



STUDIES ON BIOPROSPECTING OF ENDOPHYTIC BACTERIA FROM THE MEDICINAL PLANT OF *ANDROGRAPHIS PANICULATA* FOR THEIR ANTIMICROBIAL ACTIVITY AND ANTIBIOTIC SUSCEPTIBILITY PATTERN

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ABSTRACT

Medicinal plants have many traditional claims including the treatment of ailments of infections origin. In the observation of traditional claims, scientific research is important. The objective of the study was to determine the presence of antibacterial activity in the extract of endophytes isolated from the medicinal plant *Andrographis paniculata*. In this preliminary investigation, the leaves were used for isolation of endophytic bacteria and extract were collected and subjected to screening against six human pathogenic bacteria and two fish pathogenic bacteria. In this method carried out standard protocol of well diffusion method. The antibacterial activities were assessed by the presence or absence of inhibition zones. The endophytic extract was showed activity against both Gram positive and Gram negative bacterial pathogens. The antibiotic susceptibility test was done for all endophytic bacteria using six different antibiotics by Kirby-Bauer disc diffusion method. The finding forms a basic for further studies on endophytic bacteria from medicinal plant for antimicrobial activities.

Keywords: Endophytic bacteria, *Andrographis paniculata*, Antimicrobial activity, Antibiotic susceptibility and Human pathogenic bacteria.

INTRODUCTION

The need for new and useful compounds to provide resistance and relief in all aspects of human conditions is over growing. Natural products have been the traditional path finder compounds offering an untold diversity of chemical structures. Even with untold centuries of human experience behind us and a movement into a medium era of misty and automation natural product based compounds have an immerse impact on modern medicine since about 40% of prescription drugs are based on them¹

The phrase Bioprospecting is today most frequently used to describe the collection and screening of biological material for commercial purposes².

Molecules derived from natural products, particularly those products by plant and microbes have an excellent record of providing novel chemical structural compounds for development of new pharmaceutical products. It was not until Pasteur discovered that fermentation is caused by living cells that people seriously began to investigate microorganisms as a source of high value metabolites. Then scientific and the power of observation provide the impetus to Fleming to user in the antibiotic era via the discovery of penicillin from the fungus *Penicillium notatum*.

Since, then people have been engaged in the discovery and application of microbial metabolites with the activity against both plant and human pathogens. Further most the discovery of plethora of microbes for application that span a broad spectrum of utility in medicine, agriculture and industry is now practical³.

Since, the discovery of endophytes by Darnal, Germany in 1904⁴. Various investigations had done on endophytes in different ways, which is usually dependent on the prospective from which the endophytes were being isolated and subsequently examined. Endophytic bacteria are bacteria that live in plant tissue without doing substantive harm or gaining benefit other than residency^{5,6}. It's give an inclusive and widely accepted definition of endophytes – "Microbes that colonize living, internal tissues of plants without carrying any immediate over negative effects"⁷

A review of published endophytic bacteria was reported⁸, but the list is no longer complete, as there is much interest in this area and new endophytes are continuously being reported. Remarkable *Salmonella* strains have been detected as endophytes in Alfalfa sprouts. Outbreak with these bacteria in Alfalfa sprouts have been

recorded in North America, Asia and Europe since 1995. It has been proposed that alfalfa plants and seeds be colonized with safe bacteria to out compete human pathogens. For example; *Enterobacter absuriae* was found to eliminate *Salmonella entanica* and the enterohemorrhagic *Escherichia coli* from *Arabidopsis thaliana* seeds. It is a drawback that there may be human or opportunistic pathogens among plant endophytes.

Secondary metabolites produced by endophytes provide a variety at fitness enhancements such as increased resistant to herbivora, parasitism trough as well as growth enhancements. Endophytic antibacteria isolated from healthy cereal plants were arrested for their ability to control fungal root pathogens at cereal crops both *in vitro* and in planta^{9,10}. A study indicated that endophytic antibacterial may provide an advantage as biological control agents for use in fields, where other failed due to their ability to colonize the internal tissues of the host plane. *In vitro* tests showed that muscodor albas volatiles inhibited and killed wide range of storage pathogens belonging to species Of *Botrytis*, *Collectotrichum*, *Geotrichum*, *Penicillium* and *Rhizopus*. Since muscodor albas is a sterile mycelium and does not require direct contact with the crops to be treated, it could be an attractive biological fumigant for controlling post harvest diseases¹¹.

Generally plant growing in unique environmental settings having special ethnobotanical uses having extreme age or interesting endemic locations possess novel endophytic microorganisms which can supply new leads. About 51% of biologically active substances isolated from endophytic fungi were previously unknown¹². Recently many known as well as new endophytic bioactive metabolites, possessing a wide variety of biological activities as antibiotic, antiviral, anticancer, anti-inflammatory, antioxidant etc., have been identified¹. Microbial endophytes may be very interesting for biotechnological production of bioactive substances as medicinally important agents. Pactitaval a highly functionalized ditropenoid is found in each of the words new (*Taxus*) species. Tias compound is world first billion dollar anticancer drug which produces tuberculin molecules from depolymerizing during the process of cell division. The presence of paelitaxel in few species prompted the study of their endophytes. By the early 1990's however, no endophytic fungi have been isolated from any of the world few species. After several years of efforts a novel paelitaxel producing endophytic fungus. *Taxomyces andreanae* was discovered in *T. Brevitolia*¹. Later interesting compounds were reported to produce by endophytes in particular fungi^{13,14}.

Based on Strobel and Daisy 2003 there should be some Rationale for plant selection

- Plants from unique environmental settings especially those with an unusual biology.
- Plants that have an ethnobotanical history.
- Plant that are endemic that has an unusual longevity.
- Plant growing in areas of great bio diversity also here the prospect of housing endophytes with great biodiversity.

Andrographis paniculata

Andrographis paniculata, the Kalmegh of the Ayurveda, is an erect annual herb extremely bitter in taste in each and every part of the plant body. The plant is known in north-eastern India as 'Maha-tita', literally 'king of bitters', and known by various vernacular names. It is also known as 'Bhui-neem', since the plant, though much smaller in size has a similar appearance and has the same bitter taste as that of Neem (*Azadirachta indica*). In Malaysia, it is known as 'Hempedu Bumi' literally means 'bile of earth' since it is one of the bitterest plants that are used in traditional medicine. In Tamil it is called 'Sirunangai' or 'Siriyanangai'. The genus *Andrographis* consists of 28 species of small annual shrubs essentially distributed in tropical Asia. Only a few species are medicinal, of which *A. paniculata* is the most popular. *A. paniculata* is used in traditional Siddha and Ayurvedic systems of medicine as well as in tribal medicine in India and some other countries for multiple clinical applications like enzyme induction, antihepatotoxic, hepatoprotective. A recent study conducted at Bastyr University, confirms the anti-HIV activity of andrographolide.

MATERIALS AND METHODS

Sample collection

For the isolation of endophytic bacteria, healthy leaves of *Andrographis paniculata* plant were collected from A.K.Padavedu,

Purification, selection and preservation of endophytic bacteria

After incubation, morphologically different bacterial colonies were selected and streaked on nutrient agar plates and incubated at 28°C for 48 hours. After incubation morphological characteristics such as colony colour, size, margin, consistency and texture of all the isolates was observed.

From the total isolates, based on the different in colony morphology, limited numbers of representative isolates were selected from all the plates for further investigation all the selected isolates were sub cultured in Nutrient agar slants and preserved in refrigerator at 4°C.

Preliminary characterization of endophytic bacteria

Phenotypic characteristics such as microscopic characterization (Gram reaction, Endospore staining, Capsule staining, Motility, Catalase and Oxidase activity of all the isolates was performed) by adopting standard procedures.

Preliminary screening

Antimicrobial activity

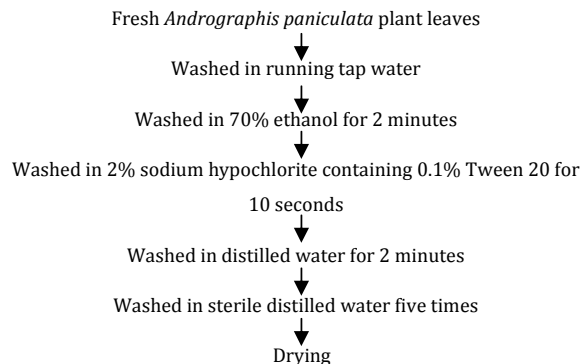
For the preparation of 18 hrs culture, nutrient broth was prepared and all the bacterial isolates were inoculated and incubated at 28°C for the production of antimicrobial compounds by using no 3 medium¹⁵ was prepared and about 10% of inoculums was transferred into it. All the test tubes were incubated in rotary shaker with 95 rpm for 120 hours at 28°C. After incubation, 2 ml of culture broth was taken and separated by centrifugation at 10,000 rpm for 10 minutes. After centrifugation, the culture supernatant was collected and used for antimicrobial activity testing.

Antimicrobial activity of culture supernatant was tested by agar well diffusion method using nutrient agar medium. Test bacterial strains used in this study include human pathogens *Staphylococcus aureus*, *Escherichia coli*, *Proteus species*, *Klebsiella species*, *Pseudomonas species*, *Salmonella typhi* and fish pathogens such as *Vibrio species* and *Salmonella species*. All the cultures were obtained from

Thiruvannamalai District, TamilNadu. All the samples were collected in sterile plastic bags and transported aseptically to the laboratory.

Sample Pretreatment and isolation of endophytic bacteria

For the pretreatment of leaf samples and isolation of endophytic bacteria, the method was adopted with some modifications. All the leaf samples were excised and subjected to a three step surface sterilization procedure.



After pretreatment, the leaves were crushed with sterile distilled water using sterile mortar and pestle. About 1 ml of crushed sample was serially diluted up to 10.5 dilutions using 12.5mM potassium phosphate buffer (pH 7.1) (phosphate buffer). Nutrient agar medium was prepared and used for the isolation of endophytic bacteria about 0.1 ml of aliquot from 10⁻² to 10⁻⁵ dilutions were taken and spreaded on nutrient agar medium using sterile L-rod. Plating was done in duplicates and all the plates were incubated at 28°C for 120 hours.

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18 hours broth cultures of test organism were inoculated into Nutrient agar plates using sterile cotton swab. About 5 mm size well was made and 100 µl of culture supernatant was added into it. All the plates were observed for zone of inhibition after incubation at 37°C for 24 hours.

Effect of sodium chloride on growth of endophytic bacteria

To study the effect of sodium chloride on the growth of endophytic bacteria, nutrient agar medium was prepared by supplementing with different concentration of NaCl and the endophytic bacterial isolates were inoculated into it. All the plates were incubated at 28°C for 5 days and observed for every 24 hours.

Antibiotic susceptibility Patten of endophytic bacteria

Antibiotic susceptibility pattern of endophytic bacterial isolates was determined by adopting Kirby-Bauer disc diffusion method (1961) broth culture of endophytic bacteria was prepared using nutrient agar and adjusted to 0.5 McFarland standards. All the cultures were inoculated into nutrient agar plates using sterile cotton swab. Standard antibiotic disc viz., Ampicillin (30mg), Gentamycin (30mg), Chloramphenicol (10mg), Enythromycin (10mg), Amikacin (30mg), Ciprofloxacin (30mg) were placed on nutrient agar plates and incubated 37°C for 24 hours. After incubation, antibiotic susceptibility pattern was determined by measuring the zone of inhibition.

RESULTS

Isolation of endophytic bacteria

Nutrient agar plates inoculated with *Andrographis paniculata* samples showed morphologically different bacterial colonies. Totally 20 bacterial colonies were recovered from one *Andrographis paniculata* plant sample (Table-1). All isolates were selected for further investigations.

Table 1: Number of bacterial endophytes recovered from selected *Andrographis paniculata*

Plant name	Pigmented colonies	Non-pigmented colonies	Total number of colonies
<i>Andrographis paniculata</i>	8	12	20

Characterization of endophytic bacteria

Cultural characteristic of all the endophytic bacterial isolates were given in table- 2 out of 20 isolates 8 pigmented and 12 non-pigmented organisms were identified. Microscopic characteristics of all the endophytic bacteria are given in table 3. Out of 20 isolates, 11 Gram positive cocci and 9 Gram positive rods were observed. Totally 7 isolates showed positive result for Endospore staining and they were suspected as *Bacillus species*. The other isolates were remained to be identified.

Tables 2: Cultural characterization of endophytic bacteria

S. no	Culture no.	Colour	Size	Margin	Consistency	Texture
1.	C1	Colourless	Small	Irregular	Creamy	Raised
2.	C2	Colourless	small	Irregular	Creamy	Raised
3.	C3	Colourless	Small	Irregular	Creamy	Flat
4.	C4	Colourless	Small	Circular	Slimy	Flat
5.	C5	Colourless	Small	Irregular	Creamy	Raised
6.	C6	Colourless	Small	Irregular	Slimy	Raised
7.	C7	Orange	Small	Circular	Mucoid	Raised
8.	C8	Colourless	Small	Irregular	Slimy	Flat
9.	C9	Orange	Small	Circular	Mucoid	Raised
10.	C10	Colourless	Large	Irregular	Slimy	Flat
11.	C11	Colourless	Small	Circular	Creamy	Raised
12.	C12	Yellow	Small	Circular	Slimy	Raised
13.	C13	Green	Large	Regular	Mucoid	Raised
14.	C14	Yellow	Small	Circular	Slimy	Raised
15.	C15	Greenish yellow	Large	Irregular	Mucoid	Raised
16.	C16	Orange	Small	Circular	Mucoid	Raised
17.	C17	Colourless	Large	Irregular	Mucoid	Raised
18.	C18	Colourless	Small	Irregular	Creamy	Flat
19.	C19	Colourless	Small	Circular	Slimy	Raised
20.	C20	Green	Large	Irregular	Mucoid	Flat

"+" = Positive, "-" = Negative

Table 3: Microscopic characterization of endophytes

S. no	Culture no.	Gram staining	Motility	Catalase	Oxidase	Spore staining
1.	C1	G+ve, cocci.	-	+	-	-
2.	C2	G+ve rods.	+	+	-	+
3.	C3	G+ve, rods.	+	+	-	+
4.	C4	G+ve, cocci.	-	+	-	-
5.	C5	G+ve, rod.	+	+	+	-
6.	C6	G+ve, rods.	-	+	+	+
7.	C7	G+ve, cocci.	-	+	-	-
8.	C8	G+ve, rods.	-	+	-	+
9.	C9	G+ve, cocci.	-	+	+	-
10.	C10	G+ve, rods.	+	+	+	-
11.	C11	G+ve, cocci.	-	+	-	-
12.	C12	G+ve, cocci.	-	+	-	-
13.	C13	G+ve, cocci.	-	+	-	-
14.	C14	G+ve, cocci.	-	+	-	-
15.	C15	G+ve, cocci.	-	+	-	-
16.	C16	G+ve, cocci.	-	+	-	-
17.	C17	G+ve, rods	+	+	+	+
18.	C18	G+ve, rods.	+	+	-	+
19.	C19	G+ve, rods.	+	+	-	+
20.	C20	G+ve, cocci.	-	+	-	-
Total			7	20	4	7

'+' = Positive, '-' = Negative

Biological activity of endophytic bacteria**Antibiotic susceptibility pattern of endophytic bacteria**

Antibiotic susceptibility of endophytic bacteria was given in table 4. Out of 20 isolates, more than 3 isolates were sensitive to Ampicillin, 14 isolates were sensitive to Gentamicin, 16 isolates were sensitive to Chloramphenicol, 6 isolates were sensitive to Erythromycin, 13

isolates were sensitive to Amikacin and 6 were sensitive to Ciprofloxacin.

Antimicrobial activity

Antibacterial activity of endophytic bacteria against human pathogens and fish pathogens were given in table 5. Out of 20 isolates 8 showed inhibition against any of the bacterial strains

tested. Number of endophytic showed activity against different human and fish pathogens.

Out of 20 isolates, 6 strains showed broad spectrum activity. pH of culture filtrate isolates were given in table 5. Totally 20 culture filtrates, 7 filtrates were shows acidic pH, 10 filtrates were shows neutral pH and 4 filtrates were shows alkaline pH.

Effect of sodium chloride on the growth of endophytic bacteria

Growth of endophytic bacteria at different sodium chloride concentration was given in table 6. All the 20 isolates showed good growth at 0% to 5% NaCl concentration. Only 14 isolates showed till 12.5% NaCl concentration. The growth rate is decreased when NaCl concentration is increased.

Table 4: Antibiotic susceptibility pattern of endophytic bacteria: (Zone size in mm)

S.No	Culture No.	Ampicilin		Gentamicin		Chloramphenical		Erythromycin		Amikacin		Ciprofloxacin	
1.	C1	0	R	22	S	16	I	30	S	12	R	23	S
2.	C2	10	R	27	S	28	S	24	S	26	S	20	S
3.	C3	31	S	25	S	24	S	30	S	26	S	20	S
4.	C4	0	R	28	S	25	S	26	S	25	S	0	R
5.	C5	0	R	10	R	25	S	0	R	0	R	0	R
6.	C6	0	R	24	S	23	S	27	S	25	S	14	R
7.	C7	0	R	26	S	18	S	14	I	22	S	12	R
8.	C8	23	S	27	S	25	S	0	R	27	S	10	R
9.	C9	0	R	11	R	23	S	0	R	10	R	0	R
10.	C10	0	R	11	R	22	S	11	R	10	R	0	R
11.	C11	0	R	12	R	27	S	0	R	0	R	0	R
12.	C12	0	R	13	I	28	S	0	R	26	S	17	S
13.	C13	0	R	24	S	28	S	22	I	24	S	0	R
14.	C14	0	R	29	S	0	R	20	I	28	S	16	R
15.	C15	0	R	11	R	23	S	0	R	0	R	0	R
16.	C16	29	S	28	S	23	S	30	S	26	S	19	R
17.	C17	0	R	19	S	27	S	0	R	0	R	0	R
18.	C18	0	R	20	S	0	R	22	I	25	S	0	R
19.	C19	0	R	25	S	15	I	0	R	28	S	0	R
20.	C20	0	R	24	S	22	S	10	I	25	S	0	R
Total		3		14		16		6		13		6	

R- Resistant, S- Sensitive, I- Intermediate

Table 5: Antimicrobial activity of endophytic bacteria

S.No	Culture no.	pH	Human pathogens				Fish pathogens				
1.	C1	7	-	-	-	-	-	-	-	-	-
2.	C2	8	-	-	-	-	-	-	-	-	-
3.	C3	8	-	-	-	-	-	-	-	-	-
4.	C4	7	-	-	-	-	-	-	-	-	-
5.	C5	8	-	-	-	-	-	-	-	-	-
6.	C6	7	-	-	-	-	-	-	-	-	-
7.	C7	7	-	-	-	-	-	-	-	-	-
8.	C8	7	-	-	-	-	-	-	-	-	-
9.	C9	7	-	-	-	-	-	-	-	-	-
10.	C10	7	-	-	-	-	-	-	-	-	-
11.	C11	7	-	-	-	-	-	-	-	-	-
12.	C12	7	-	-	-	-	-	-	-	-	-
13.	C13	4	+	+	+	+	+	+	+	-	-
14.	C14	8	-	-	-	-	-	-	-	-	-
15.	C15	6	+	+	+	+	-	-	-	-	-
16.	C16	6	+	+	-	+	+	+	+	-	+
17.	C17	4	+	+	-	+	+	-	-	-	+
18.	C18	6	+	+	-	+	+	-	-	-	+
19.	C19	4	+	+	+	+	+	+	+	-	+
20.	C20	4	+	+	+	+	+	+	+	-	+
Total			7	7	4	7	6	3	4		5

'+' = Positive, '-' = Negative

DISCUSSION

Various investigators reported endophytic microbes from various plant exists in different ecosystems. It is not worthy that of the nearly 3, 00,000 plant species that exists on earth each individual plant is host to one or more endophytes. Only a few these plants have ever been completely studied relative to their endophytic biology. Consequently the opportunity to find new and interesting microorganism among myriads of plants in different settings and ecosystems is great¹.

Since the number of plant species in the world is so great creative and imaginative strategies must be used to quickly narrow the search for endophytes displaying various bioactivities. With this

view by considering the several reasonable hypothesis. The present investigation was attempted for bioprospecting of endophytic bacteria from selected *Andrographis paniculata* plant A.k. Padavedu, Thiruvannamalai district, Tamilnadu.

In the present study *Andrographis paniculata* plant leaves were collected from A.k.padavedu selective pretreatment is a prerequisite for the isolation of endophytic microbes. Totally 20 bacterial strains were recovered from leaves collected for selected *Andrographis paniculata* plants. All the bacterial strains are markable different from terrestrial bacterial isolates. Pinpointed to larger spread colonies were observed so far these are numerous reports is available on endophytic fungi in mangroves¹⁶. In India also countable number of report showed on diversity of endophytic

bacteria, fungi in medicinal plants¹⁷, but from available literature there is no report on endophytic bacteria from *Andrographis paniculata* particularly in Tamil nadu. In general endophytic bacteria occur as lower population densities than rhizospheric bacteria or bacterial pathogens¹⁸. Based on the visible morphological differences totally 20 bacterial isolates were selected from *Andrographis paniculata* plant samples for further investigation.

Endophytes are the chemical synthesizers with in plants. Many of them are capable of synthesizing bioactive compounds that can be used by plants for defense against pathogens and some of

these compounds have been proved for useful drug discovery. Up to now most of the natural products from endophytes are antibiotics, anticancer agents, biological control agents antivirals, antidiabetic agents and other bioactive compounds by their different functional roles¹⁹. In the present study antimicrobial activity of endophytic bacteria were tested by agar well diffusion method. Out of 20 isolates, 6 showed broad spectrum activity against clinical and fish pathogens. In particular endophytic bacteria from selected *Andrographis paniculata* showed antimicrobial activity.

Table 6: Effect of sodium chloride on the growth of endophytic bacteria

S.No	Culture No.	0% NaCl	2.5% NaCl	5%NaCl	7.5%NaCl	10%NaCl	12.5%NaCl
1.	C1	+++	+++	+++	+++	++	+
2.	C2	+++	+++	+++	+++	++	+
3.	C3	+++	+++	+++	+++	++	+
4.	C4	+++	+++	+++	+++	++	+
5.	C5	+++	+++	+++	+++	++	+
6.	C6	+++	+++	+++	-	-	-
7.	C7	+++	+++	+++	+++	++	+
8.	C8	+++	+++	+++	+++	++	+
9.	C9	+++	+++	+++	-	-	-
10.	C10	+++	+++	+++	+++	++	+
11.	C11	+++	+++	+++	+++	++	+
12.	C12	+++	+++	+++	+++	++	+
13.	C13	+++	+++	+++	-	-	-
14.	C14	+++	+++	+++	+++	++	+
15.	C15	+++	+++	+++	+++	++	+
16.	C16	+++	+++	+++	+++	++	+
17.	C17	+++	+++	+++	-	-	-
18.	C18	+++	+++	+++	+++	++	+
19.	C19	+++	+++	+++	-	-	-
20.	C20	+++	+++	+++	-	-	+
Total		20	20	20	14	14	14

+ = Poor, ++ = Moderate, +++ = Good

Emergence of antibiotic resistance among the pathogenic microorganisms limits treatment options. Antibiotic resistant genes, in addition to clinical pathogens, are also present in environmental isolates which are horizontally transferred to other microorganisms. In the present study susceptibility pattern of endophytic bacteria were studied using 6 different antibiotics. Most of the isolates (3-16) are sensitive to Ampicillin, Ciprofloxacin, Erythromycin, Chloramphenicol, Amikacin, and Gentamycin but some are resistant to all antibiotics.

In the present study salt tolerance of endophytic bacteria were tested in which growth was observed up to 12.5% NaCl concentration but growth rate was decreased when the salt concentration increased.

The present study concludes that this work may be the first report on endophytic bacteria from *Andrographis paniculata* in our state (Tamilnadu). This study evidenced that *Andrographis paniculata* are the potential but under exploited resources for bioactive endophytic bacteria since the exploited bacteria isolated from *Andrographis paniculata* in this study showed promising antimicrobial, enzymatic, plant growth promoting substance, Biodegrading activities and biosurfactant activities. Detailed investigations on *Andrographis paniculata* plant endophytic bacteria were needed to prove its potential further and if will leads to the discovery of numerous high value metabolites. This is suggested for further work.

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