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Studies on the biodiversity of endophytic fungi from *Ruta graveolens* and screening for their antimicrobial activities

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ABSTRACT

Aims: The main objective of the present study was to study the diversity of endophytic fungi from *Ruta graveolens*, an important medicinal plant. The alkaloids produced by this plant have been used in many medical applications. The endophytic fungi that inhabit the plants are also recognized as rich sources of secondary metabolites. This study was aimed to isolate, identify and study the diversity of endophytic fungi in *R. graveolens* and to screen the isolates for their antimicrobial activities.
Methodology and results: A total of 12 different fungal genera were isolated from *R. graveolens* collected from various sites in and around Bangalore. The species richness and colonizing frequency of endophytic fungi in this plant are comparatively less than other plants. This may be due to the secretion of the plant's phytochemicals, as it has antimicrobial activity and more than 120 phytochemicals in it. Screening of antimicrobial activity of all 10 isolates was done by agar well diffusion method, of which 80% of the fungal isolates could produce antimicrobials.
Conclusion, significance and impact of study: To conclude *R. graveolens* being a good medicinal plant along with its rich source of endophytes and their medicinal properties, can be exploited for the therapeutic applications.

Keywords: Antibacterial activity, *Epicoccum sorginum*, *R. graveolens*

INTRODUCTION

Ruta graveolens has been utilised in traditional medicine in ancient nations. It is indigenous to southern Europe, northern Africa and the Mediterranean region, and known for its antioxidant (Sailani and Moeini, 2007), anti-inflammatory (Ratheesh and Helen, 2007), anti-tumor (Preethi *et al*., 2006; Réthy *et al*., 2007); anti-arrhythmic (Khori *et al*., 2008); antimicrobial (Ojala *et al*., 2000; Alzoreky and Nakahara, 2003; Ivanova *et al*., 2005; Khouri and El-Akawi, 2005; Harat *et al*., 2008) properties. The phytochemical studies of *R. graveolens* have revealed the presence of more than 120 natural compounds, including flavonoids, rutin, quercetin, furocoumarin, furanoacridones, coumarins-ruamarin, rutacultin, kokusaginine, 6-methoxydictamnine and edulinine (Steck *et al.*, 1971); quioline, quinolone, an essential oil that contains undecan-2 (Kirtikar and Basu, 2003).

Endophytic organisms have received considerable attention as they protect their host against insect pests and pathogens (Webber, 2000). They also play a crucial role in promoting the growth of host plants and give resistance to environmental stress (Webber, 2000). Paul and Jayashree (2014) has reported seven fungal species such as *Aspergillus flavipes*, *Bipolaris nodulosa*, *Aspergillus niger*, *Cunninghamella blacksleena*, *Rhizopus nodusus*, *Nigrospora sphaerica* and *Fusarium avenaceum*. A review of the literature suggests that endophytic fungi can produce novel bioactive compounds, which could be used in the discovery of new antimicrobial and anticancer agents (Chandra, 2012; Gutierrez *et al.*, 2012). Réthy *et al.* (2019) synthesized a silver nanoparticle from *Alternaria tenussima* an endophytic fungus from *R. graveolens* and studied its antimicrobial and antioxidant activities. And, through a review of the literature, it was found that very little research has happened in studying the endophytic fungi in *R. graveolens*, an important medicinal plant. Hence, this study was aimed to explore the diversity and antibacterial activity of endophytic fungi in *R. graveolens* collected from various locations.

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MATERIALS AND METHODS

Isolation, identification and diversity of endophytic fungi from *R. graveolens*

Plant collection

Healthy, fresh plant samples were collected from different places, including nurseries and natural habitats in and around Bangalore, Karnataka, in February 2019, brought to the laboratory (Figure 1) and used to isolate endophytic fungi from it. The rational selection of host plants is vital to increase the chances of isolation of novel microorganisms which may produce new bioactive compounds (Al-Shuneigat *et al*., 2015).

Isolation of endophytic fungi from R. graveolens

For the isolation of fungal endophytes, the fresh root, stem, leaves and flowers of *R. graveolens* were used as explants. Plant parts were rinsed in running water to remove dust debris. After proper washing, parts of the plants were cut into small bits. Isolation of endophytic fungi was done according to the method described by Arnold *et al.* (2000). The samples' surface sterilisation was accomplished by washing with 70% ethanol for 2 min, 3% sodium hypochlorite solution for 3-5 min and rinsing in sterile distilled water. These segments were then placed in a test tube containing sterile water and the plant sample was crushed, serially diluted and inoculated using the pour plate technique.

Petri plates containing potato dextrose agar media supplemented with kanamycin to inhibit the growth of bacteria. All the inoculated plates were kept for incubation at 25-30 °C and observed for the growth of endophytic fungi.

After the incubation period, the plates were observed for the growth of fungus on the explants. The fungus was identified based on morphological (microscopic and culture characteristics) features like colony characterization, growth of fungi (slow-growing or fast-growing), the colour of the colony (front and reverse), conidial development, size and shape of conidia, the shape of the conidial head and attachment of conidia. Barnett and Hunter (1998) and Gilman's (1957) classification concept was used for identifying fungal isolates. The pure cultures of all the isolated and identified fungal isolates were maintained on PDA slants and stored in 15% glycerol stock and used for further research.

Extraction of the secondary metabolite from endophytic fungi

The production of the metabolites from fungal isolates was done as suggested by (VanderMolen *et al.*, 2013); the pure culture of the fungal isolates was inoculated into Czapek's Dox broth under aseptic condition and incubated at 25-30 °C for 15 days in an orbital shaker. The extraction of the secondary metabolite was done as

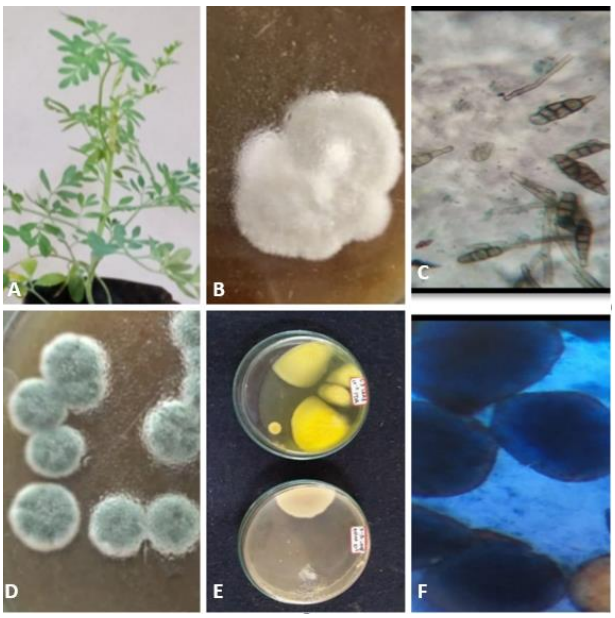

Figure 1: (A) *Ruta graveolens*; (B-F) Endophytic fungi isolated from *R. graveolens.*

described by Dos Santos *et al.* (2015) and Deepthi *et al*. (2018). The fermented broths were filtered through filter paper and the filtrates were extracted 2-3 times with an equal volume of ethyl acetate using a separation funnel. The ethyl acetate extract was separated from the aqueous extract. The mycelial mat obtained was washed, dried and homogenized with ethyl acetate pooled together and dried using a rotary vacuum evaporator. The residue obtained was used for further analysis.

Screening for antimicrobial activity of endophytic fungi isolated from *R. graveolens*

Kirby-Bauer agar diffusion method is used to determine the antibacterial activities of the fungal extract, according to NCCLS standards (Bauer *et al*., 1966; NCCLS, 2000) against seven bacterial cultures (*Bacillus subtilis*, *Escherichia coli*, *Micrococcus luteus*, *Citrobacter freundii*, *Klebsiella* sp., *Staphylococcus aureus* and *Serratia marcescens*) obtained from the Department of Microbiology, RCASC, Bangalore, were used in the present study. All the bacterial cultures were grown in nutrient broth and incubated at 37 °C for 24 h nutrient agar of about 20 mL was poured into sterile Petri plates aseptically and allowed to solidify. Six (6) mm diameter was made on the surface of the medium with the help of a sterilized cork borer. The bacterial suspensions were made and swabbed on the solidified media using sterilized swabs in the respective plates. The crude fungal extract was dissolved in dimethyl sulfoxide and the suspension was sterilized by filtration through a membrane filter (Turkoglu *et al*., 2007). The crude fungal extract of about 100 µg/mL was filled into the wells of the agar plates and the methanolic extract of *R. graveolens*

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was also screened for antimicrobial activity. Penicillin and Tetracycline were used as standard antibiotics. All the inoculated plates were incubated at 37 °C for 24 h to 48 h to observe the zone of inhibition around the sample or antibiotic disc.

RESULTS

Isolation, identification and diversity of endophytic fungi from *R. graveolens*

Ruta graveolens were collected from a different location in Bangalore. The root, stem and leaf of *R. graveolens* were the samples used to isolate fungal endophytes (Arnold *et al.*, 2000; Ivanova *et al*., 2005). The endophytic fungi isolated were identified based on morphological (microscopic and culture characteristics) features like colony characterization, growth of fungi, the colour of the colony (front and reverse), conidial development, size and shape of conidia, the shape of a conidial head and attachment of conidia (Gilman, 1957; Barnett and Hunter, 1998).

A total of 12 distinct fungal genera were isolated from *R. graveolens* that had been collected from various locations. Of these, 11 belonged to the subdivision Deuteromycotina (*Fusarium* sp., *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., *Phoma* sp., *Chaetomium* sp., *Alternaria* sp., *Oospora* sp., *Curvularia* sp., *Epicoccum* sp.) and 01 belongs to division Zygomycotina (*Rhizopus* sp.) and one *Mycelia sterilia* (Figure 2). *Cladosporium* sp. showed the highest colonizing unit, followed by *Alternaria* sp., *Penicillium* sp. The diversity index of endophytic fungi in *R. graveolens* has been elaborated on in Table 1. Species richness was more in *R. graveolens* collected from GKVK, followed by Nelamangala, Vijayanagar and comparatively less in a sample collected from Lalbagh, Bangalore. The percentage of colonization, species richness and diversity index of endophytic fungi was more in the sample collected from its natural habitat. It has been observed that the plant sample collected from nurseries has less diversity of endophytic fungi may be due to ecological variation. Leaf tissue showed more biodiversity of Endophytic fungi followed by root and stem. A similar report had been reported by Rajeswari *et al*. (2016). This study suggests that endophytic fungi are both host and tissue-specific. "It also confirms that despite the ecological variation, there were little differences in the species richness of fungal endophytes recovered from plants in all sites" (Aharwal *et al*., 2018). Medicinal plants have fewer endophytes compared to non-medicinal plants, maybe due to phytochemicals present in the medicinal plants where they have some role in the colonization of endophytic fungi since they contain

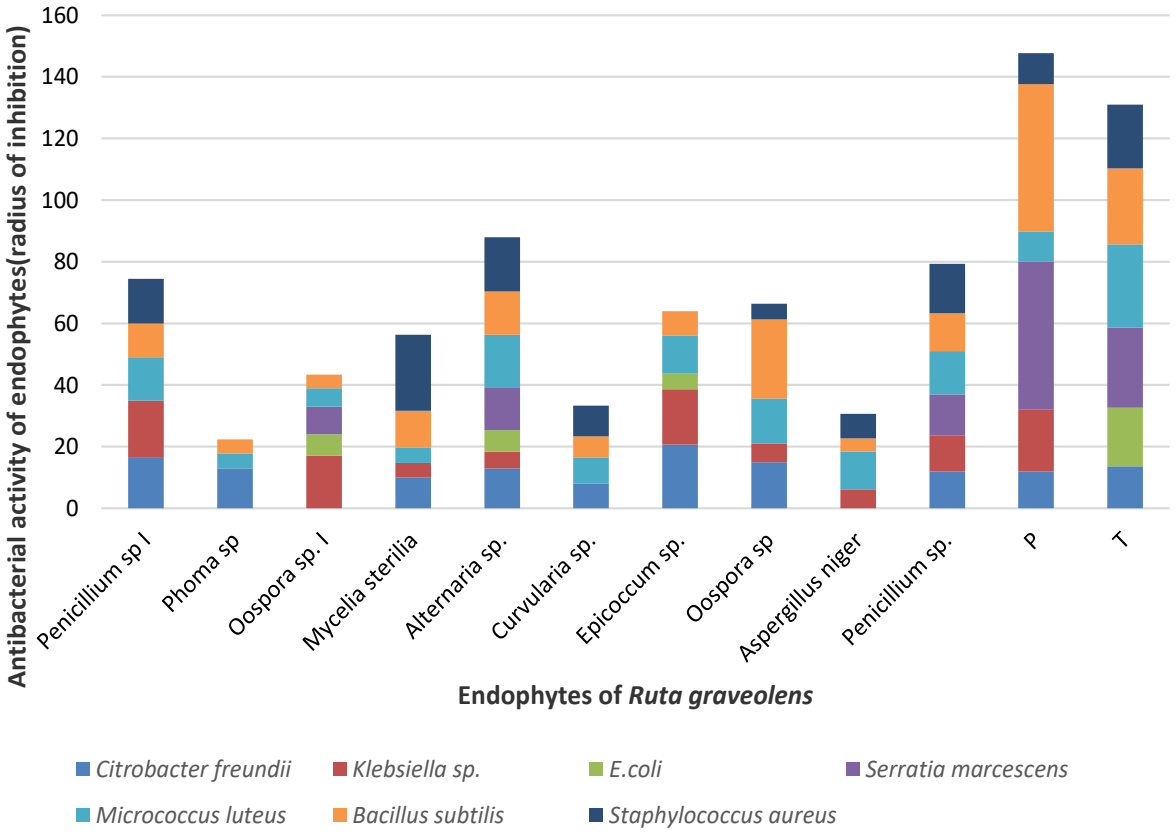

Figure 2: Antibacterial activity of endophytic fungi isolated from *R. graveolens*.

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Table 1: Diversity Index of different endophytic fungi from *R. graveolens* form localities in and around Bangalore.

	GKVK	Vijayanagar	Nelamangala	Lalbagh
No. of species	6	4	5	3
Total no. of individuals	56	26	103	26
Simpson's diversity index	0.8365	0.9035	0.632	0.724
Shannon's diversity index	0.569	0.403	0.579	0.253
Evenness	0.321	0.338	0.287	0.178

antifungal compounds (Balasubramanian *et al*., 2010). The percentage of colonization, species richness and diversity index of endophytic fungi was more in the sample collected from its natural habitat. It has been observed that the plant sample collected from nurseries has less diversity of endophytic fungi may be due to ecological variation. Leaf tissue showed more biodiversity of endophytic fungi followed by root and stem. A similar research finding has been reported by Paul and Jayashree (2014), and Rajeswari *et al*. (2016). This study suggests that endophytic fungi are both host and tissue-specific. "It also confirms that despite the ecological variation, there were little differences in the species richness of fungal endophytes recovered from plants in all sites" (Aharwal *et al.*, 2018). Medicinal plants have fewer endophytes compared to non-medicinal plants, maybe due to phytochemicals present in the medicinal plants where they have some role in the colonization of endophytic fungi since they contain antifungal compounds (Balasubramanian *et al.*, 2010).

Antibacterial activity of endophytic fungi isolated from *R. graveolens*

The pure cultures of the isolated fungi were used for mass production. The pure culture of the fungal isolate was inoculated into the sterilized Czapek Dox broth and incubated at 25 to 30 ° for 2 to 3 weeks, as shown in Figure 3C. The pure culture was extracted by maceration with ethyl acetate with a ratio of 1:1 for 24 h and filtered. The filtrate was evaporated to produce the extract. The extract obtained was used to screen for antimicrobial activity (Tayung and Jha, 2010). Kirby-Bauer agar diffusion method is used to determine the activities of the fungal extract, according to NCCLS standards. Among 12 endophytes isolated, 09 of the isolates showed antimicrobial activity against test organisms, as shown in Figures 3A and 3B. 88% of fungal isolates showed antimicrobial activity against *C. freundii* of which *Epicoccum* sp. showed maximum activity, followed by *Penicillium* sp., *Alternaria* sp., *Phoma* sp., *Curvularia* sp., *Oospora* sp. and *Klebsiella* sp. were found to be sensitive to 88% of fungal isolates, of which *Epicoccum* sp. showed maximum activity, followed by *Penicillium* sp. However, all other isolates showed very little action.

Only 30% of the fungal isolates showed positive activity against *E. coli*, of which *Epicoccum* sp., *Oospora* and *M. sterilia* showed positive activity. Thirty-three (33) % of isolated endophytes from *R. graveolens* were found to be sensitive to *S. marcescens* of which *Penicillium* sp. showed the maximum activity. However, compared to that

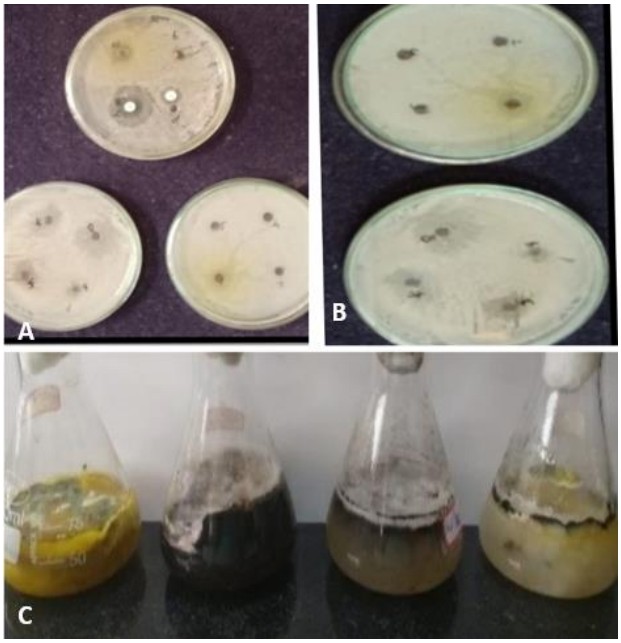

Figure 3: (A-B) Antibacterial activity of endophytic fungi; (C) Mass production of fungal isolates.

standard Penicillin, the activity was much less. A hundred (100) % of crude extracts of all endophytic fungi showed antimicrobial activity against *M. luteus*, where *Epicoccum* sp., *Penicillium* sp. and *Oospora* sp. showed good activity when compared to that of standard antibiotics Penicillin and Tetracycline. 90% of the fungal extract showed antimicrobial activity against *B. subtilis*, of which *Oospora* sp. was showing maximum activity on par with that of standard Tetracycline. 60% of the isolated endophytes could show antimicrobial activity against *S. aureus* of which *M. sterilia* and *Penicillium* sp. showed maximum activity when compared to other isolates. However, out of 10 isolates, *Epicoccum* sp. and *Penicillium* sp. showed an excellent response to most bacterial cultures. It was also observed that these two isolates produced extracellular products that diffused into the media.

DISCUSSION

Many fungal isolates produce extracellular products that have been found to have an antimicrobial activity of which *Penicillium* sp. is predominant (Rančić *et al.*, 2006; Petit *et al*., 2009). Raper and Thom (1949) reported the antimicrobial activity of *Penicillium chrysogenum*, similarly

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Bentley (2000) reported penicillins from *Penicillium brevicompactum*. Gharaei-Fathabad *et al*. (2014) reported the antimicrobial activity against *Candida albicans*, *B. subtilis*, *S. aureus*, *Salmonella typhi* and *E. coli*. Numerous useful bioactive substances, including antibacterial and antifouling polyketides, are produced by *Penicillium* sp. (Bao *et al*., 2013; Wong *et al*., 2015) has documented the antimicrobial activity of *Penicillium* sp. isolated as an endophyte from coastal brown seaweed against *E. coli* and *Bacillus* sp. Isolated *Penicillium* sp. from marine Fijian sponge *Melophlus* sp. displayed antibacterial against multidrug-resistant infections. Govindappa *et al*. (2011) have also reported antimicrobial activity of *Penicillium* sp., *A. niger* and *Alternaria alternata* isolated from *Loranthus* sp. Mallea *et al*. (1991) reported antifungal and antibacterial activities of several strains of *Epicoccum purpurascens* isolated from Quercus ilex, which inhibited the growth of *S. aureus* and *Trichophytoton mentagrophytes*. Bamford *et al*. (1961) and Eka (1970) has reported flavipin (3,4,5-trihydroxy-6-methylphthaldehyde) from the pigments of *Epicoccum* sp., which showed strong antimicrobial activity. Vaz *et al*. (2009) reported the antimicrobial activity of *Epicoccum* sp. and *Alternaria* sp. isolated from plants belonging to the family Orchidaceae.

CONCLUSION

In conclusion*, R. graveolens* is a rich source of secondary metabolites and has antimicrobial activity. The endophytic fungi isolated form *R. graveolens* have good antimicrobial properties. However, the fungal diversity is comparatively less, maybe because of phytochemicals present in the host plant.

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