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Isolation and identification of endophytic fungi from medicinally important plant *Hibiscus surattensis* L.

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ABSTRACT

The study was aimed to localize, isolate and identify endophytic fungi in an ethno medicinally important plant *Malvaceae* species *Hibiscus surattensis* L. The study plant was collected from Pondicherry and Marakkanam region and its taxonomic position was authenticated. Localization of endophytic fungi in the transverse section of leaves stems and roots were done after proper staining with Lacto phenol Cotton Blue. Exact locus of the endophytic fungi in the *Hibiscus surattensis* L. was found out through light microscope and scanning electron microscope. Endophytic fungi were found in all plant parts examined; root and stem showed higher incidence of endophytes. Plant segments were surface sterilized and kept in four different culture media. Identification of fungal colonies grown in each plant segments were primarily identified based on colony morphology and reproductive characters. Totally 172 endophytic fungal colonies were recorded out of 216 plant parts segment (leaf, root and stem) tested. Nine species with higher occurrence and active were identified using MALDI TOF MS technique. *Aspergillus niger* and *Aspergillus versicolor* were two species recorded in stem, leaf and root of *H. surattensis* L.

KEYWORDS: Endophytic fungi-*Hibiscus surattensis* L-SEM

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INTRODUCTION

Endophytes are microorganism found in living tissues of various plant parts having mutual relationship without causing any diseases.^{1,2} Endophytic fungus was first identified by Freeman in 1904 in *Lolium persicum* Persian darnel.³ Asymptomatic microbial infections are known to occur in the twigs and leaves of several vascular plants.^{4, 5 & 6} Interestingly, endophytic fungi are alternate medicinal source for many diseases and potential source for bio prospecting. In the recent years, it is gradually recognized that endophytic fungi or endophytes play a very important role in influencing quantity and quality of crude drugs in medicinal plants through fungus-host interaction.⁷ More importantly majority of natural products occurring in endophytic microorganisms have antimicrobial activity and involved in defending host plant against pathogenic microorganisms.⁸

Many plants particularly medicinal and ornamental plants such as *Terminalia*⁹, *Spondias*¹⁰, *Eucalyptus*¹¹, *Azadirachta*^{12,13}, *Musa acuminata*¹⁴, *Aegle marmelos*¹⁵, palms, orchids and thallophytes have been explored for endophytic fungi. In that pursuit, present study was carried out to localize, isolate and identify endophytes in medicinal plant *H. surattensis* L.

Intensive literature survey revealed that Malvaceae member *Hibiscus surattensis* L. has not been screened for its endophytic fungi except for ethno medicinal use of this plant. The flowers of *H. surattensis* L were consumed for treatment of hypertension in Nigeria¹⁶; Stems and leaves were used for the treatment of ureteritis and venereal diseases.¹⁷ In West Africa; Leaves were used for treatment of malarial disease.¹⁸ Crushed leaves used for wound healing, abscess and gonorrhoea in Tanzania.¹⁹ Whole plant used for stomach ache in Nigeria.²⁰ Camphor, β -caryophyllene, methyl salicylate and menthol were reported to be the main compounds present in Leaves of *Hibiscus surattensis* L.²¹ Preliminary phytochemical tests on *Hibiscus surattensis* L. revealed the presence of sterols, carbohydrates, phenolic compounds and flavones in dry leaves.²² Plants having an ethnobotanical history and/or used by local people provide us the best opportunities to isolate novel endophytic fungi and bioprospecting.²³ With this rationale, the medicinal plant *Hibiscus surattensis* L. was chosen for the isolation of endophytic fungi from its leaf, stem and root part as there is no report on these lines.

MATERIALS AND METHODS

Kingdom : *Plantae*
Phylum : *Tracheophyta*
Class : *Magnoliopsida*
Order : *Malvales*
Family : *Malvaceae*
Genus : *Hibiscus L.*
Species : *Hibiscus surattensisL.*



Figure 1:*Hibiscus surattensis L.*

The study plant, *Hibiscus surattensisL*(Figure 1) is an indigenous scrambling annual commonly known as Wild Sour distributed throughout Africa and Asia. Leaves and stems are densely pubescent, with silvery hairs visible to the naked eye. Leaf margins are serrated and often appear reddish, suggesting the presence of anthocyanin compounds. Flowers are bisexual, pentamerous. The ability of *Hibiscus surattensisL.* to self-pollinate²⁴ is considered as an evolutionarily advanced trait.*Hibiscus surattensisL.* is growing abundantly in Pondicherry and Marakkanam regions located at 12.0219°N, 79.8575°E and 12.1899°N, 79.9249°E along the Coromandel Coast of India. Fresh and healthy plants were collected and identified by the experts at Pondicherry French institute and Pondicherry University, Puducherry.

LOCALIZATION OF ENDOPHYTIC FUNGI

The transverse section (T.S.) of roots stems and leaves of *Hibiscus surattensisL.* were stained following the method described by Mishra et al.,²⁵ Each section was stained with Lacto phenol cotton blue for 1 minute and put cover slip on it after surface sterilization aseptically. To remove the excess stain, the sections were washed in distilled water 4-5 times. The stained T.S of the plant sections were observed under Phase contrast microscope camera attached (Zeiss, 415500-1812-000) and images focused on fungal spores and hyphae were photographed at different magnifications 10X and 100X.

To get more details, Scanning Electron Microscopic (SEM) studies of plant materials were processed and images were taken in Hitachi, Model: S-3400N available at Central Instrumentation Facility, Pondicherry University. The plant materials were washed under tap water. Samples were processed within 6 hours of collection. Leaf, stem and root were cut into thin sections using sterile

blade and again cleaned by distilled water. Pieces of 3 to 10 mm size were cut and cut sections were mounted on SEM stubs after sealing the cut surface with glue and images were taken under SEM.²⁶

Surface sterilization and isolation of endophytic fungi

The plant material was washed repeatedly under running tap water to remove soil particles and debris completely followed by distilled water. For culture purpose the plant materials were dipped in 70% ethanol for 30 sec, followed by 0.5% sodium hypochlorite (NaOCl) for 2–3 mins and again rinsed in 70% ethanol for 1-2 mins and finally with distilled water for 2 times. The surface sterilized plant materials were dried using sterile filter papers under aseptic condition in the laminar air flow chamber.²⁷

Totally four different fungal growth media were used to get maximum number of fungi as the type of growth medium also influence fungal growth viz. PDA, Semi-synthetic PDA, Czapek Dox Agar and Fungal Agar. The surface sterilized plant was cut (aseptically) into segments of 0.5-1cm long and transferred to petri dishes with fungal growth media. Six segments of each plant part viz. Leaf, Stem and Root were placed onto petri dishes containing media amended with 500mg/l of chloramphenicol as antibacterial drug. Unsterilized plant segments were also kept as a positive control. Inoculated petri dishes were coded and sealed with Para film and then incubated at 27°C for 3–6 days. All the works were done under laminar air flow chamber.²⁷

Preparation of Sub culture and identification

For raising Sub cultures, tips of fungal hyphae were carefully transferred to new potato dextrose agar plates without the addition of antibiotics to obtain pure cultures. The purified endophytic fungi isolates were then separately transferred to PDA slants and maintained at 4°C till further use. The each tube was labelled with code and date of isolation.²⁷

Identification of endophytes

Morphological identification of fungi was done based on colony, hyphae morphology and characteristics of the spores. Active and most frequently occurred endophytic fungi were isolated. Tips of the hyphae of the fungal colonies were placed on to the clean glass slide then stained with 1-2 drops of Lacto Phenol Cotton blue stain. Thus prepared slides with cover slips were viewed under phase contrast microscope with digital camera (Model: P95 M37/52×0.75) available at Department of Microbiology, Pondicherry University and images of conidiophore, hyphae, and mycelia were photographed.²⁷ Based on morphological and reproductive characteristics of fungi, totally 9 strains that

showed higher frequency of occurrence, were sub cultured. The isolated fungal species were confirmed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry MALDI-TOFMS.²⁸ Colonization frequency (CF) was calculated as described by Hata et al.,²⁹

$$\text{CF\%} = \frac{\text{Number of segments colonized by endophytes}}{\text{Total number of segments examined}} \times 100$$

RESULTS:

Isolation of endophytic fungi

Totally 216 plant parts segments were examined. Endophytic fungi emerged from 172 plant parts segments (Table1). The initial growth of fungi were observed on 2nd day and continued up to 6th day of incubation. Result showed that endophytic fungal colonies were high in stem and root segments whereas leaf had less endophytic fungal colonies.

The colonization frequency of endophytic fungi varies with the media used. The highest colonization frequency was observed in PDA medium compared with other media viz. Czapek Dox Agar, Fungal Agar and Semi-synthetic PDA. Among the endophytic fungi, predominant were belonged to genus *Aspergillus* and other genera were *Aureobasidium*, *Rhizomucor*, *Schizophyllum* and *Scopulariopsis*. The identified endophytic fungal species were given in Figure 11-13(g, h & i). Among fungal species identified, *Aspergillus niger* showed higher colonization frequency (29.1) and *Aspergillus clavatus* (4.7) showed least occurrence (Figure 14).

Localization of endophytic fungi

Endophytic fungi were detected in the T.S of root of *Hibiscus surattensis*L. in and around the phloem region at 10X were shown in the (Figure 2). T.S of *Hibiscus surattensis*L. stem in 100x showing endophytic fungal found in parenchyma tissues were seen in (Figure 3).

Root Sectioning of *Hibiscus surattensis*L. species showing endophytic fungal spores in and around the xylem region under SEM were seen in (Figure 4). T.S of stained (LPCB) stem *Hibiscus surattensis*L. at 10X showing endophytic fungi found in the intracellular space of the parenchyma tissues and also found in xylem and phloem region were seen in (Figure 5). T.S of stem of *Hibiscus surattensis*L. at 100X showing endophytic fungal in intracellular space of the parenchyma tissues were seen in (Figure 6). Stem sectioning of *Hibiscus surattensis*L. species showing endophytic fungal spores residing in the xylem region under SEM were seen in (Figure 7).

T.S of leaf *Hibiscus surattensis*L. in 10X showing endophytic fungi in palisade parenchyma cells (PP) were seen in (Figure 8). T.S of leaf *Hibiscus surattensis*L. in 100x showing endophytic fungi in

epidermal cells (E) were seen in (Figure 9). Leaf sectioning of *Hibiscus surattensis* L. species showing endophytic fungal spores in the Lower epidermal region (LE) in Scanning Electron Microscope was seen in (Figure 10).

The *Hibiscus surattensis* L. were showed that sections of stem and root had maximum number of endophytic fungi and leaves showed lesser number of endophytic fungi. Microscopic and SEM images indicated maximum endophytic fungal spores in and around the xylem region of stem and root than in leaf.

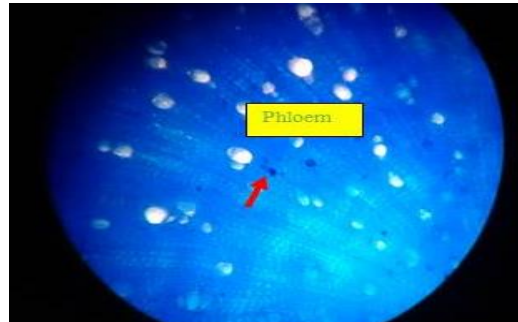


Figure 2: T.S of root of *Hibiscus surattensis* L. Showing endophytic fungal in phloem

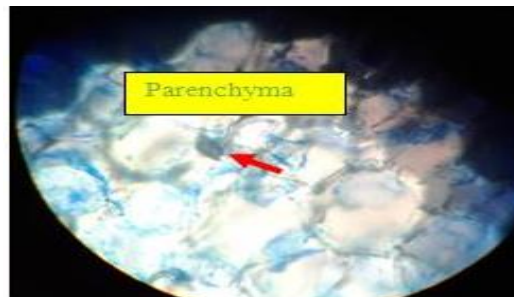


Figure3: T.S of root of *Hibiscus surattensis* L. showing endophytic fungi in intercellular space of the



Figure4: SEM image of T.S. of Root of *Hibiscus surattensis* L. showing endophytic fungal spores in and



Figure5: T.S of stem of *Hibiscus surattensis*L. Showing endophytic fungi in the intracellular space of the parenchyma



Figure 6: T.S of stem of *Hibiscus surattensis* L. showing endophytic fungal in parenchyma



Figure7: SEM image of T.S. of Stem of *Hibiscus surattensis* L. showing endophytic fungal

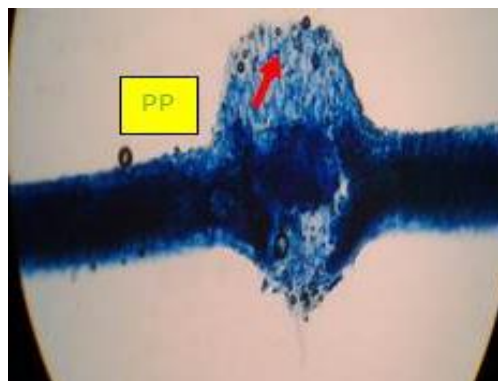


Figure8: T.S of leaf of *Hibiscus surattensis* L. Showing endophytic fungi in palisade parenchyma cells (PP) (10X)

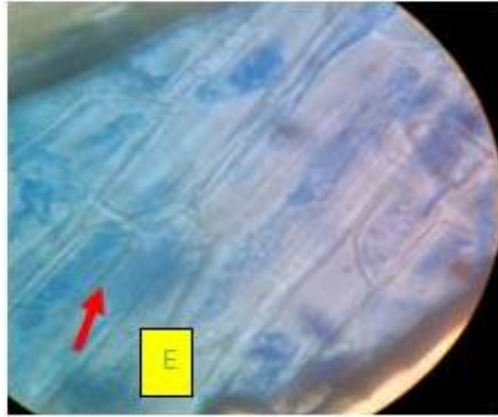


Figure9: T.S of leaf of *Hibiscus surattensis*L.showing endophytic fungi in epidermal cells (E)(100X)

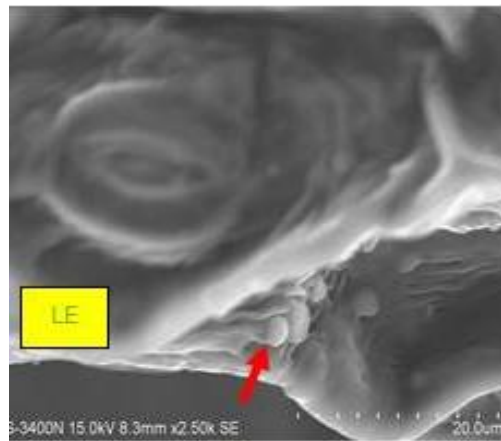


Figure10: SEM image of T.S. of Leaf of *Hibiscus surattensis* L.Showing endophytic fungal spores in the Lower epidermal region(LE)and around the xylem region

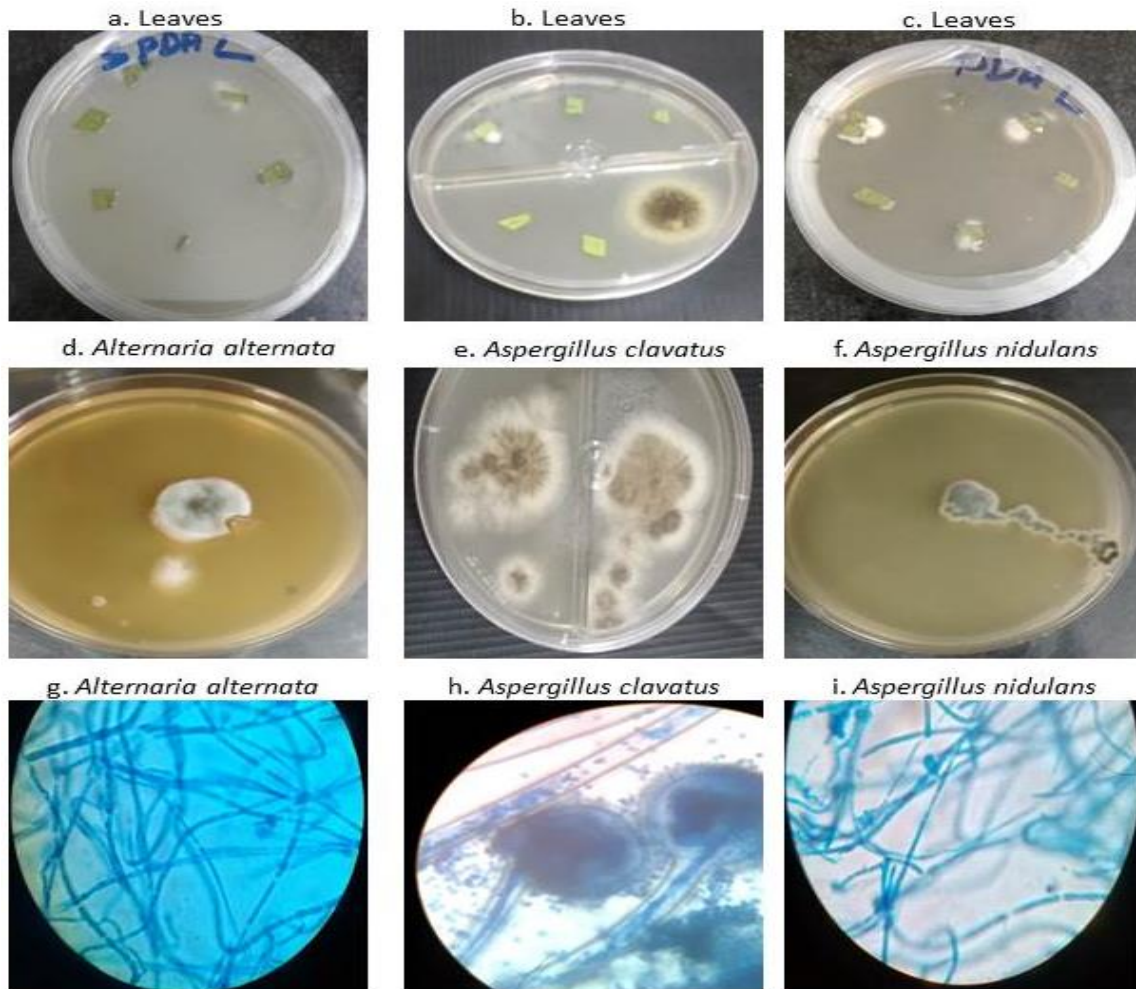


Figure 11: (a, b, & c: *Hibiscus surattensis* L. Leaves on the petri dish after surface sterilized and incubated), (d, e & f: selected endophytic fungi sub cultured plates), g, h & i: Identified endophytic fungi in microscopic view-g: *Alternaria alternata*, h: *Aspergillus clavatus*, i: *Aspergillus nidulans*

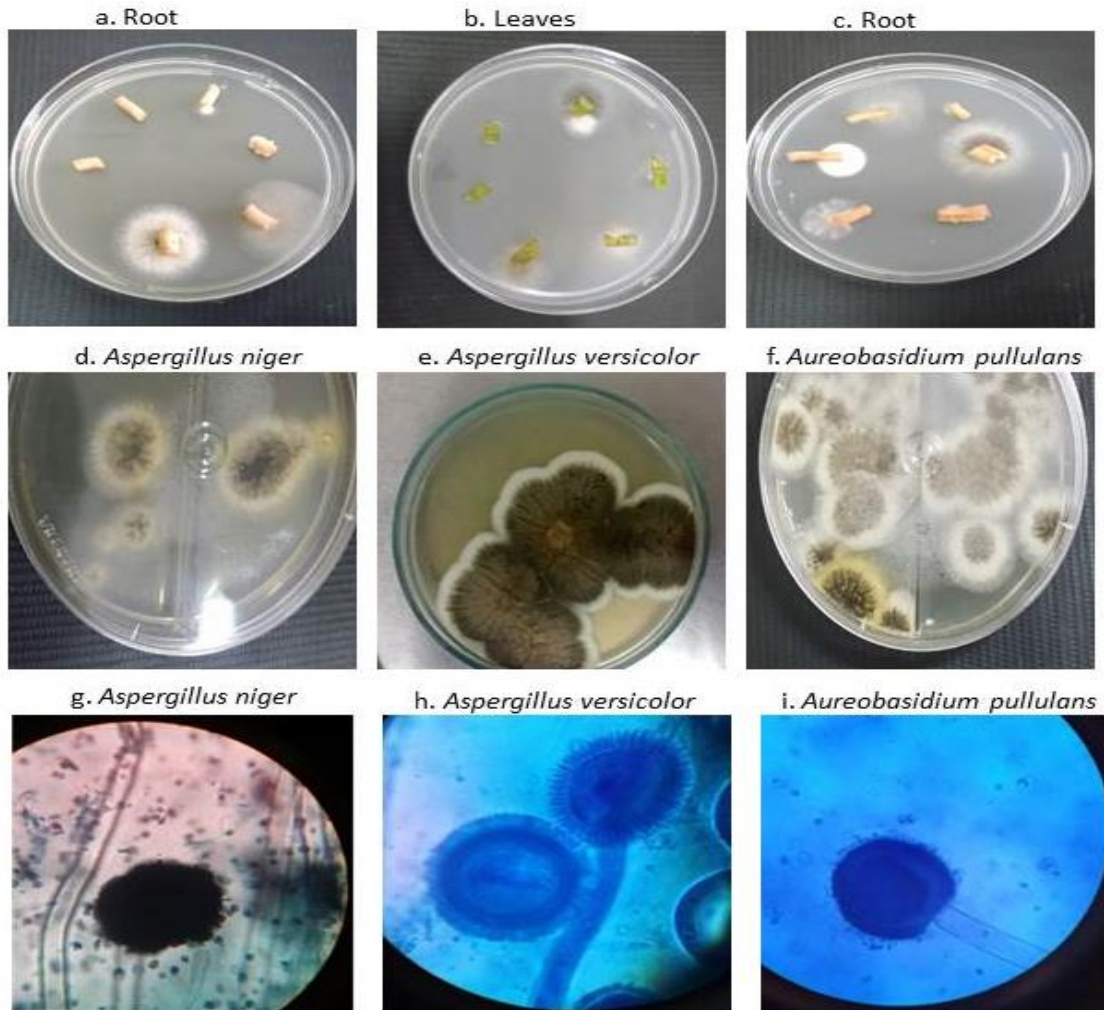


Figure 12: (a, b, & c: *Hibiscus surattensis* L. Leaves and roots on the petri dish after surface sterilized and incubated), (d, e & f: selected endophytic fungi sub cultured plates), g, h & i: Identified endophytic fungi in microscopic view-g: *Aspergillus niger*, h: *Aspergillus vesicolor*, i: *Aureobasidium pullulans*

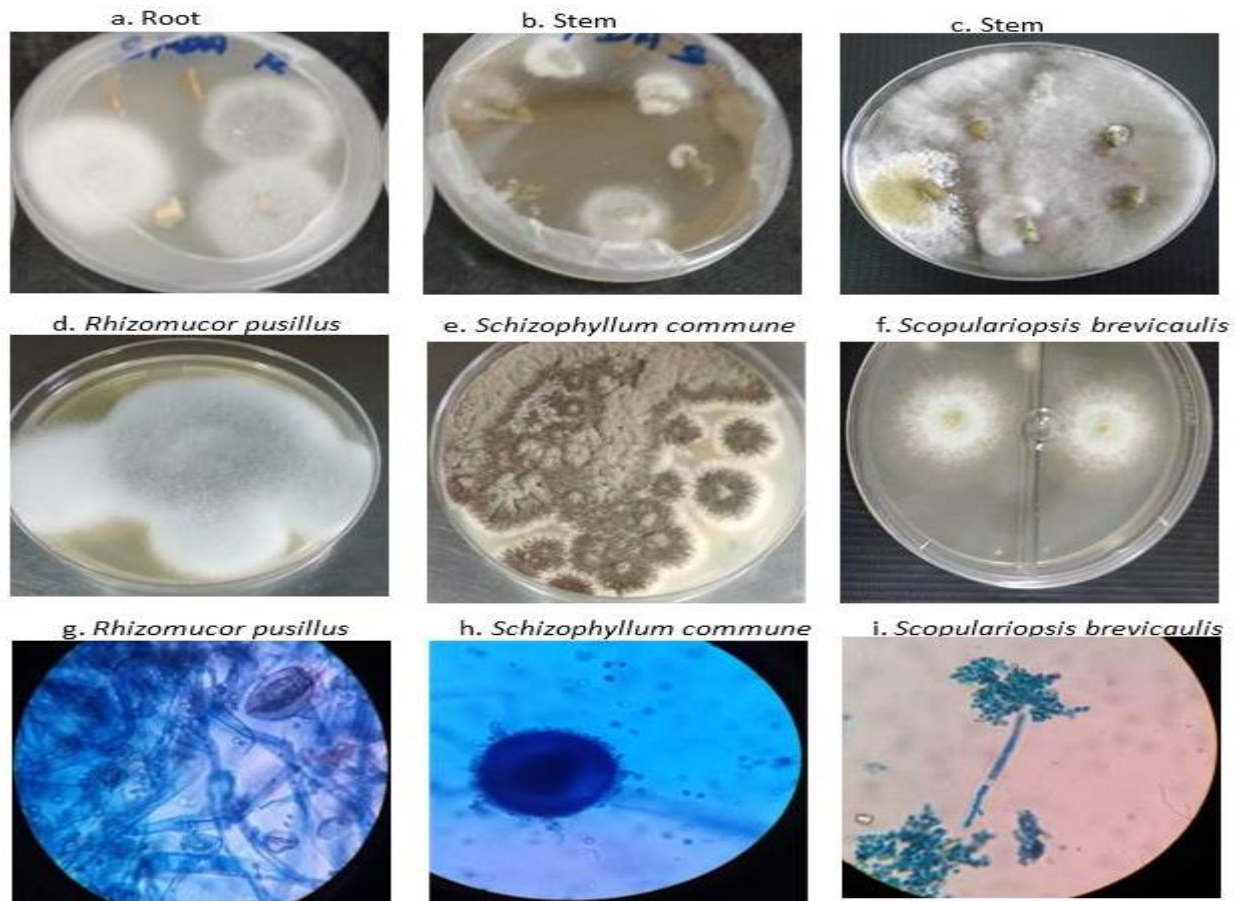


Figure 13: (a, b, & c: *Hibiscus surattensis* L. Root and stems on the petri dish after surface sterilized and incubated), (d, e & f: selected endophytic fungi sub cultured plates), g, h & i: Identified endophytic fungi in microscopic view-g: *Rhizomucor pusillus*, h: *Schizophyllum commune*, i: *Scopulariopsis brevicaulis*

Table 1: Endophytic fungi growth from different parts of *Hibiscus surattensis* L. in four growth media

| S. No. | Plant parts | Total no. of plant segments examined | Total no. of segment with endophytic fungal growth | % occurrence |
|--------|-------------|--------------------------------------|--|--------------|
| 1 | Leaf | 72 | 40 | 79.62 |
| 2 | Stem | 72 | 69 | |
| 3 | Root | 72 | 63 | |
| | Total | 216 | 172 | |

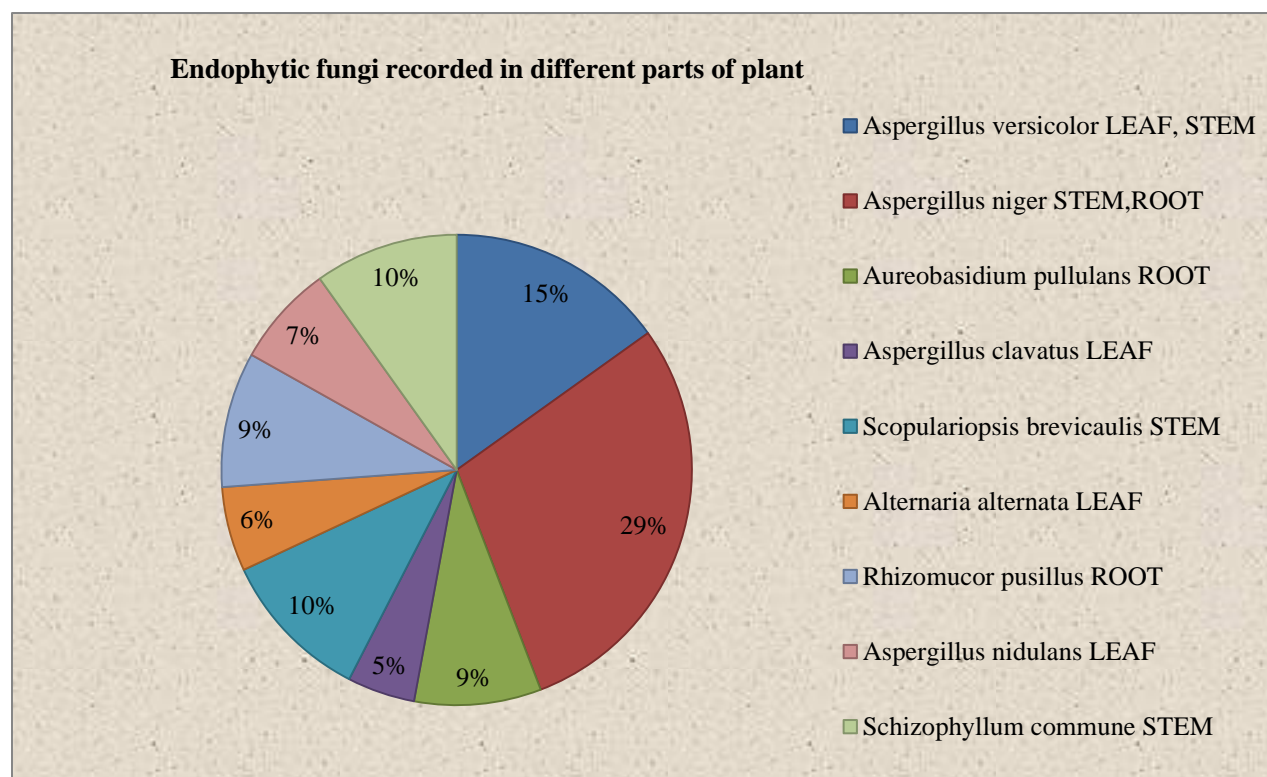


Figure 14: Colonization frequency of endophytic fungi in different parts of *Hibiscus surattensis*L.

DISCUSSION

A plant having an ethnobotanical history is the finger print providing the scientists the best opportunity directly isolate novel endophytic fungi and to make use of them to derive novel bioactive compounds. In that pursuit, *H. surattensis* L is reported to possess ethno medical history, studies on endophytic fungi present in the study plant will be of more useful for its further pharmacological screening. With this rationale, different parts of the ethno medically important plant species *Hibiscus surattensis*L. was examined for endophytic fungal species.

A total of 216 segments (root, stem and leaf) in four different fungal growth media were examined. Out of 216 segments 172 endophytic fungi colonies were recorded shown in Table 1. Among 172 endophytic fungal species belonged to *Alternaria alternata* from the leaves of 10(5.8%), *Aspergillus clavatus* from the leaves of 8(4.7%), *Aspergillus nidulans* from the leaf of 12(7.0%), *Aspergillus niger* from the stems and roots of 50(29.1%), *Aspergillus versicolor* from the leaves and stems of 26(15.1%), *Aureobasidium pullulans* from the roots of 15(8.7%), *Rhizomucor pusillus* from the roots of 16(9.3%), *Schizophyllum commune* from the stem of 17(9.9%) and *Scopulariopsis brevicaulis* from the stems of 18(10.5%). The highest colonization frequency of

endophytic fungal species was *Aspergillus niger* 29.1% and Lowest colonization frequency of endophytic fungal species was *Aspergillus clavatus* 4.7% were shown in the (Figure 14).

Four species of genus *Aspergillus* viz., *A. niger*, *A. nidulans*, *A. clavatus* and *A. versicolor* were isolated from leaves of *Hibiscus surattensis* L. Species of *Aspergillus* and *Penicillium* were isolated from banana leaves and roots.³⁰ Further, *Alternaria* and *Rhizomucorpusillus* were isolated from roots of *Hibiscus surattensis* L. Patil³¹ isolated and identified endophytic fungi viz. *Alternaria* spp., *Rhizopus* spp., *Curvularia* spp. and *Trichoderma* spp. from different medicinal plants.

Endophytic fungi found in the intercellular and intracellular spaces of parenchyma tissues and in xylem and phloem region of leaf, stem and roots in *Hibiscus surattensis* L (Figure 2-10). Occurrence of endophytes colonize in the intercellular spaces as well as intracellular space in xylem and phloem cells.³² Kumari and Chandra³³ reported maximum occurrence of endophytes was observed in phloem tissue of stem in *Stevia rebaudiana* (Bert). Many organic and inorganic nutrients, present in the intracellular spaces are able to support endophytic fungi and hence most of the fungi were found in the vascular system of the plant.³⁴ Bernardi-Wenzel³⁵, using light microscopy and SEM images reported endophytic fungi in the inter and intracellular spaces in leaves of *Luheadivaricata*. Durán et al.,³⁶ recorded fungal hyphae on palisade parenchyma in leaves of *Citrus limon*. Our SEM results showed occurrence of endophytic fungal spores/hyphae in lower epidermal region of leaves of *Hibiscus surattensis* L. This indicated that the colonization of endophytic fungi was significantly determined by the type of plant tissues producing different organic substances.

Therefore, present report on occurrence and abundance of endophytic fungi in an ethnomedically important plant species *H. surattensis* L. would form a strong platform for bio prospecting the secondary metabolites and further drug development based on ethnomedical knowledge and practice. Thus, present contribution opens up new vistas for further research and development not only in the field of drug discovery and bioprospecting but also enlighten our understanding on evolution of such endophytes in diversified plant species.

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