. Besides antibiotics, they are being explored for the production of enzymes, plant growth promoting substances and other important molecules such as enzyme inhibitors and immunomodifiers. In this respect, future success relies on our ability to isolate novel actinomycetes from tissue environments. Developing methods for the culture of currently unculturable or rare actinomycetes would represent a unique and promising source for the discovery of novel secondary metabolites. Endophytic actinomycetes in higher plants, particularly those associated with medicinal plants and being used traditional medicines, are more likely to contain bioactive compounds. Based on the hypothesis that endophytes produce bioactive compound(s), which might have some pharmaceutical potential, the aim of the study is to isolate and identify endophytic actinomycetes from medicinal tree *Terminalia arjuna* and production of bioactive secondary metabolites.

CONCLUSION

In this present study, endophytic actinomycete was isolated from *Terminalia arjuna* and maintained on Starch casein agar plates. The isolated 1 endophytic actinomycete was initially identified though colony morphology and microscopic examination such as size, shape, arrangement and gram reaction of isolated endophyte. Crude extract of endopytic actinomycete shows anti-bacterial activity against Gram positive (*Bacillus subtilis, Staphylococcus aureus*) and Gram negative (*Escherichia coli, Pseudomonas aeruginosa*). Crude extract B-1 shows maximum zone of inhibition against bacterial pathogen as compared to B-2 crude. The morphological characterization, microscopic observations and biochemical test results confirm that the isolated strain belonging to actinomycetes genus. Further work on characterization is required for detailed understanding of actinomycetes biology and mode of action as well as biotechnological potential.

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