

Bactericidal Potential of Actinomycetes Isolated from the Rhizosphere Soil of *Withania somnifera*

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Abstract:

Withania somnifera is a medicinal plant and the root extract was mainly used in Ayurveda for antistress activity and neurological complications. 15 Rhizosphere soil samples of *Withania somnifera* were processed for enumeration of bacteria and fungi on Nutrient agar and Potato dextrose agar, and the soil is processed for isolation of Actinomycetes on a selective medium, Actinomycete isolation agar and Humic acid vitamin agar. The bacterial and fungal colonies were higher in the 3-month-old plants - 9.56×10^5 CFU/g and 1.15×10^5 CFU/g, when compared with one-month-old and five-month-old plants of *Withania somnifera*. 10 Actinomycete isolates (A1-A10) were obtained and some Actinomycetes produced powdery white colonies and some produced tough leathery colonies with pigments like yellow and brown. The isolates were tested for antibacterial activity by preliminary screening - Cross-streak method and agar overlay method. The test bacteria were *Escherichia coli* MTCC 452, *Salmonella enterica* abony NCTC 6017, species, *Pseudomonas aeruginosa* MTCC 424, *Staphylococcus aureus* and *Bacillus cereus* MTCC 430. The secondary screening of Actinomycetes for antibacterial activity was done by the Agar well diffusion method. Actinomycete isolates A1, A6, A7, A8, A9 and A10 inhibited the growth of *Pseudomonas aeruginosa*. A4, A6, A7 and A8 inhibited *E. coli*. A6 and A7 inhibited *Salmonella enterica* abony. A3 and A4 inhibited *Bacillus cereus*. A6 and A7 inhibited the growth of the tested pathogens - *E. coli*, *Salmonella enterica* and *Pseudomonas aeruginosa*. The Rhizosphere of *Withania somnifera* contains active Actinomycetes that produced metabolites against human pathogens.

Keywords: Actinomycetes, Agar well diffusion method, Antibacterial activity, *Withania somnifera*, Rhizosphere.

INTRODUCTION

Microorganisms act as a good source of pharmaceutical compounds like antibiotics. Nowadays, Pathogenic bacteria are gaining resistance to many antibiotics and are said to be multi-drug resistant. Hence the search for new metabolites that could kill multidrug-resistant bacteria is required. Actinomycetes are the most significant source of biologically active microbial products among all known microorganisms, and they include many antibiotics that are both medically and commercially significant [1]. The broad-spectrum antibiotics, Vancomycin which is effective against methicillin-resistant *Staphylococcus aureus*, and, Rifampicin against Tuberculosis and Leprosy are obtained from numerous species of Actinomycetes [2].

One of the most prevalent phyla of bacteria on nearly natural substrates is Actinobacteria, which contain a high percentage of guanine and cytosine (greater than 55 %) [3]. Actinomycetes play an important role in the carbon cycle and the breakdown of organic molecules. Because actinomycetes have filamentous, branching development that resembles a fungal kind of morphology, their taxonomy has been the subject of ongoing debate [4]. Actinomycetes produce a large range of extracellular enzymes and antibiotics, and they make up a significant

component of the soil microbial biomass [5]. The majority of naturally occurring antibiotics are made by actinomycetes [6]. Additionally, these are a significant source of new antibiotics, which explains why they are of great pharmacological and economic importance, notably in the management of infectious diseases [7,8]. Most soil actinomycetes are members of the *Streptomyces* genus, which produces 75% of the physiologically active chemicals [9]. Plant rhizosphere soil is inhabited by actinomycetes, which generate active chemicals [10].

The World Health Organization defines "a medicinal plant" as any plant that contains compounds that have therapeutic value or that serve as building blocks for the creation of effective medications (WHO) [11]. The biologically active chemicals found in plants may possess antibacterial, antipyretic, and antioxidant capabilities that underlie their therapeutic qualities [12]. *Withania somnifera* is an important medicinal plant, in which leaves and roots possess active secondary metabolites proven to contain pharmacological properties [13,14]. *Withania somnifera* is also commonly called Ashwagandha and the meaning of *Somnifera* indicates inducing sleep. Its root is used in Ayurvedic medicine and it contains an anti-inflammatory compound used in treating tumors, Scrofula, and Rheumatism. The

root extract possessed anti-stress activity [15]. *Withania somnifera* plant was also used to treat bacterial infections and hypothyroidism [16,17].

Medicinal plants release unique secondary metabolites in the root exudates and possess distinctive microbiomes in the rhizosphere [18]. Up to 1011 microbial cells, representing more than 30,000 distinct prokaryotic species, can be found in the rhizosphere for each gram root [19]. Because the different primary and secondary metabolites secreted by roots can shape, interfere with, or transmit signals to change the rhizosphere microflora, recruit and promote beneficial microorganisms, and resist harmful microorganisms, root exudates play a significant role in the formation of rhizosphere microorganisms [20]. Root exudates have been found to contain several kinds of organic materials, comprising ethers, olefins, acids, aldehydes and ketones, esters, alkanes, ureas, phenols, and alcohols [21]. Root exudates act as an energy as well as a nutrient source for the growth of these microorganisms [22]. The kind of plant, its stage of growth, and environmental elements including pH, soil type, humidity, temperature, and the presence of microbes all influence the composition of the root exudates [23].

The discovery of new drugs is rare. So active Actinomycetes from different sources paved the way for discovering new antibiotics [24]. Various soil samples are screened for effective Actinomycetes that could produce secondary metabolites that kill the pathogenic bacteria. However, only a small portion of the earth's soil is screened for Actinomycetes [25].

Drugs obtained from bacteria have several advantages over those obtained from medicinal plants. Bacterial cultures can be easily controlled standardized, and grown in large quantities. Bacterial compounds can be easily purified, and bacteria can be used in fermentation to produce large amounts of bioactive compounds. Bacterial fermentation can be more environment-friendly and sustainable than large-scale plant cultivation and harvesting. In most studies related to Rhizosphere bacteria, Rhizosphere bacteria from various plants were tested for their plant growth-promoting properties. Medicinal plants may produce unique metabolites in the root exudates and harbour specific active Actinomycetes bacteria which may possess various pharmacological properties. Still, Rhizobacteria have not been much tested for their antimicrobial properties against human pathogens and multi-drug-resistant bacteria, the present study aims to isolate Actinomycete from the Rhizosphere soil of the medicinal plant, *Withania somnifera*.

MATERIALS AND METHODS

Soil Sampling

The Rhizosphere soil samples of *Withania somnifera* were collected from the CTRI Research Station, Veda sandur, Dindigul district, Tamil Nadu, India. The actively growing Medicinal plant, *Withania somnifera*

was uprooted and after being shaken, the loose soil attached to the root region was detached and the rhizosphere soil was collected carefully using a sterile spoon and then transferred to a sterile plastic container. 5 Rhizosphere soil samples from one-month plants, 5 soil samples from 3 months plants, and 5 soil samples from 5 months plants were collected. 15 Rhizosphere soil samples were collected from the 15 plants of *Withania somnifera*.

Enumeration of Bacteria and Fungi

The dilution plate count is one of the most widely used methods for enumerating microbes [26]. 1 g of each Rhizosphere soil sample was suspended in 9 ml of sterile distilled water in a test tube and shaken well for the dispersal of microbes. This gives 10⁻¹ dilution. Then the soil suspension was serially diluted up to 10⁻⁷ using the standard Serial dilution method and plated by Pour plate technique. 1 ml of the diluted sample was first placed in sterile Petri dishes and then molten Nutrient agar was added and swirled to mix with the sample for the enumeration of bacteria and 1 ml of the diluted sample was plated on Potato Dextrose agar (PDA) for the enumeration of fungi. Two or three replicate pour plates per dilution tube are done. Nutrient agar plates were incubated at 37°C for 48 hours and PDA plates were incubated at 30°C for 4-7 days. Colonies were counted.

Isolation of Actinomycetes

The soil samples collected from the rhizosphere of *Withania somnifera* were air-dried at room temperature for 7 days. Then the soil samples were pulverized using a mortar and pestle. The conventional serial dilution technique was followed to isolate actinomycetes [27]. In 9 ml of sterile double-distilled water, 1 g of soil was suspended. Up to 10⁻⁵ dilutions were used in the serial dilution. Using an L-rod, aliquots (0.1 ml) of 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵ were spread out in the Humic acid Vitamin agar, Starch- Casein agar, and ISP-2 agar for Actinomycete isolation. 100 µg nystatin/ml, 100 µg cycloheximide/ml, and 50 µg nalidixic acid/ml were added to all the media [28]. The plates were incubated for 7–14 days at 28 °C [29]. Individual colonies were sub-cultured on ISP-2 agar. Colony morphology was observed.

Morphological Identification by Gram's staining

The isolated Actinomycetes were smeared on a clean microscopic glass slide and stained by Gram's Staining and observed under oil immersion objective and the morphology was observed for each isolate [30].

Antibacterial activity of Actinomycetes

Preliminary Screening

The preliminary screening of Actinomycetes for antibacterial activity was done by the Cross-streak method [31,32,33]. Mueller Hinton agar was prepared, sterilized and 15 ml was poured into sterile Petri plates, and allowed to set. The actinomycete isolates were single-streaked in the middle of the agar plates and

incubated at 28°C for 7 days. Then the test bacterial pathogens such as *Escherichia coli* MTCC 452, *Staphylococcus aureus*, *Salmonella enterica* abony NCTC 6017, species, *Pseudomonas aeruginosa* MTCC 424, and *Bacillus cereus* MTCC 430 were streaked perpendicular to the Centre streak. The distance between the test pathogens was 1 cm apart and streaked it was 2mm apart from the side of the Actinomycete isolate. The inhibitory activity of the Actinomycete isolate was determined by measuring the distance in mm from the growth of the test pathogen to the Actinomycete growth. The agar overlay method is another method of testing the antibacterial activity of Actinomycetes [34,35]. The Actinomycete isolates were spot inoculated on the Mueller Hinton agar plate and incubated at room temperature (28 – 30 °C) for 7 days. 3 ml of soft agar inoculated with the test bacteria (108 CFU/ml) was overlaid on the Mueller Hinton agar with the grown Actinomycete and incubated at 37 °C for 24 hours. The plates were observed for the zone of inhibition around the Actinomycete colony.

Extraction of metabolites from Actinomycete

In 250 ml of conical flask, 100 ml of ISP 1 broth was prepared and sterilized by Autoclaving at 121 °C at 15

lbs for 15 minutes. Actinomycete isolates were inoculated in a separate broth and incubated in a rotary shaker at 150 rpm for 7 days at 28-30 °C. Then the culture was centrifuged at 10,000 RCF for 10 minutes. The supernatant was collected and treated with an equal amount of ethyl acetate and the content was mixed well. The aqueous layer was removed and the organic layer containing the compounds was concentrated by keeping it in a Rotary evaporator with a temperature of 40 °C [34].

Secondary screening by Agar well Diffusion method

The Actinomycete isolates were screened for antibacterial activity using the Agar well diffusion method. Test bacterial strains were grown in Nutrient broth and about 0.1 ml of test bacterial strains adjusted to 0.5 McFarland standard (108 CFU/ml) were spread on MHA plates. Then wells with a diameter, of 6 – 8 mm were made using 1000 µl sterile microtips and 20 - 100 µl of ethyl acetate extract of desired concentration (stock 1 mg/ml) was added into bored wells. Then the Plates were incubated for 24 h at 37 °C. The zone of inhibition of growth around the well was measured in mm [36].

RESULTS

Table 1. Enumeration of Bacteria and Fungi from Rhizosphere soil samples of Withania somnifera

S.No	Plant age of <i>Withania somnifera</i>	Soil sample	Bacterial count (cfu/g) of Rhizosphere soil	Fungal Count (X10 ⁵ cfu/g)
1.	1 month	1MWS1	2.9 x10 ⁴	6.6 x10 ³
2.		1MWS2	4.4 x10 ⁴	2.4 x10 ⁴
3.		1MWS3	4.84 x10 ⁵	7.2x10 ³
4.		1MWS4	6.6 x10 ⁴	1.4x10 ⁴
5.		1MWS5	1.53 x10 ⁵	1 x10 ⁴
6.	3 months	3MWS1	5.04x10 ⁵	6.3x10 ⁴
7.		3MWS2	4.58x10 ⁵	8.1x10 ⁴
8.		3MWS3	6.64 x10 ⁵	1.13 x10 ⁵
9.		3MWS4	9.56 x10 ⁵	1.15x10 ⁵
10.		3MWS5	4.64 x10 ⁵	0.8x10 ⁴
11.	5 months	5MWS1	2.01 x10 ⁵	1.6 x10 ⁵
12.		5MWS2	5.5 x10 ⁴	1.6 x10 ⁴
13.		5MWS3	1.61 x10 ⁵	1.4 x10 ⁴
14.		5MWS4	8.1 x10 ⁴	9 x10 ³
15.		5MWS5	1.81 x10 ⁵	7.5 x10 ⁴

The bacterial population was highest in the Rhizosphere soil of a three-month-old plant of *Withania somnifera*, 9.56 x10⁵ CFU/g, and lowest in a one-month-old plant, 2.9 x10⁴(Table 1). The highest fungal population was observed in the rhizosphere soil of a three-month-old plant, 1.15x10⁵ CFU/g, and the lowest fungal population was observed in the one-month-old plant, 6.6 x10³ CFU/g (Table 1). The highest mean bacterial and fungal populations were recorded in the three-month-old plant of *Withania somnifera*.

Isolation of Actinomycetes

Actinomycetes were isolated on Actinomycete isolation agar (Figure 1). A total of 10 Actinomycete isolates were obtained from Rhizosphere soil samples of *Withania somnifera*. The colony morphology and biochemical characteristics were recorded (Table 2). The morphological features were observed by Gram's staining (Figure 2 and Table 2)

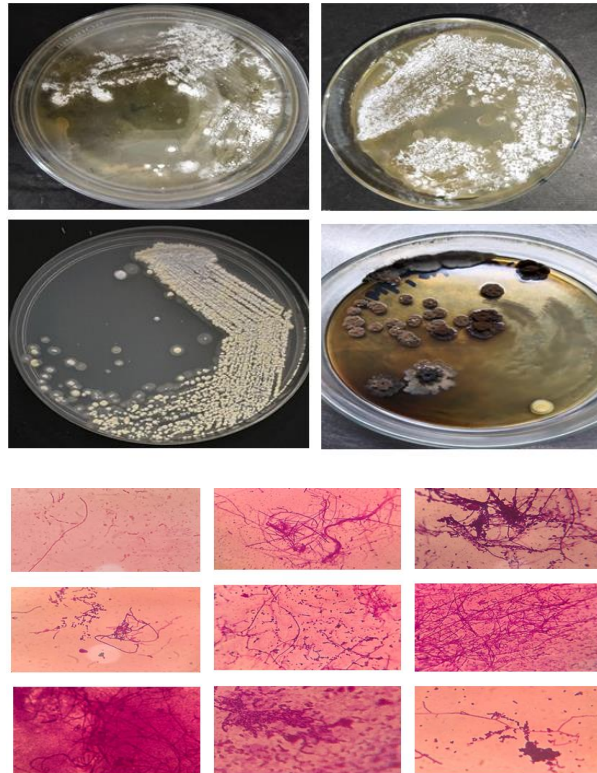


Figure 2. Gram's Stained Actinomycetes isolates

Table 2: Colony Morphology, Morphological, and Biochemical Characteristics of 10 Actinomycete isolates from Rhizosphere soil of Withania somnifera

S.No	Actinomycete isolate	Colony Morphology of Actinomycete on isolation agar	Gram's Staining	Catalase	Oxidase	Starch hydrolysis
1	A1	White powdery colony	Gram Positive filamentous rod	-ve	-ve	-ve
2	A2	White powdery colony	Gram Positive rod	+ve	-ve	-ve
3	A3	yellow powdery colony.	Gram Positive rod	+ve	-ve	-ve
4	A4	white powdery colony.	Gram Positive rod	+ve	-ve	-ve
5	A5	white powdery colony.	Gram Positive rod	+ve	-ve	-ve
6	A6	white-colored powdery colony.	Gram Positive rod	+ve	-ve	+ve
7	A7	brown-pigmented powdery colony.	Gram Positive rod	+ve	-ve	+ve
8	A8	yellow-colored powdery colony	Gram Positive rod	+ve	-ve	+ve
9	A9	Brown-coloured tough leathery colony	Gram Positive rod	+ve	-ve	-ve
10	A10	Bright yellow-colored powdery colony	Gram Positive rod	+ve	-ve	-ve



The antibacterial activity of the Actinomycete isolates was tested initially by the cross-streak method. Even after performing the test several times, only some Actinomycete isolates could inhibit a few tested bacteria like *Salmonella enterica* and *E. coli* and had not inhibited *Pseudomonas* species and *Bacillus* species (Figure 3) in the preliminary test. The agar overlay method was also carried out and it did not produce satisfactory results. So, after many repeated tests of Cross-streak and agar overlay method, Agar well diffusion method was carried out to determine the antibacterial activity of 10 Actinomycete isolates against the test bacteria.

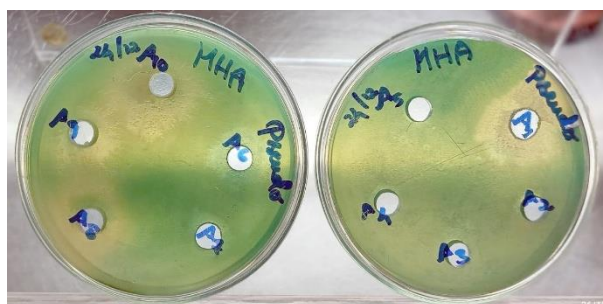


Figure 4. Antibacterial activity of ethyl acetate extract of Actinomycetes by Agar well diffusion method

Table 3: Antibacterial activity of Actinomycete isolates (A1-A10)

Isolates	Escherichia coli MTCC 452	Salmonella enterica abony NCTC 6017	Pseudomonas aeruginosa MTCC 424	Bacillus cereus MTCC 430	Staphylococcus aureus
A1	-	-	30	-	-
A2	-	-	-	-	-
A3	-	-	-	20	-
A4	31	-	-	23	-
A5	-	-	-	12	-
A6	27	30	19	-	-
A7	28	29	14	-	-
A8	30	-	25	-	-
A9	-	-	25	-	-
A10	-	-	30	-	-

The isolates A1, A8, A9, and A10 showed stronger inhibitory activity against *Pseudomonas aeruginosa* than A6 and A7 (Figure 4). Some isolates have also inhibited *Escherichia coli* and *Salmonella enterica* species and showed less zone of growth inhibition against Gram-positive bacteria, *Bacillus cereus*. All Actinomycete isolates

showed no inhibitory activity against *Staphylococcus aureus* (Table 3). Actinomycete isolates showed strong antibacterial activity against the tested Gram-negative bacteria than Gram-positive bacteria.

DISCUSSION

The highest mean bacterial and fungal population was observed from the rhizosphere soil of three-month-old plant of *Withania somnifera* (Table 1) when compared with rhizosphere soil of one-month and five-month-old plant. As plants mature, microbial activity rises and falls, most likely due to the plants secreting lower quality and quantity exudates that may contain antimicrobial compounds [37]. The Bacterial population was found to be higher than the fungal population. This result was consistent with the results of Paringamalai et al [38]

Actinomycetes were grown on the selective medium, Actinomycete isolation agar and some were found to produce pigments (Table 2). *Streptomyces* species when grown on a more suitable medium like Actinomycete isolation agar, it produces a bright yellow colony with a powdery white aerial mycelium. *Streptomyces* also produces a wide variety of pigments. Actinomycete show tough leathery colonies [30].

All 10 Actinomycete isolates were Gram-positive filamentous rods with Catalase positive and oxidase negative. Similar results for Catalase and oxidase test were observed for Actinomycete isolated from

Sunderbans Mangrove ecosystem by Paringamalai et al [39].

Khamna et al., 2009 isolated 445 Actinomycetes isolates from medicinal plant rhizosphere soil and reported that, 89% of the isolates belonged to Streptomyces species [28]. Different species of the plant and the root exudates containing organic matter and different secondary metabolites determine the diversity of Actinomycetes.

The antibacterial activity of Actinomycetes in the present study correlates with the study of Barakate et al. 2002. In this case, ethyl acetate extracts of Actinomycetes had a stronger zone of inhibition of growth against Gram-negative bacteria than Gram-positive bacteria. Vijayakumar et al also reported that ethyl acetate extracts of Streptomyces species showed antibacterial activity against Gram-negative bacteria, Proteus vulgaris, and less activity against Staphylococcus aureus.

Sengupta et al isolated 56 Actinomycete isolates from the Sunderbans Mangrove ecosystem, only 9 isolates showed antimicrobial activity against tested bacteria and fungi. One Actinomycete isolate showed stronger inhibitory activity against Pseudomonas aeruginosa than Gram-positive bacteria and fungi. Pathalam et al reported only one strain among 44 isolates showed broad spectrum activity against Gram-positive bacteria like S. aureus, M. luteus, E. faecalis, and B. subtilis, and Gram-negative bacteria like K. pneumoniae, Shigella flexneri, and Proteus vulgaris [29].

The study of Singh et al., 2016 stated that Actinomycetes isolated from different soil samples in India, when tested for antibacterial activity showed greater inhibitory activity against Gram-positive bacteria than Gram-negative bacteria [32]. This difference in the antibacterial activity of Actinomycetes could be due to the different type of Actinomycetes that exist in different soil sample. Rhizosphere soil acts as a unique zone where diverse Actinomycetes occur due to the organic material released by roots.

Atsede et al 2018 stated in their study that some of the Actinomycetes inhibited the tested bacteria like Pseudomonas aeruginosa in the secondary screening, Agar well diffusion method but not in the preliminary screening, Cross-streak method. Similar differences in the preliminary and secondary screening of antibacterial activity were also reported by Vadakkan et al [40].

CONCLUSION

The rhizosphere soil of Withania somnifera enhances the activity of Actinomycetes as root exudates are rich in organic matter and release unique secondary metabolites like withanolides. Bacterial and fungal counts were higher in the rhizosphere soil of 3-month-old plant. Actinomycetes were isolated on Actinomycete isolation agar with varied pigment production like white, yellow, and brown. Agar well diffusion method for antibacterial

activity gave the best results when compared with cross-streak method and Agar overlay method. Actinomycetes isolated from the Rhizosphere soil of Withania somnifera inhibited the growth of Gram-negative bacteria like Pseudomonas aeruginosa, E. coli and Salmonella enterica abony when compared with the tested Gram-positive bacteria like Bacillus subtilis and Staphylococcus aureus. The Rhizosphere soil of the Medicinal plant, Withania somnifera acts as a good source of different Actinomycetes with varied antimicrobial activity.

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Conflict of Interest

The authors declare no competing interest.

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