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STUDY OF *AEGLE MARMELOS L.* FOR PRESENCE OF POTENTIAL ENDOPHYTES

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ABSTRACT

The well known medicinal tree, *Aegle marmelos L.* is known to be rich in numerous bioactive substances. Its extracts have been used as an effective remedy for many disorders. It has been demonstrated that endophytes of various plants produce bioactives associated with those plants. Hence, we have proceeded to isolate endophytes from Bael and screened them for their ability to produce some of the active substances associated with this plant. Production of these bioactives via their endophytes would provide an economical and rapid means of producing them in large quantities and in a purer form. Bacterial endophytes were isolated from *Aegle marmelos L.* and identified on the basis of their biochemical, morphological and cultural characteristics. They were further screened for their ability to produce various bioactive compounds. Some of these isolates demonstrated the ability to produce phenolic compounds. These compounds are being identified and assessed for their medicinal properties.

KEYWORDS: Endophytes, medicinal plants, *Aegle marmelos* L., Bioactive compounds.

1. INTRODUCTION

1.1. Endophytes

The most significant associations have been the mutually beneficial associations between plants and microorganisms. Bacteria in plants often stimulate defense responses. It is hypothesized that the bacterial endophytes stimulate plant growth and/or prevent competing organisms from colonizing the plant. The bacteria isolated from the rhizosphere of the plants also share a mutualistic relation with the plant. They provide the plant with requisite nutrients and benefit with the root exudates. Such bacteria and fungi have been isolated from several groups of plants. Many bacterial species of genera including *Azoarcus, Herbaspirillium,*

Azospirillium, Gluconacetobacter, Klebsiella, Serratia and *Burkholderia* have been found in important crops plants like rice, maize, wheat, sugar beet, sweet potato, coffee, cotton, squash, sweet corn and some C-4 plants like alfalfa, and temperate and tropical grasses. [1,2] Endophytic organisms inhabit vast and diverse niches within plants without any apparent and observable symptoms. They are mostly harmless and benefit the plant in a mutual association. These organisms may be localized at a point of entry or spread throughout the plant residing within cells in the intercellular spaces or in the vascular system. Bacterial endophytes, both gram positive and gram negative have been isolated from surface sterilized plant tissues or extracted from internal plant tissues. The endophytic populations in plants vary with plant source, plant age, tissue type, time of sampling and environment. The endophytic load is generally higher in roots and lower in stems and leaves.[3]

1.2. Production of bioactive compounds

Many of the isolated endophytes have been found to be beneficial to the host plant in a numerous ways such as promoting plant growth and rendering the host plant resistant to pathogens. Some endophytes discovered in medicinal plants produce compounds with medicinal properties. The medicinal compounds from plants have been actually found to be produced by the residing endophytes themselves. Isolation of endophytes from some plants has led to discovery of new compounds which have potential pharmaceutical and agricultural applications. Following are some of the endophytes isolated from different plants.

Table 1: Endophytic isolates and their sources

Source	Endophytes	Benefits	References
Elm trees (Ulmus species)	*Phomopsis oblonga*	Biocontrol agent (Protection against the beetle *Physocnemum brevilineum*)	Azevedo J. L. et. al.
Scotch pine, citrus, crotalaria	*Methylobacterium* species	Nitrogen fixation	Araujo W. L. et. al.
Typha australis	*Klebsiella oxytoca*	IAA production and Phosphate solublization	Jha P. N. et. al.
Piper nigrum L.	*Bacillus* species, *Psuedomonas* species	Biocontrol against *Phytopthora capsici*	Aravind R. et. al.
Wheat (*Triticum aestivum*)	*Klebsiella pneumonia*	Plant growth promoter	Sachdev D. P. et. al.
Glycine max, Glycine soja	*Tsukamurella inchonensis Bacillus fastidiosus*	Phytohormones production	Hung P. Q. et. al

1.3. Endophytes in Medicinal plants.

Aegle marmelos L. family Rutaceae is commonly known as Bael or Bilva tree. It is popularly held sacred by Hindus and offered in payers and is also known as Shivaduma (the tree of Shiva). This tree has originated in Eastern ghats and central India. It is indigenous to Indian subcontinent. *Aegle marmelos L.* is a slow growing, medium sized (12 to 15 m tall) deciduous tree. It has a short trunk, thick, soft, flaking bark, and spreading branches which sometimes bear spines. It has alternate leaves, borne singly or in 2's and 3's and are composed of 3 to 5 oval, pointed, shallowly toothed leaflets 4-10 cm long, 2-5 cm wide, the terminal one with a long petiole. The fruits are oblong, yellowish green with small dots on outer surface.[9] The phytochemical profile of this plant shows presence of varied classes of chemicals such as alkaloids, coumarins, terpenoids, fatty acids etc. In addition certain important compounds associated with the plant are γ-sitosterol, aegelin, lupeol, rutin, marmesinin, β-sitosterol, flavones, glycosides, Oisopentenyl halfordiol, marmelin and phenylethyl cinnamides.[9] The presence of these phytochemicals explains the wide use of this plant in traditional Indian medicinal systems. The tree shows various pharmacognistic activities like antifungal, anti-microfilarial, antioxidant, analgesic, hepatoprotective, radioprotective, anti-depressant, anti-inflammatory, anti-pyretic, hypoglycaemic, anti-ulcer, hypolipidemic and immuno modulatory activity.[10] Bael leaves are used in treatment of jaundice, leucorrhoea, conjunctivitis and deafness. The fruit has carminative and astringent properties and is used in thyroid related disorders. Some other therapeutic uses are cardiac stimulant, swollen joints, pregnancy trouble, typhoid, coma and irritable bowel syndrome. [11]

2. MATERIALS AND METHODS

2.1. Collection of leaves

Young leaves of *Aegle marmelos L.* were carefully selected and pruned from the tree and transported to the lab for investigation within 2 hours of collection. These leaves were washed clean with tap water, dried and then screened for the presence of endophytes.

2.2. Screening of leaves for endophytes.

The leaves were initially cleaned with sterile distilled water, followed by treatment with 70% ethanol for 3 minutes. This was followed by another wash with sterile distilled water. The leaves were then treated with 3.5% (v/v) sodium hypochlorite for 2 minutes and then rinsed with sterile distilled water three times for 1 minute.[12, 13]

The lower surface of the leaves was gently scrapped with a sterile scalpel to expose the inner cortex and then the leaves were laid on the nutrient agar plates with the exposed cortex facing

downwards and in contact with the agar. The plates were then incubated at room temperature for a week.

To ensure that the leaf surface was thoroughly sterilized, the water from last wash given to the leaves was plated and incubated separately.

2.3. Study of endophytic isolates

The endophytic isolates were obtained on the plates which were further purified and sub cultured on nutrient agar slants. The isolates were then subjected to biochemical tests for identification. They were also tested for production of phenolic compounds. A Qualitative test employing 10% lead acetate was carried out.[14] An assay was then performed to estimate the total phenolic content by Folin Ciocalteau method according to Chen J. C. et. al. (2007).[15]

3. RESULTS AND DISCUSSION

Endophytic growth was obtained along the periphery of the leaf after the incubation of 24 hours (Figure 1). The growth was streaked on nutrient agar plates and subcultured further till pure strains were isolated. Two types of bacterial strains were isolated from the endophytic growth. The gram staining and microscopic examination of the isolates confirmed that the isolates were bacterial. The plates were incubated further for a week to allow the growth of additional slow growing endophytes if any. However, no additional endophytes were obtained.

The isolated endophytic bacterial strains are gram positive rods with endospores. Their biochemical characteristics are listed in the Table 2. These endophytes belong to genus Bacillus. The isolates showed different characteristics like the different colony color and different arrangement of cells observed in gram staining. Isolate 1 (Figure 2), showed cells in long chains whereas isolate 2 (Figure 3), showed cells in association of 2 or 3 cells. Hence, both the isolates were further studied for production of bioactive compounds. The culture broths of both the isolates gave white precipitate after reaction with lead acetate and hence tested positive for production of phenolic compounds. Therefore estimation of total phenolic production was done by a spectrophotometric assay performed by Folin Ciocalteau method. The assay was carried out in triplicates and the average quantity was reported. The quantity of phenolic compounds produced by the isolates is listed in Table 3.

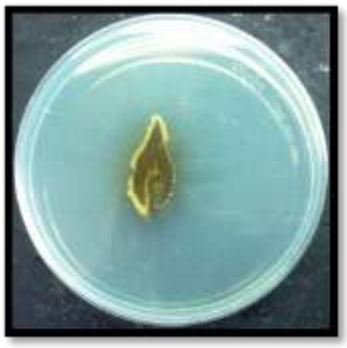

Figure 1: Endophytic growth along the leaf

Figure 2: Isolate 1: Gram positive rods in long chains

Figure 3: Isolate 2: Gram positive rods in short chains

Table 2: Biochemical characteristics of endophytes

Endophytes	Isolate 1	Isolate 2
Colony morphology	**Slightly raised, opaque, light cream coloured colonies**	**Flat, opaque, Dark cream coloured colonies**
Gram nature	Gram positive Rods in long chains	Gram positive Rods in clusters and chains of twos and threes
Capsule	Present	Present
Motility	Non motile	Non motile
Catalase	Positive	Positive
Oxidase	Negative	Negative
NaCl tolerance	6%	6%
Starch hydrolysis	No	No
Genus	Bacillus	Bacillus

Table 3: Total phenolic contents produced by endophytic isolates in a liquid media

Isolates	Average total phenolic content (µg/ml)
Isolate 1	12.83
Isolate 2	11.33

4. CONCLUSION

This study demonstrates the presence of bacterial endophytes inside the tissues- leaves, of *Aegle marmelos L*. The endophytes also showed a significant production of phenolic compounds. The phytochemical profile of the plant under study shows presence of several phenolic compounds. It remains to be studied whether the phenolic compound produced by the endophytic isolates is one of those assumed to be produced by the plant. Hence this study could be further extended to identification of the phenolic compounds produced by the endophytes and their possible application in the fields of agriculture and pharma. The study has thus opened a new avenue for research of renewable, faster and eco friendly resources.

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