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Isolation and Characterization of Endophytes from *Justicia Adhatoda* as a Source of Novel Antibiotics

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Abstract: World human population is increasing with an alarming rate; and a variety of new types of health issues are popping up. For instance, increase in number of drug-resistant bacteria is a cause of concern. Research on antibiotics and other microbial natural products is pivotal in the global fight against the growing problem of antibiotic resistance. It is necessary to find new antibiotics to tackle this problem. The use of therapeutic plant species in traditional medicine is as old as mankind; and currently, it is strongly believed that all types of plant species across the plant kingdom do harbour Endophytic Bacteria (EB). The Endophytes derived natural products such as Ecomycins, Pseudomycins, Munumbicins and Xiamycins are antibacterial, antimycotic and antiplasmodial. Some of these natural products have been reported to possess even antiviral (including Human Immunodeficiency Virus (HIV) properties. Ndophyrtic bacteria could serve as a potential source of novel antibiotics. This study aimed to isolate and characterize plant Endophytes and antimicrobial activity of endophyte was investigated against selected pathogenic strains.

Keywords: Antibiotics, Endophytes, Antibacterial, Antimycotic

I. INTRODUCTION

Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Mahesh B et.al). It has been established that up to 25% of the drugs prescribed in conventional medicines are allied directly or indirectly to natural substances mostly of plant origin. In recent years, pharmaceutical companies have spent a lot of time and money in developing natural products extracted from plants, to produce more cost-effective remedies that are affordable to the population (Doughier JH). Traditionally used medicinal plants produce a variety of compounds of known therapeutic properties. In recent years, antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. However, very little information is available on such activity of medicinal plant (Ahmad I et.al).

Considering the vast potentiality of plants as sources for antimicrobial drugs with reference to antibacterial and antifungal agents, a systematic investigation was undertaken to screen the antibacterial and antifungal activity from *J. adhatoda. Justicia adhatoda* L. (Family Acanthaceae) is a shrub, Widespread throughout the tropical regions of Southeast Asia (K halekuzzaman M et.al). The name, *J. adhatoda* L. is commonly known as Vasaka or Malabar nut. It is a perennial, evergreen and highly branched shrub (1.0 m to 2.5 m height) with unpleasant smell and bitter taste. It has opposite ascending branches with white, pink or purple flowers. It is a highly valuable Ayurvedic medicinal plant used to treat cold, cough, asthma and tuberculosis (Dhankhar S et.al).

Its main action is expectorant and antispasmodic (bronchodilator) (K arthikeyan A et.al). Moreover, the importance of Vasaka plant in the treatment of respiratory disorders can be understood from the ancient. Indian saying, "No man suffering from phthisis needs despair as long as the Vasaka plant exists". Thus the frequent use of *J. adhatoda* has resulted in its inclusion in the WHO manual "The Use of Traditional Medicine in Primary Health Care" which is intended for health workers in south-east Asia to keep them informed of the restorative utility of their surrounding flora[5].The major alkaloids of the plant, vasicine and vasicinone, have been found to be biologically active and are the area under discussion of many chemical compounds and pharmacological studies. The source of the drug 'Vasaka' is well known in the indigenous system of medicine for its beneficial effects, particularly in bronchitis (K umar A et.al).

In the present study, the antimicrobial activity of leaflet explants of *J. adhatoda* was determined against Gram negative pathogenic bacteria along with vasicine and reference antibiotics using disc diffusion method.

II. REVIEW OF LITERATURE

Some of these observations have helped in identifying the active principle responsible for such activities and in the developing drugs for the therapeutic use in human beings. Adams, C. 1972. Flowering plants of Jamaica. Aldén, B., S. Ryman & M. Hjertson. 2009. Chinese Academy of Sciences. 1959–. Flora republican popularize sinicae. Council of

Scientific and Industrial Research, India. 1985–1992. The wealth of India: a dictionary of Indian raw materials and industrial products. Raw materials (revised edition). New Delhi. However, not many reports are available on the exploitation of antifungal or antibacterial property of plants for developing commercial formulations for applications in crop protection. Emergence of multi drug resistance in human and animal pathogenic bacteria as well as undesirable side effects of certain antibiotics has triggered immense interest in the search for new antimicrobial drugs of plant origin. Pseudomonas aeruginosa was the most resistant strain of all the bacteria used in this study. Hara, H. et al. 1978–1982.An enumeration of the flowering plants of Nepal. In fact, Gram negative bacteria, especially P. aeruginosa are frequently reported to have developed multi drug resistance to many of the antibiotics. Therefore, it is not surprising to learn that P. aeruginosa is the least responding bacterial strain to the tested plant extract. When comparing the antimicrobial activity of the tested samples with the reference antibiotics, the inhibitory potency of tested extracts could mostly be considered as important (Table 1) and Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the in vivo antibacterial activity assay. Many reports are available on the antiviral, antibacterial, antifungal, antihelminthic, antimolluscal and anti-inflammatory properties of plants. Some of these observations have helped in identifying the active principle responsible for such activities & in the develop drug for therapeutic use in human beings. However, not many reports are available on the exploitation of antifungal or antibacterial property of plants for developing commercial formulation for application in crop protection. Emergence of multi drug resistance in human & animal pathogenic bacteria as well as undesirable side effects of certain antibiotics has triggered immense interest in the search for new antimicrobial drugs of plant origin. Pseudomonas aeruginosa was the most resistant strain of all the bacteria used in this study.

III. MATERIALS AND METHODS

3.1 Surface Sterilization:

- Washed plant material/ explants in tap water and immersed it in 5% solution of liquid detergent 10-15 min .
- Wash the material thoroughly in tap water and finally distilled water.
- Dip the explants in70% ethyl alcohol for 30 sec.
- Then Keep it in 0.1% mercuric chloride (HgCl2) for 2 min.
- After 2 min decant the sterilant and wash the explants thoroughly with D.W. to remove all traces of sterilant.

3.2 Isolation of Endophytes:

- Make a Nutrient Agar and Potato Dextrose agar plates.
- Keep a leaflet Disc of *A. justicia* L. on NA and PDA plates.
- Plates should be incubated overnight at an incubation temperature of 37°C (98.6°F).[3]
- Make A pure culture on other plates.

3.3 Colony Characterization:

- Observe the size, shape, colour, opacity, margin and consistency of colony which is observed on NA plates.
- Gram staining:
  1. Prepare a smear of bacterial sample and heat-fix smears.
  2. Stain the slides as follows:
     1. Flood the crystal violet for one minute.
     2. Pour off excess dye and wash gently in tap water and drain the slide against a paper towel.
     3. Expose the smears to Gram's iodine for one minute by washing with iodine, then adding more iodine and leaving it on the smear until the minute is over.
     4. Wash with tap water and drain carefully. (Do not blot.)
     5. Wash with 95% alcohol for 30 seconds.
     6. Wash with tap water at the end of the 30 seconds to stop the decolourization. Drain.
     7. Counterstain with 0.25% safranin for 30 seconds.
     8. Wash, drain, blot, and examine under oil.
     9. Draw the cells showing morphology, grouping, and relative sizes. Color a few of the cells of each bacterial species to show the Gram reaction.

a) CATALASE TEST:

Principle:

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen (such as vegetables, fruit or animals). It catalyzes the decomposition of hydrogen peroxide to water and oxygen.[1] It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS). Likewise, catalase has one of the highest

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turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second.[2]
Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long.[3] It contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide.

Procedure:

1. Spread the bacteria on an agar plate and incubate the plate over night (18-24 h) under appropriate conditions.
2. Collect bacteria from one colony with a sterile inoculating loop (of plastic or platinum) and apply the bacteria in a test tube.
3. Add one drop of 3% H_2O_2 to the bacteria and observe the suspension. Be careful with the handling of H_2O_2 which is corrosive!!!

b) Oxidase Test:

Principle:

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless.

Procedure:

1. Soak a small piece of filter paper in 1% Kovács oxidase reagent and let dry.
2. Use a loop and pick a well-isolated colony from a fresh (18- to 24-hour culture) bacterial plate and rub onto treated filter paper
3. Observe for color changes.

3.4 Disc Diffusion Method:

Incubation Procedure

1. Using an aseptic technique, place a sterile swab into the broth culture of a specific organism and then gently remove the excess liquid by gently pressing or rotating the swab against the inside of the tube.
2. Using the swab, streak the nutrient Agar plate to form a bacterial lawn.
3. To obtain uniform growth, spread the plate with the swab.
4. Allow the plate to dry for approximately 5 minutes.
5. Use an Antibiotic Disc Dispenser to dispense disks containing specific antibiotics onto the plate.
6. Using a flame-sterilized forceps, gently press each disc to the agar to ensure that the disc is attached to the agar.
7. Plates should be incubated overnight at an incubation temperature of 37°C (98.6°F).[3]

Biochemical Tests:

- **Gelatine hydrolysis:** The purpose is to see if the microbe can use the protein gelatine a source of carbon and energy for growth. Use of gelatin is accomplished by the enzyme gelatinase**.**

Method;

1. Prepare the gelatin medium plate.
2. By using the sterile inoculating loop pick up in inoculums from the culture tube of the unknown bacterium.
3. Immediately streak or spread the inoculums into the nutrient gelatin medium plate.
4. Keep the inoculated plate into the incubator at 37^0C.

- **Starch Hydrolysis:** A medium containing starch is used. After inoculation and overnight incubation, iodine reagent is added to detect the presence of starch. Iodine reagent complexes with starch to form a blue-black color in the culture medium. Clear halos surrounding colonies is indicative of their ability to digest the starch in the medium due to the presence of alpha-amylase.

Method:-

1. Prepare the starch medium plate.
2. By using the sterile inoculating loop pick up in inoculums from the culture tube of the unknown bacterium.
3. Immediately streak or spread the inoculums into the nutrient starch medium plate.
4. Keep the inoculated plate into the incubator at 37^0C.

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IV. RESULTS

1. Colony Characterization:

Size	0.1mm
Shape	Circular
Colour	Cream
Opacity	Opaque
Margin	Entire
Consistency	Smooth
Gram staining	Gram –ve rod
Catalase Test	Positive
Oxidase Test	Positive

2. Catalase Test: Positive

After addition of bacterial culture air bubbles are get formed of oxygen due to decomposition of H_2O_2

3. Oxidase Test: Positive

As the color changes to dark purple within 5 to 10 seconds.

4. Biochemical tests:

Sr.no.	Biochemical test	Result
1.	Gelatin hydrolysis	Positive
2.	Starch hydrolysis	Negative

So, the organism isolated is *P.aeruginosa*

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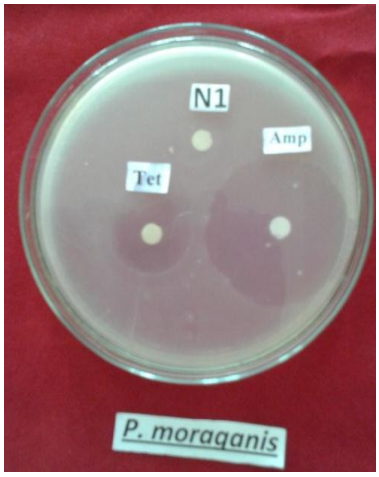
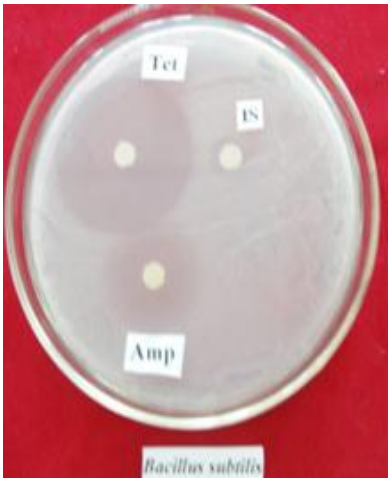
5. Antimicrobial activity of Endophytes:



Proteus moraganis

***Xanthomonas axonopodis* pv. Citri**



Bacillus subtilis

Antibacterial activity of endophyates against different bacteria

Sr. No.	Name of the bacterium	Diameter of inhibitory zone (mm)
1	*Proteus morgani* NCIM 2719	21.0
2	*Xanthomonas citri*	20.5
3	*Bacillus subtilis* NCIM 2063	19.0

V. CONCLUSION

A diverse microbial community was isolated from *Justicia Adhatoda*, with notable inhibitory activities against gram positive and gram-negative bacteria. From these results, it can be concluded that endophytic derived crude extracts isolated from plant produce potential bioactive compounds which should be explored further for their biological activities. Therefore, to deal with increasing number of drug-resistant pathogens Endophytes could serve as a potential source of novel antibiotics. The natural therapeutic compounds produced by Endophytes do have several potential applications in pharmaceutical industry.

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