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Sachin Jain
Department of Veterinary Pharmacology & Toxicology, College of Veterinary Science and A.H., Jabalpur, Madhya Pradesh, India

RK Sharma
Department of Veterinary Pharmacology & Toxicology, College of Veterinary Science and A.H., Jabalpur, Madhya Pradesh, India

Vidhi Gautam
Department of Veterinary Pharmacology & Toxicology, College of Veterinary Science and A.H., Jabalpur, Madhya Pradesh, India

K Shrman
Department of Veterinary Pharmacology & Toxicology, College of Veterinary Science and A.H., Jabalpur, Madhya Pradesh, India

Arpita Shrivastav
Department of Veterinary Pharmacology & Toxicology, College of Veterinary Science and A.H., Jabalpur, Madhya Pradesh, India

Alka Sawarkar
Department of Veterinary Pharmacology & Toxicology, College of Veterinary Science and A.H., Jabalpur, Madhya Pradesh, India

Corresponding Author:
Sachin Jain
Department of Veterinary Pharmacology & Toxicology, College of Veterinary Science and A.H., Jabalpur, Madhya Pradesh, India

Morphological characterization and enzymatic activity of endophytic bacteria from *Aegle marmelos* leaves

Sachin Jain, RK Sharma, Vidhi Gautam, K Shrman, Arpita Shrivastav and Alka Sawarkar

Abstract
The present study was conducted for morphological characterization and detection of extracellular hydrolytic enzyme activity of endophytic bacteria isolated from *Aegle marmelos* i.e. common Indian plant Bael. In this study, total fifteen bacterial isolates of *Aegle marmelos* leaves of which were collected from three different areas of Jabalpur *viz*. Jawaharlal Nehru Krishi Vishwa Vidyalaya (J.N.K.V.V), Tropical Forest Research Institute (T.F.R.I.), State Forest Research Institute (S.F.R.I), Jabalpur. Five bacterial isolates were collected from the leaves of Bael of each area. All fifteen bacterial isolates were identified by morphological and enzymatic activity tests. Growth characteristics of endophytic bacteria isolated from *Aegle marmelos* on King's B media showed that 60 per cent of isolates were irregular in shape while 40 per cent were circular in shape, 73.33 per cent showed flat elevation on petriplate while 26.67 per cent were of raised elevation, margin of 73.33 per cent colonies were undulated while 26.67 per cent showed entire, the surface of the growth was smooth for the undulated colonies and 80 per cent growth were opaque and white in colour. The endophytic bacteria isolated from *Aegle marmelos* were evaluated for the presence of active hydrolytic enzymes including cellulase, amylase and protease. No cellulolytic, amylolytic and proteolytic activity was observed as there was no clearing of zones in agar plates observed for the endophytic bacterial isolates of *Aegle marmelos.*

Keywords: Endophytic bacteria, *Aegle marmelos*, bacterial isolates, enzymatic activity

Introduction
Plants produce a diverse range of bioactive molecules making them a rich source of different types of medicines (Stuffness and Douros, 1982) [18]. Higher plants as sources of medicinal compounds have continued to play a dominant role in the maintenance of human health care since ancient times. Over 50 per cent of all modern clinical drugs are of natural origin. Hence, they play a vital role in modern drug development in the pharmaceutical industry (Baker *et al*., 1995) [4]. Plants with possible antimicrobial activity should be tested against an appropriate microbial model to confirm the activity and to ascertain the parameters associated with it. The effects of plant extract on bacteria have been studied by a very large number of researchers in different parts of the world (Ates and Erdogrul, 2003) [2].

The need for new natural and useful compounds to provide assistance and relief in all aspects of human and animal health is ever growing with the passage of time. Due to this, there is a general call for constructive interactions with pharmaceutical industry for ensuring and encouraging the development of new antibiotics and chemotherapeutic agents which have low toxicity and are highly effective with negligible environmental impact (Pal and Paul, 2013) [12]. Many of the recent researches are pointing towards yielding novel natural pharmaceutically active compounds from various plant sources (Bahgat *et al*., 2014) [3].

The term "endophyte" is derived from the Greek, endon = within and phyte = plant. It was first introduced in 1866 by de Bary (Arnold, 2008) [1]. Endophytes refer to the microorganisms (mostly fungi and bacteria) colonising the intercellular and intracellular regions of healthy plant tissues at a particular time, whose presence is unobtrusive and asymptomatic. Sometimes, the medicinal properties of plants result from the endophytes present in the host plant and the type of biologically secondary metabolites that are produced by those endophytes (Schulz and Boyle, 2006) [15].

Endophytes are microorganisms that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host (Schulz and Boyle, 2006) [15].

Endophytes are intra/intercellular organisms, often fungi and bacteria that reside in living plant cells. The relationship that they establish with the plant varies from symbiotic to pathogenic. Endophytes establish a mutualistic symbiosis with their hosts because they are selectively favoured. They are classified according to their colonization strategy. Obligatory endophytes are unable to proliferate outside the plant. Facultative endophytes live in the soil and colonize the plant when the opportunity arises. Passive endophytes colonize the plant through open wounds in the root hairs (Rosales *et al*., 2017) [13]. Endophytic bacteria seem to be distributed in most plant species and have been isolated from roots, leaves and stems and a few from flowers, fruits and seeds (Lodewyckx *et al*., 2002) [10]. Endophytes existing in plants have a wide range of antimicrobial strains, which are the important potential sources of antimicrobial substances. Some endophytes could excrete antimicrobial compounds that may be involved in a symbiotic association with a host plant. Bacterial endophytes have been shown to prevent disease development through endophyte-mediated *de novo* synthesis of novel compounds and antifungal metabolites. Investigation of the biodiversity of endophytic strains for novel metabolites may identify new drugs for effective treatment of diseases in humans, plants and animals (Strobel *et al.*, 2004) [17].

Aegle marmelos belongs to the family Rutaceae, commonly called as Bael (English), and is found throughout India. Bael is a medium sized decidous tree bearing strong axillary thorns. The leaves, bark, roots, fruits and seeds are used extensively in the Indian traditional system of medicine, the Ayurveda, and in various folk medicines to treat myriad ailments. Bael Leaves with 3 or 5 leaflets, are extremely useful for treating diabetes, jaundice, cholera and asthma. Bael leaves are made into a poultice and used in the treatment of ophthalmic disorder. Bael leaf poultice is applied to inflammations with black pepper for reducing edema (Kirthikar and Basu, 1999) [9].

Materials and Methods

Location and place of work

The proposed work was conducted in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, N.D.V.S.U., Jabalpur (M.P.).

Collection of leaves

Fresh mature leaves of *Aegle marmelos* (Plate 01) were procured from three different places of Jabalpur *viz*. Jawaharlal Nehru Krishi Vishwa Vidyalaya (J.N.K.V.V), Tropical Forest Research Institute (T.F.R.I.), State Forest Research Institute (S.F.R.I), Jabalpur. Samples were immediately brought to laboratory and were used within 24 hrs and finally processed for isolation of endophytic bacteria.

Plate 1: Fresh mature leaves of *Aegle marmelos*

Sterilization of leaves

Sterilization of leaves and isolation of endophytic bacteria from the leaves (Plate 02) was done according to Mahajan *et al.* (2014) [11] with some modifications.

Sterility check

To confirm that the surface of leaves were effectively sterilized, 1 ml of the sterile distilled water that was used in final rinse of surface sterilization procedures was plated on to nutrient agar media and incubated at 37°C for 24 hrs. Then bacterial growths were observed.

Preparation and sterilization of media

King's B (KB) media (HiMedia), Mueller Hinton media (HiMedia), Blood agar media (HiMedia) and BHI broth (HiMedia) were prepared by adding agar into the distilled water. Hot plate was used for the proper mixing of media and autoclaved at 121°C for 15-20 minutes at 15 lbs (Plate 2)

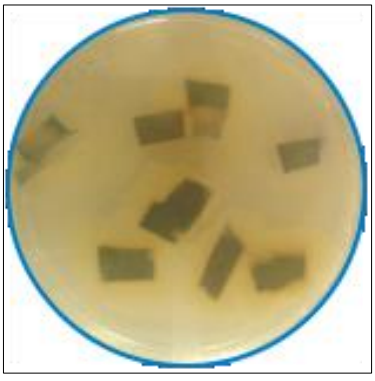


Plate 2: Sterilization of leaves and isolation of endophytic bacteria

Morphological characterization
Elevation, margin, surface, opacity and chromo genesis of isolated endophytic bacterial colonies were noted.

Enzymatic activity test
The agar diffusion method was used to detect extracellular hydrolytic enzyme activity. The isolates were grown on different indicator media including cellulase activity indicator medium, amylase activity indicator medium and protease activity indicator medium. Bacterial cultures were incubated at 30°C for 48 h. Clearing zones in the medium, indicating positive enzymatic activity, were recorded (El-Deeb *et al*., 2013) [6].

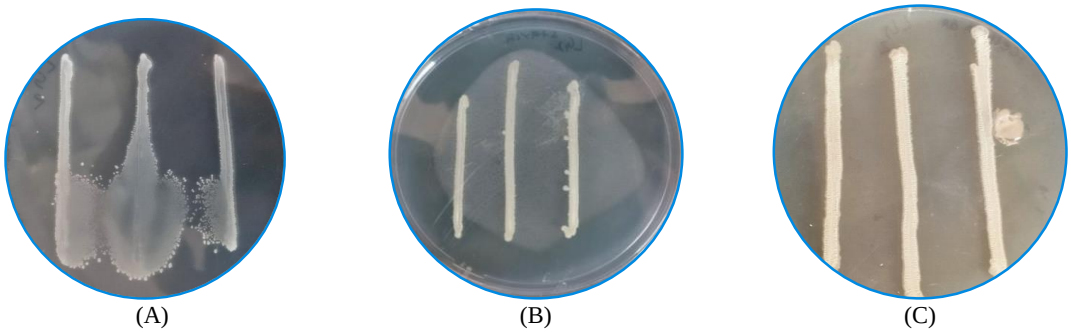
Results and Discussion
The present study was conducted to detect extracellular hydrolytic enzyme activity by agar diffusion method. In this study, total fifteen bacterial isolates of *Aegle marmelos* leaves of which were collected from three different areas of Jabalpur *viz*. Jawaharlal Nehru Krishi Vishwa Vidyalaya (J.N.K.V.V), Tropical Forest Research Institute (T.F.R.I.), State Forest Research Institute (S.F.R.I), Jabalpur. Five bacterial isolates were collected from the leaves of Bael of each area. All fifteen bacterial isolates were identified by morphological and enzymatic activity tests.
The sterilized leaves of *Aegle marmelos* were placed on the King's B media and incubated at 37°C for 24 hrs. The morphological characterization of endophytic bacterial isolates exhibited diverse colonies, texture, shapes and margins including round to irregular colonies which were white in colour, mucoid, soft with wavy and irregular margins. (Table - 01)
The population density of endophytic bacteria varied from 10^2 to 10^9 which depends on many factors, including the plants being studied, the part under analysis, developmental stage of the plant cultivar (genotype) and the interaction with other organisms, as well as other environment related factors (Costa *et al*., 2012) [5].

Table 1: Growth of endophytic bacteria isolated from leaves of *Aegle marmelos* on King's B media

Isolate No.	Form	Elevation	Margin	Surface	Opacity	Chromo-genesis
J1	Circular	Flat	Undulated	Smooth	Opaque	Absent
J2	Irregular	Flat	Undulated	Smooth	Opaque	Absent
J3	Circular	Flat	Undulated	Smooth	Opaque	Absent
J4	Irregular	Raised	Entire	Smooth	Opaque	Absent
J5	Irregular	Flat	Undulated	Smooth	Glistening	Absent
T1	Irregular	Flat	Undulated	Smooth	Opaque	Absent
T2	Circular	Raised	Entire	Smooth	Opaque	Absent
T3	Irregular	Flat	Undulated	Smooth	Glistening	Absent
T4	Irregular	Flat	Undulated	Smooth	Opaque	Absent
T5	Circular	Flat	Entire	Smooth	Opaque	Absent
S1	Irregular	Raised	Undulated	Smooth	Opaque	Absent
S2	Circular	Flat	Undulated	Smooth	Opaque	Absent
S3	Irregular	Flat	Undulated	Smooth	Opaque	Absent
S4	Circular	Raised	Entire	Smooth	Glistening	Absent
S5	Irregular	Flat	Undulated	Smooth	Opaque	Absent

1J where, J=J.N.K.V.V., S= S.F.R.I, T= T.F.R.I.

Enzymatic activity test
The endophytic bacteria isolated from *Aegle marmelos* were evaluated for the presence of active hydrolytic enzymes including cellulase, amylase and protease. No cellulolytic, amylolytic and proteolytic activity was observed as there was no clearing of zones in agar plates observed for the endophytic bacterial isolates of *Aegle marmelos* (Table - 02).The present findings were very near to the work of Soman (2018) [16] where she found that endophytic bacteria isolated from different varieties of babool leaves did not show enzymatic activity reaction (Cellulase activity, amylase activity and protease activity). Khanam and Chandra (2015) [8] conducted a study in which the isolates from the dye yielding plant *Beta vulgaris* did not show any enzymatic activity reaction. Kewat (2019) [7] found that endophytic bacteria isolated from *Moringa oleifera* and *Cymbopogon citrates* did not show any enzymatic activity reaction. Sawarkar (2021) [14] also found that endophytic bacteria isolated from *Tamarindus indica* did not show any enzymatic activity reaction. However, El-Deeb *et al.*, (2013) [6] found that endophytic bacteria isolated from *Plectranthus tenuiflorus* had exhibited extracellular enzymatic activity (Plate 3).



(A) (B) (C)

Plate 3: Enzymatic activity (A) Cellulase activity, (B) Amylase activity and (C) Esterase activity of endophytic bacteria isolated from leaves of *Aegle marmelos*

Table 2: Enzymatic activity test reaction of endophytic bacteria isolated from leaves of *Aegle marmelos*

Isolate No.	Cellulase activity	Amylase activity	Protease activity
J1	Negative	Negative	Negative
J2	Negative	Negative	Negative
J3	Negative	Negative	Negative
J4	Negative	Negative	Negative
J5	Negative	Negative	Negative
T1	Negative	Negative	Negative
T2	Negative	Negative	Negative
T3	Negative	Negative	Negative
T4	Negative	Negative	Negative
T5	Negative	Negative	Negative
S1	Negative	Negative	Negative
S2	Negative	Negative	Negative
S3	Negative	Negative	Negative
S4	Negative	Negative	Negative
S5	Negative	Negative	Negative

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