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STUDY OF ENDOPHYTIC BACTERIA FROM *Aegle marmelos*

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ABSTRACT

Man cannot survive on earth for long time without plants. A number of traditional herbal medicinal plants are used for management of various diseases. Therapeutic use of medicinal plants has become popular because of its inability to cause side effects and combat antibiotic resistance in microorganisms. *Aegle marmelos* is one of the most important medicinal plants. All parts of plant such as leaves, root, flower, fruit, and seeds have medicinal properties. *Aegle marmelos* shows anticancer, antidiabetic, antiulcer, anti-inflammatory activities. The present study was carried out on the endophytic bacteria of this plant. Endophytic bacteria are one of the most potential biological control agents in disease protection. These endophytes are also used to obtain the bioactive compounds and applied in biotransformation process. They improve the phyto-remediation of water soluble, volatile organic pollutants. Endophytic colonizing bacteria from *Aegle marmelos* (Balefal) were isolated and characterized. Their colony and Gram characteristics were studied. Antibacterial and anti-oxidant properties of the cultures were analyzed and results of the same are presented. The secondary metabolites produced by these cultures were further studied by extracting these compounds. Thus in the near future, *Aegle marmelos* (Baelfal) extract could be further exploited as a source of useful phytochemical compounds & may play a very important role in modern system of medicine.

KEYWORDS: Endophytic bacteria, *Aegle marmelos*, antibacterial, antifungal, anti-oxidant.

INTRODUCTION

Endophytes are microorganisms (bacteria) which inhabit in healthy living plant tissue for all or part of their life cycle without causing apparent harmful symptoms to the host. Endophytes are the microbes which colonize living internal tissues of plants without causing any

immediate, overt negative effects.[1] Many of the endophytes are known to produce bioactive compounds that can be used by the host plant for their defense against different phytopathogens. Some of these compounds have been proven for novel drug discovery.[2]

Aegle marmelos is important medicinal plant. It is subtropical species. Belongs to family Rutaceae and subfamily Aurantioideae. Also known as golden apple, Bengal quince, stone apple, etc. This species is native to India and is sacred to Hindus. *Aegle marmelos* is slow growing, medium sized tree, up to 12-15 m tall with short trunk, thick, soft, flaking bark, sometime spiny branches the lower ones drooping the fruit, round, pyriform, oval, or oblong, 5-20 cm in diameter, may have thin, hard, woody, shell, gray-green until the fruit is fully ripe, when it turns yellowish. Inside, there is a hard central core & 8-20 faintly defined triangular segments, with thin, dark orange walls, filled with aromatic, pale orange, pasty sweet pulp. These plant was selected because every part of plant such as leaves, root, flower, fruit, seed is useful because they shows bioactivity, antidiabetic activity, antioxidant activity, antimalarial activity, anticancer activity, antiviral, antifungal activity.[3,4] Leaves are used in treatment of dyspepsia and sinusitis.

Work on isolation and studies on the endophytic fungi of *Aegle marmelos* have been reported earlier. Gond *et al*. (2007) [5] have isolated and characterized a total of 79 endophytic fungi belonging to 21 genera from *Aegle marmelos*. *Bartaliniarobillardoides*, a coelomycetous fungus, isolated as an endophyte from the leaves of *Aegle marmelos* was shown to produce the taxol, an anticancer drug. However, a lacunae of information exits as far as the bacterial endophytes of *Aegle marmelos* are concerned.[6]

MATERIALS AND METHODS

Isolation of endophytic bacteria from *Aegle marmelos* fruit

Fruits were obtained from trees growing in Thiba palace area of Ratnagiri city, Maharashtra (India). The fruits were washed with tap water and wiped dry. Then wiped with alcohol, broken and aseptically opened. The pulp was added in sterile 10ml saline. Then the pulp was crushed & mixed well. 0.1 ml of the mixture was taken and spread on sterile nutrient agar plate & incubated at 37 °C for 24 hours. The colonies obtained were studied for colony morphology. The colonies were further purified by streaking and maintained on nutrient agar slants.

Antibacterial activity

Loopful of endophytic bacterial culture from slant, was inoculated into sterile nutrient broth and kept on shaker at 120 rpm for 24 hours. The culture broth was centrifuged at 5000 rpm for 15 minutes. 0.1ml overnight grown culture of *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus and Bacillus subtilis* were spread plated on sterile nutrient agar plates. Then wells were bored on plates with the help of sterile cork borer. 100 µl of supernatant was added into each well. The plates were placed in fridge for 10 minutes and then transferred to incubator at 37°C for 48 hours. The zones of inhibition around each well were measured.

Photochemical screening[7,8]

Phytochemical screenings were performed using standard procedures as follows.

a.Test for phenols

To 0.5 ml each of the culture supernatant, 2 ml of ferric chloride was added. A reddish brown coloration at the interface indicates the presence of phenols.

b.Test for terpenoids (Salkowski test)

To 0.5 ml each of the culture supernatant, 2 ml of chloroform was added. Concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown coloration at the interface indicates the presence of terpenoids.

c.Test for flavonoids

Three methods were used to test for flavonoids.

First, diluted ammonia (5 ml) was added to a portion of supernatant followed by addition of concentrated sulphuric acid (1ml). A yellow coloration that disappears on standing indicates the presence of flavonoids.

Second, a few drops of 1% aluminium solution were added to a portion of the supernatant. A yellow coloration indicates the presence of flavonoids. Third, a portion of the culture supernatant was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of diluted ammonia solution. A yellow coloration indicates the presence of flavonoids.

d.Test for saponins

To 0.5 ml each of the culture supernatants, 5 ml of distilled water was added in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing

was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

e.Test for tannins

To 0.5 ml each of the culture supernatants a few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

f.Test for alkaloids

To 5 ml of the culture supernatant, 2 ml of diluted ammonia was added followed by addition of 5 ml of chloroform and shaken gently to extract the alkaloid base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids

Extraction of secondary metabolite

The culture supernatant of the endophytes was subjected to solvent extraction using ethyl acetate. Three times the volume of ethyl acetate was added to separating funnel containing acidified culture supernatant and shaken well. After standing for 10 minutes, the organic phase was collected. This was evaporated to concentrate the product and used for further analysis. The extract was scanned on UV-Vis spectroscopy between 250-700nm.

Thin layer chromatography

The ethyl acetate extract of culture supernatants was spotted on silica gel TLC plates and run in solvent system Toluene: Ethyl acetate (4:1). The plates after the run were developed in iodine chamber. The number of spots obtained were noted and the Rf values were calculated.

RESULTS AND DISCUSSION

De Bary (1866)[9] first introduced the term epiphytes for fungi that live on the surface of their host and endophytes for those living inside the plant tissue. Microbial extracts of endophytes have been and continue to be a productive source of new biologically active molecules for drug discovery.[10]

In the present study, *Aegle marmelos* was selected for studying its endophytic composition and characterization of its active biomolecules produced by these endophytes. This is the first work to be carried out and reported, on *Aegle marmelos* from Western Maharashtra.

Four endophytic bacterial isolates were obtained from *Aegle marmelos.* Morphological characterization of the colonies is summarized in Table 1 and in Fig. 1. All the isolates were Gram positive cocci in nature. No Gram negative isolates were obtained.

Table 1: Colony morphology of the *Aegle marmelos* endophytes

Endophyte	Size	Shape	Colour	Margin	Elevation	Opacity	Consistency	Gram nature	Morphology
Bael 1	3-4 mm	Circular	Green	Entire	Flat	Opaque	Mucoid	Gram positive	Cocci
Bael 2	1-2 mm	Circular	Red	Ir-regular	Raised	Opaque	Butyrous	Gram positive	Cocci
Bael 3	4-5 mm	Irregular	White	Irregular	Flat	Opaque	Mucoid	Gram positive	Cocci
Bael 4	1-2 mm	Circular	Orange	Entire	Flat	Opaque	Mucoid	Gram positive	Cocci

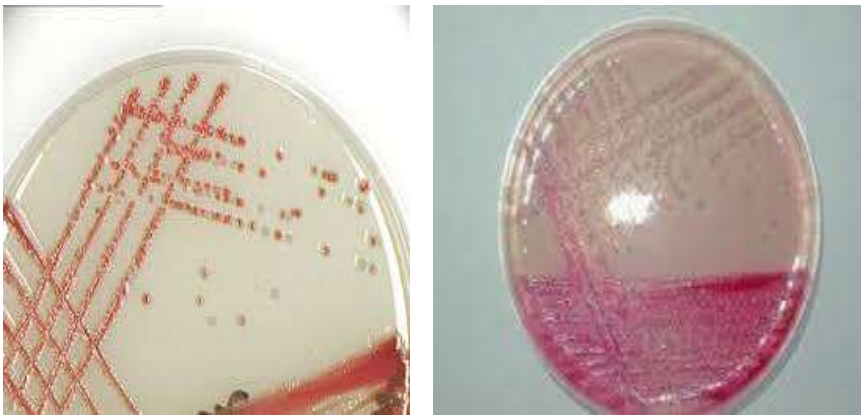

Fig 1: Endophytic bacteria isolated from *Aegle marmelos*

Endophytic bacteria isolates from *Aegle marmelos* showed the zone of inhibition against the test organisms: *Bacillus subtilis, Klebsiella pneumonia, Staphylococcus aureus & E.coli* (Table 2)

Table 2: Zones of inhibition of *Aegle marmelos* endophytes against bacterial culture

Endophyte	*Bacillus subtilis*	*Klebsiella pneumonia*	*Staphylococcus aureus*	*E.coli*
Bael 1	-	15 mm	-	-
Bael 2	15 mm	13 mm	20 mm	-
Bael 3	20 mm	20 mm	-	-
Bael 4	20 mm	-	-	20 mm

Most of the isolates showed inhibition against test organism *Bacillus subtilis*, endophyte Bael 1 showed zone of inhibition only against the test organism *Klebsiella pneumoniae*. Endophyte Bael 2 showed antimicrobial activity against all the test organisms used accept for

E. coli while Bael 4 did not inhibit the growth of *Klebsiella pneumonia* and *Staphylococcus aureus. Bacillus subtilis* was inhibited by endophytes Bael 2,3,,4 while *Klebsiella pneumonia* was inhibited by Bael 1,2,3. Only Bael 2 could inhibit the growth of *Staphylococcus aureus* and *E. coli* was inhibited by Bael 4 (Table 2). Thus it is evident that Bael endophytes have antibacterial activity and the mechanism of action may be the blockage of protein synthesis either at transcription or translation level and/or peptide-glycan synthesis at membrane level. The results of the present investigation on antimicrobial activity revealed a broad spectrum of antibacterial property. The culture supernatents of these endophytes exhibited potent antibacterial activity both against Gram positive and Gram negative bacteria. The antibacterial activity of endophytic bacteria has been well documented.[11]

Antifungal assay of isolated endophytes from *Aegle marmelos*

All the 4 isolates were screened against *Candida albicans, Aspergillus* and *Penicillium*. Bael 2 and Bael 3 does not show the zone of inhibition against to test fungal cultures i.e. *Candida albicans, Aspergillus* sp. *and Penicillium* sp. Only Bael 1 gave the positive result i.e culture showed the zone of inhibition against to the *Candida albicans* and *Aspergillus* sp. of 22 mm and 25 mm, respectively.

Table 3: Zones of Inhibition of *Aegle marmelos* endophyte against fungal culture

Endophyte	*Candida albicans*	*Aspergillus sp.*	*Penicillium sp.*
Bael 1	22mm	25 mm	-

Endophytic bacteria from *Aegle marmelos* the showed antifungal activity against to the *Candida albicans and Aspergillus.* Endophytic bacterial components may interfere with the ca +2 - dipicolonic acid metabolism pathway and possibly inhibit spore germination. Thus it exhibit the antifungal activity by lowering the vegetative fungal body inside the host or in solid medium that means the play important role in controlling the fungal infection such as candidiasis, nail infection.[12]

These isolated endophytic bacteria also showed the antioxidant property mainly due to phenolic and flavonoid content. During oxidation free radical are generated and are implicated for many disease including diabetes mellitus, cancer, aging, arthritis, etc. In treatment of these conditions, antioxidant therapy using antioxidant compounds, that are pharmacologically potent and have low or no side effect, has gained most importance. The phenolic compounds found in bael extracts possess potent antioxidants which helped in

reducing gastric ulcers. Presence of alkaloids in fruits of *Aegle marmelos* has been reported.[13-16]

In case of *Aegle marmelos* endophytic bacterial cultures i.e. Bael 1, 2 and 3 showed the presence of phenol, terpenoids and tannin except Bael 3 for tannin test.

Table 4: Phytochemical screening of *Aegle marmelos* endophytes

Endophyte	Phenol	Terpenoids	Tannin
Bael 1	+	++	+
Bael 2	+	+	+
Bael 3	+	+	-

TLC the ethyl acetate extract of culture supernatant was carried out using mobile phase Toluene: ethyl acetate and Rf value for the spot obtained from culture Bael1 was found to be 0.30 (Fig.2). TLC spot of ethyl acetate extract was scraped and eluted in ethyl acetate and scanned using UV-Vis spectrophotometer. Peaks obtained are tabulated in Table 5.

Fig. 2: TLC profile of ethyl acetate extract of culture supernatant of endophyte Bael 1

Table 5: UV-Vis spectrum of ethyl acetate extract of culture supernatant of endophyte Bael 1.

ID	WL(nm)	ABS
1	528	0.714
2	480	0.679
3	381	0.952
4	341	1.068
5	332	1.015

After taking the UV Visible spectrophotometer analysis culture showed the maximum peak in the range of 200-300 nm. This means extracted secondary metabolites contain phenolic group, their nucleus have the ring structure.

Presence of antioxidant properties in the extracts of *Aegle marmelos* have been reported by number of workers.[7,8] The properties have been attributed to the presence of flavonoids and polyphenols in the plant. The endophytic bacterial isolates of the fruit of *Aegle marmelos* have antioxidant properties as shown by the host plant and furthermore the presence of phenols in the culture supernatants of the endophytic bacterial cultures strengthen the findings.

From the results, the potential value of investigating metabolite production by endophytic bacteria from indigenous plants were demonstrated. Since they are a source promising antimicrobial compounds against various human pathogenic microbes. It would be of interest to find out which functional group is responsible for the bioactivity and also whether any of them is a novel compound which would make it a promising candidate for the production of new antimicrobials. Further work on these metabolites will reveal this and will also aid in gaining insight of synergism among the different functional groups. Thus in the near future, Bael fruit endophytic bacteria *e*xtracts could be further exploited as a source of useful compounds and may play a very important role in modern system of medicine.

REFERENCES

1. Bacon C.W. and White J. F. 2000 Microbial Endophytes. Marcel Dekker Inc Ny
2. Guo B., Wang Y., Sun X. and Tang K. 2008 Bioactive Natural Products From Endophytes: A Review. Appl. Biochem. Microbiol., 44: 136.
3. Maity P., Hansda D., Bandyopadhyay U. and Mishra D.K. 2009 Biological Activities Of Crude Extracts And Chemical Constituents Of Bael, *Aegle marmelos* (L.) Corr. Indian J Exp Biol., 47(11): 849-61.
4. Rahman S. and Pravin R. 2014. Therapeutic potential of *Aegle marmmelos* (L) – An Overview. Asian Pacific Journal of Tropical Disease., 4: 71-11
5. Gond, S., Verma V., Kumar A., Kumar V. and Kharwar R. 2007 Study Of Endophytic Fungal Community From Different Parts Of *Aegle marmelos* Correae (Rutaceae) From Varanasi (India) World J Microbiol Biotechnol., 23: 1371–5.
6. Gangadevi V. and Muthumary J. 2008 Taxol, An Anticancer Drug Produced By An Endophytic Fungus *Bartaliniarobillardoides tassi*, Isolated From A Medicinal Plant, *Aegle marmelos* Correa Ex Roxb. W J Microbiol Biotechnol., 24: 717- 724.

7. Rajan, S. Gokila, M. Jency, P. Brindha P.and Sujatha R. K. 2011 Antioxidant And Phytochemical Properties Of *Aegle marmelos* Fruit Pulp. International Journal Of Current Pharmaceutical Research., 3: 65-70.
8. Vanitha Reddy P., Sahana N. and Ashna U. 2012 Antioxidant Activities Of *Aegle marmelos* And *Psidium guajava* Leaves. Int. J. Med. Aromatic Plts., 2: 155-160.
9. De Bary, A. 1866. Morphologie und Physiologie Pilze, Flechten, und myxomyceten. Hofmeister's Handbook of Physiological Botany. Vol. 2. Leipzig.
10. Arunachalam C. and Gayathri P. 2010 Studies On Bioprospecting Of Endophytic Bacteria From The Medicinal Plant Of *Andrograrphis paniculata* For Their Antimicrobial Activity And Antibiotic Susceptibility Pattern. International Journal Of Current Pharmacology., 4: 63-68.
11. Pimentel M. R, Molina G, Dion´Isio A. P, Junior M.R.M and Pastore G.M. 2010 The Use Of Endophytes To Obtain Bioactive Compounds And Their Application In Biotransformation Process. Biotechnol Res Int., 2011; 1-12
12. Dhankhar S., Ruhil S., Balhara M., Dhankhar S. and Chhillar A. 2011 *Aegle marmelos* (Linn.) Correa: A Potential Source Of Phytomedicine. J Med Plant Res., 5: 1497–1507.
13. Sharma, B.R. Rattan R.K. and Sharma P. 1981. Marmeline, An Alkaloid, And Other Components Of Unripe Fruits Of *Aegle marmelos*., 20 11: 2606–2607.
14. Govindachari, T.R. and Premila M.S. 1983. Some Alkaloids From *Aegle marmelos*. Phytochem., 22 3: 755–757.
15. Riyanto, S., Sukari MA. and Rahmani M. 2001. Alkaloids From *Aegle marmelos* (Rutacea). Malaysian J Anal Sci., 72: 463–465.
16. Lanjhiyana, S., Patra K.C., Ahirwar D. et.al 2012. A Validated HPTLC Method For Simultaneous Estimation Of Two Marker Compounds In *Aegle marmelos* (L.) Corr., (Rutaceae) Root Bark. Der Pharm Lett., 41: 92–97.