ISOLATION, IDENTIFICATION AND ANTIMICROBIAL ACTIVITIESOF ENDOPHYTIC FUNGAL STRAINS FROM DIFFERENT PARTS OF *HESPERETHUSA CRENULATA* (ROXB.) ROEM.

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Abstract

In this study, ten endophytic fungal strains were isolated from the plant parts of *Hesperethusa crenulata* (Roxb.) Roem. sample 1 and six fungal strains were also isolated from the plant parts of *Hesperethusa crenulata* (Roxb.) Roem.sample2. *Hesperethusa crenulata* (Roxb.) Roem. sample 1 was collected from Ayartaw Township, Saging Region and *Hesperethusa crenulata* (Roxb.) Roem. Sample 2 was also collected from Yay Sa Kyo Township, Magway Region. The morphological characters of isolated fungal strains were conducted at Microbiology Laboratory, Department of Botany, University of Yangon and microscopic characters were investigatedat Universities' Research Center, YU. In the present study, strains HH 1, 2, 9 and 13 were identified as *Cephalosporium* sp. and strain HH 12 was *Rhizoctonia* sp.. Antimicrobial activities of all isolated strains were conducted by using paper disc diffusion assay with eight test organisms. All isolated strains were exhibited antimicrobial activity on *Agrobacterium tumefaciens, Aspergillus flavus, Bacillus subtilis, Candida albicans, Malassezia furfur, Micrococcus luteus, Salmonella typhus* and *Xanthomonas oryzae.*

Keywords: Antimicrobial activity, Endophytic fungi, *Hesperethusa crenulata* (Roxb.) Roem.

Introduction

Endophytes are organisms, often fungi and bacteria that live between living plant cells. Endophytes, found ubiquitous in all plant species in the world, contribute to their host plants by producing plenty of substances that provide protection and ultimately survival value to the plant. The natural products obtained from endophytic microbes are found to be antimicrobial, antiviral, anticancer, antioxidants, antidiabetic and immunosuppressant.

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The fungal endophytes are known to produce these types of natural products. Nonetheless, poorly explored and underutilized endophytic fungi are known to produce antibiotics in addition to other natural products. The endophytic fungi appear to be a potential source of novel antibiotics. Now the endophytic fungi seem to be a promising alternative potential source of novel antibiotics. Endophytic fungi are as a potential source of novel antibiotics (Walsh, 1992 and Strobel *et al.*, 2002).

Now a day herbal drugs are prescribed widely even when their biologically active compounds are unknown because of their effectiveness, minimal side effects in clinical experience and relatively low cost. Endophyte infected plants often grow faster than non-infected plants. They colonise plant tissue and are remained within the tissue, except that fruiting structures may emerge through the surface of the plant tissue. Indeed, leaves may be fully colonised by a variety of fungi within a few weeks of leaf emergence. The colonies remain asymptomatic and some in perennial plant parts may have a very long life. In amicrobe-plant relationship, endophytes contribute substances that possess various types of bioactivity, suchas antibacterial, antifungal, antibiotic, antitumor, antioxidant, anti-inflammatory etc. The bioactivesubstances in plants are produced as secondary metabolites (Strobel *et al.*, 2002).

The objectives of this study are to isolate endophytic fungal strains from different parts of *Hesperethusa crenulata* (Roxb.) Roem. samples 1 and 2, to investigate morphological and microscopic characters of isolated fungal strains and to evaluate antimicrobial activity of isolated fungal strains.

Materials and Methods

Collection of Plant Samples

Hesperethusa crenulata (Roxb.) Roem. sample 1 was collected from Ayartaw Township, Saging Region and *Hesperethusa crenulata* (Roxb.) Roem. sample 2 was also collected from Yay Sa Kyo Township, Magway Region.

Isolation of Endophytic Fungal Strains

Isolation of endophytic fungal strains can be carried out by the following scheme:

(1) The plants were washed in running water for fifteen minutes. **(**2) The plant parts (leaves, barks and roots) were cut into about 1 cm pieces. **(**3) These parts were sterilized by soaking in 75% ethanol for 2 min. **(**4) They were sterilized by soaking in 5.3% sodium hypochloride for 1 minute. **(**5) After that, these parts were sterilized by soaking in 75% ethanol for thirty seconds. **(**6) These parts were dried on sterilized paper and then they were placed on agar plates containing sucrose/yeast extract medium. **(**7) After 3 to 7 days the microorganisms were picked and purified by subculturing. **(**8) The pure strains are maintained in test tubes as seen in Figure 1 **(**Phay, 1997).

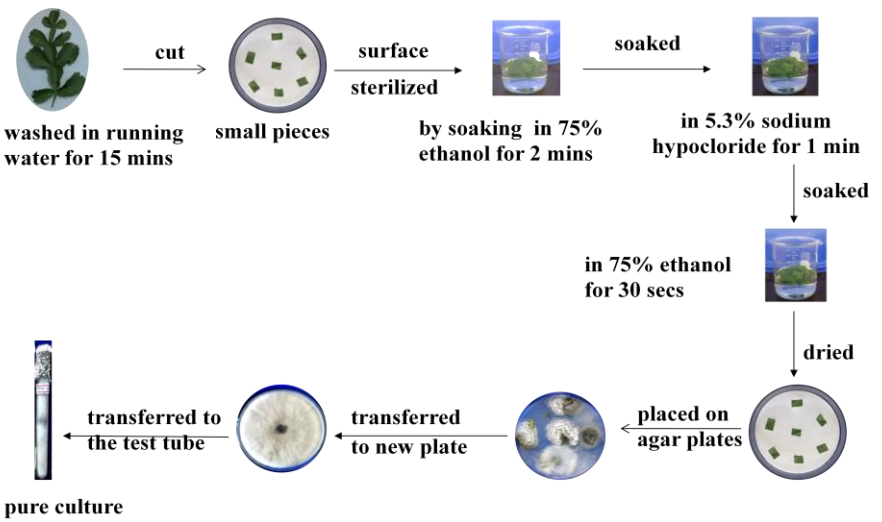


Figure 1. Isolation of endophytic fungal strains

Morphological and Microscopic Characters of Endophytic Fungi

Sixteen isolated endophytic fungi grown on slant culture were transferred into the plates containing SY medium(sucrose 1.0 g, yeast extract 0.3 g, NaCl 0.05 g, $CaCO_3$ 0.01 g, agar 1.8 g, pH7, distilled water 100 ml).

Then, these plates were incubated at 30˚C for 3-7 days.Colony forms, surfaces and reverse pigments of isolated strains were studied for morphology at Microbiology Laboratory, Department of Botany, University of Yangon. Microscopic characters of isolated fungi were investigated by using high magnification of microscope at Universities' of Research Center, YU.

Antimicrobial Activity of Isolated Fungal Strains

Fermentation of isolated strains

Isolated fungal strains grown on 5 days old slant cultures were inoculated into 16 of 50 ml conical flasks containing 25 ml of medium1 or 2 in each.There were 32 conical flasks were utilized for two fermentation media in this study. Next, they were incubated at 30˚C for 3-13 days. Two fermentation media were mentioned in the following.

Test agar plates

Test organisms were *Agrobacterium tumefaciens, Aspergillus flavus, Bacillus subtilis, Candida albicans, Malassezia furfur*, *Micrococcus luteus, Salmonella typhus* and *Xanthomonas oryzae.* Broth culture (50µl) of each test organism was added into 100 ml assay medium and then poured into plates.

Fermentation Medium 1: sucrose 1.0g, yeast extract 0.3g, NaCl 0.05g, $CaCO_3$ 0.01g, pH 7, distilled water 100ml

Fermentation medium 2: glucose 1.0g, yeast extract 0.3g, NaCl 0.05g, $CaCO_3$ 0.01g, pH 7, distilled water 100ml

Paper disc diffusion assay

After solidification, paper discs impregnated with fungal broth sampleswere applied on the test plates.These plates were incubated at room temperature 30˚C for 24 to 48 hrs.After 24 to 48 hrs, clear zones (inhibitory zones) surrounding the test discs were measured.These zones indicate the presence of the bioactive compounds that inhibits the growth of test organism (Phay, 1997).

Results

Outstanding Characters of *Hesperethusa crenulata* (Roxb.) Roem. Sample1

It is imparipinnate leaves with winged rachii, racemes of white-flowered inflorescences and the basifixed anthers. It is aromatic and compact, thin, reddish brown-streaked aromatic bark and the pubescent leavesas shown in Figure 2.

Habit

Leaves

Figure 2. Outstanding characters of *Hesperethusa crenulata* (Roxb.) Roem. sample1

Outstanding Characters of *Hesperethusa crenulata* (Roxb.) Roem. Sample 2

It has the germinate thorns, the imparipinnate leaves with winged rachii, the white-flowered cauliflorous inflorescences and the basifixed anthers. It is zig-zag young stems, the thick, whitish yellow, corky aromatic bark and the leaves which are glabrous except for the nervesas shown in Figure 3.

Habit

Leaves

Figure 3. Outstanding characters of *Hesperethusa crenulata* (Roxb.) Roem. sample 2

Isolation of Endophytic Fungal Strains from *Hesperethusa crenulata* (Roxb.) Roem.

Sixteen fungal strains were isolated from the leaves, barks, stems and roots of *Hesperethusa crenulata* (Roxb.) Roem. sample 1 and sample 2. These strains were given as temporary names HH-1 to HH-16 as shown in Table 1.

Table 1. Isolation of endophytic fungal strains from sample 1 and sample 2

Strains	**Sources**
HH-1 to HH-4	Leaves of *Hesperethusa crenulata* (Roxb.) Roem. sample1
HH-5 to HH-7	Barks of *Hesperethusa crenulata* (Roxb.) Roem. sample1
HH-8 and HH-9	Woods of *Hesperethusa crenulata* (Roxb.) Roem. sample1
HH-10	Roots of *Hesperethusa crenulata* (Roxb.) Roem. sample1
HH-11 to HH-13	Leaves of *Hesperethusa crenulata* (Roxb.) Roem. sample2
HH-14	Barks of *Hesperethusa crenulata* (Roxb.) Roem. sample2
HH-15	Woods of *Hesperethusa crenulata* (Roxb.) Roem. sample2
HH-16	Roots of *Hesperethusa crenulata* (Roxb.) Roem. sample2

Morphological and Microscopic Characters of Isolated Fungal Strains

Morphological and microscopic characters **of** sixteenfungal strains (HH-1 to HH-16) isolated from *Hesperethusa crenulata* (Roxb.) Roem. Samples 1 and 2 were shown in Table 2 and Figures 4 to 19.

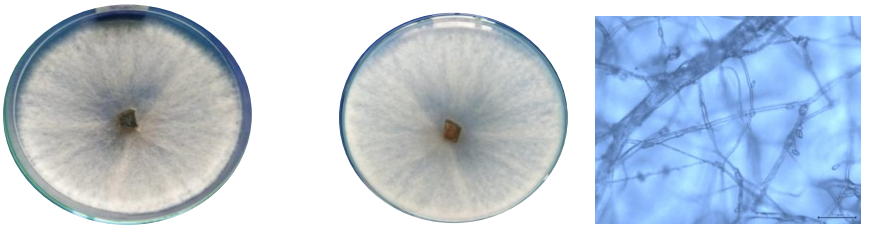
Table 2. Morphological characters of isolated strains

Cultural characters			
Strains	**Surface color**	**Reverse color**	**Hyphae**
HH1	White	White	Septate
HH2	Brownish white	Pale brown	Septate
HH3	White	White	Septate
HH4	Brown	Brown	Septate
HH5	White	White	Aseptate
HH6	White	Pink	Septate
HH7	White	White	Septate
HH8	White	Pale pink	Aseptate
HH9	White	White	Septate
HH10	White	White	Septate
HH11	White	White	Septate
HH12	White	White	Septate
HH13	White	White	Septate
HH14	White	White	Septate
HH15	White	White	Aseptate
HH16	Pink	Pink	Septate

Morphological and microscopic characters of strain HH1

The surface color and reverse color of strain HH1 was the same color white.It shows conidia (phialospores) hyaline, 1 celled, septate hyphae which are typically very fine and narrow. The phialides are separated from hyphae by a septum and taper towards their apices. They usually appear in clusters, in

balls or rarely as fragile chains. It is identified as *Cephalosporium* sp. as shown in Figure 4.

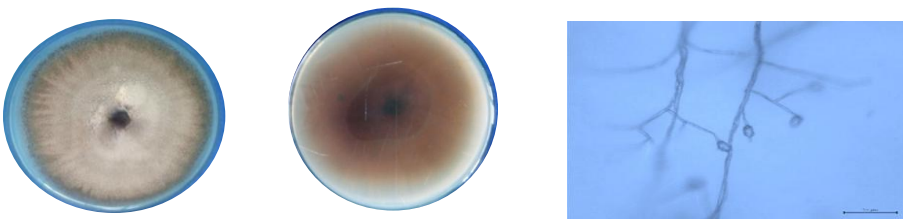


Surface view Reverse view

Figure 4. Morphological and microscopic characters of HH1 (*Cephalosporium* sp.)

Morphological and microscopic characters of isolated strain HH2

The surface color of strain HH2 was brownish white and its reverse color was pale brown. It shows conidia (phialospores) hyaline, 1 celled, septate hyphae which are typically very fine and narrow. The phialides are separated from hyphae by a septum and taper towards their apices. They usually appear in clusters, in balls or rarely as fragile chains. It is identified as *Cephalosporium* sp. as shown in Figure 5.

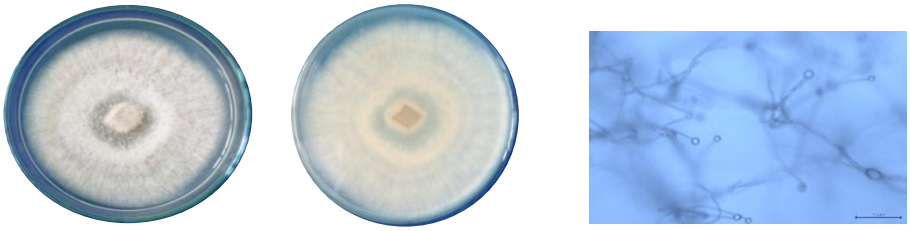


Surface view Reverse view

Figure 5. Morphological and microscopic characters of HH2 (*Cephalosporium* sp.)

Morphological and microscopic characters of isolated strain HH3

The surface color and reverse color of strain HH3 was the same color white.

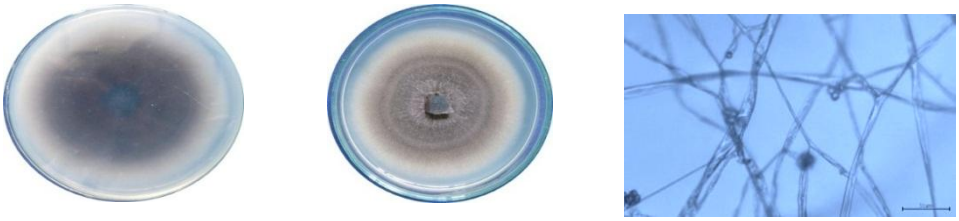


Surface view Reverse view

Figure 6. Morphological and microscopic characters of HH3

Morphological and microscopic characters of isolated strain HH4

The surface color and reverse color of strain HH4 was the same color brown.

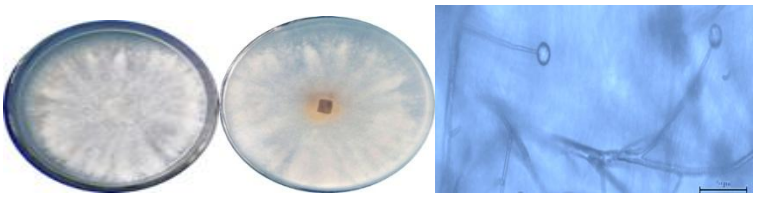


Surface view Reverse view

Figure 7. Morphological and microscopic characters of HH4

Morphological and microscopic characters of isolated strain HH5

The surface color and reverse color of strain HH5 was the same color white.

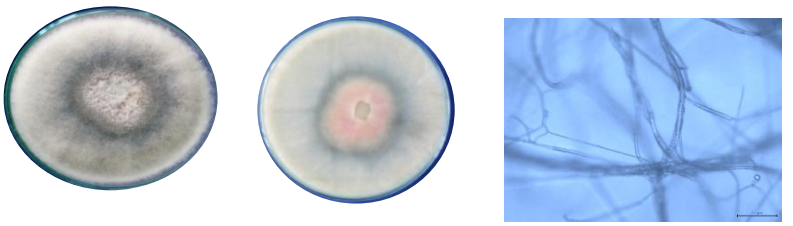


Surface view Reverse view

Figure 8. Morphological and microscopic characters of HH5

Morphological and microscopic characters of isolated strain HH6

The surface color of strain HH6 was white and its reverse color was pink.



Surface view Reverse view

Figure 9. Morphological and microscopic characters of isolated strain HH6

Morphological and microscopic characters of isolated strain HH7

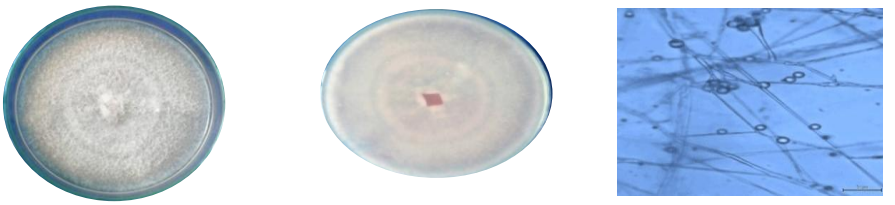
The surface color and reverse color of strain HH7 was the same color white.

Surface view Reverse view

Figure 10. Morphological and microscopic characters of isolated strain HH7

Morphological and microscopic characters of isolated strain HH8

The surface color of strain HH8 was white and its reverse color was pale pink.

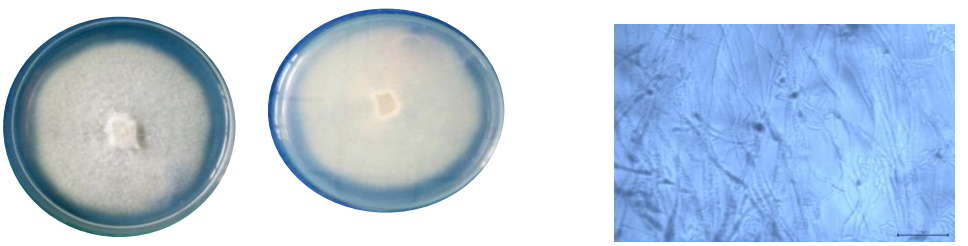


Surface view Reverse view

Figure 11. Morphological and microscopic characters of isolated strain HH8

Morphological and microscopic characters of isolated strain HH9

The surface color and reverse color of strain HH9 was the same color white. It shows conidia (phialospores) hyaline, 1 celled, septate hyphae which are typically very fine and narrow. The phialides are separated from hyphae by a septum and taper towards their apices. They usually appear in clusters, in balls or rarely as fragile chains. It is identified as *Cephalosporium* sp. as shown in Figure 12.



surface view Reverse view

Figure 12. Morphological and microscopic characters of HH9 (*Cephalosporium* sp.)

Morphological and microscopic characters of isolated strain

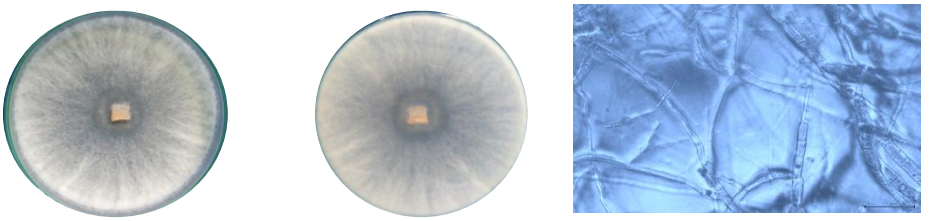
The surface color and reverse color of strain HH10 was the same color white.

surface view Reverse view

Figure 13. Morphological and microscopic characters of HH10

Morphological and microscopic characters of isolated strain HH11

The surface color and reverse color of strain HH11 was the same color white.

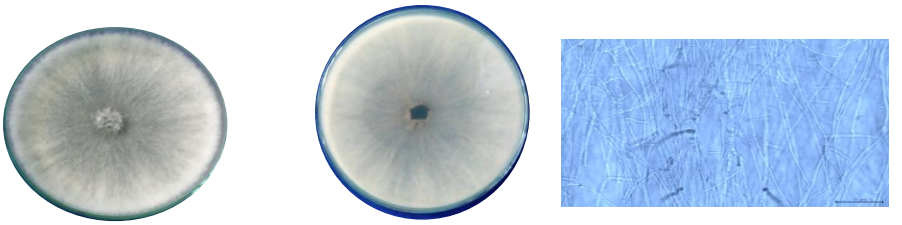


Surface view Reverse view

Figure 14. Morphological and microscopic characters of HH11

Morphological and microscopic characters of isolated strain HH12

The surface and reserve color of strain HH12 was white. Mycelium hyaline was dark. Cells of mycelium are long, septa of branches and set off from the main hyphae. Asexual fruit bodies, conidia absent, sporodocium-like bodies and chlamydospore-like cells were in chains. It is identified as *Rhizoctonia* sp. as shown in Figure 15.

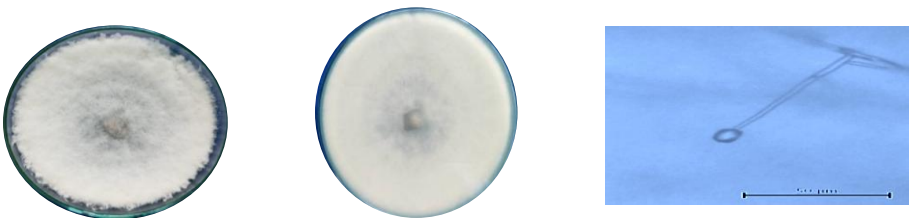


Surface view Reverse view

Figure 15. Morphological and microscopic characters of HH12 (*Rhizoctonia* sp.)

Morphological and microscopic characters of isolated strain HH13

The surface color and reverse color of strain HH13 was the same color white. It shows conidia (phialospores) hyaline, 1 celled, septate hyphae which are typically very fine and narrow. The phialides are separated from hyphae by a septum and taper towards their apices. They usually appear in clusters, in balls or rarely as fragile chains. It is identified as *Cephalosporium* sp. as shown in Figure 16.

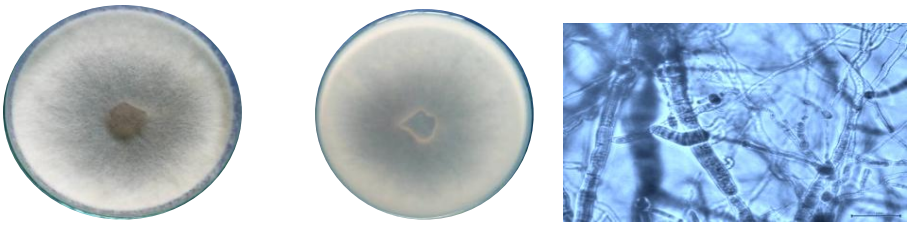


Surface view Reverse view

Figure 16. Morphological and microscopic characters of HH13 (*Cephalosporium* sp.)

Morphological and microscopic characters of isolated strain HH14

The surface color and reverse color of strain HH14 was the same color white.

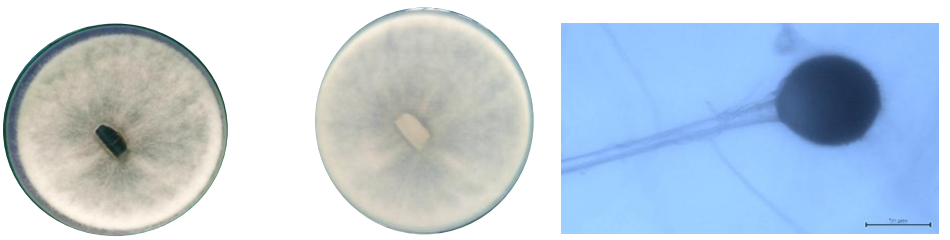


Surface view Reverse view

Figure 17. Morphological and microscopic characters of HH14

Morphological and microscopic characters of strain HH15

The surface color and reverse color of strain HH15 was the same color white.

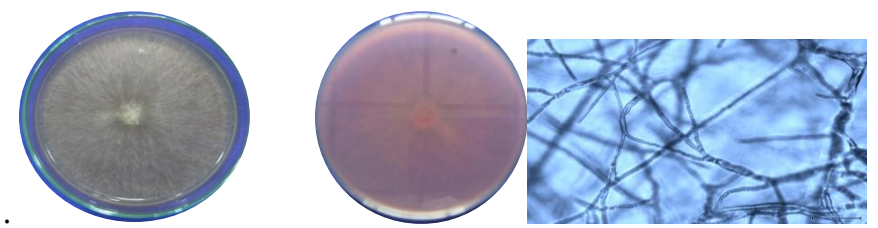


Surface view Reverse view

Figure 18. Morphological and microscopic characters of HH15

Morphological and microscopic characters of isolated strain HH16

The surface color and reverse color of strain HH16 was the same color pink as shown in Figure 19.



Surface view Reverse view

Figure 19. Morphological and microscopic characters of HH16

Antimicrobial Activity of Isolated Fungal Strains

Strain HH 9 showed very high activity against seven test organisms except *C. albicans* on medium 1 while strain HH 8 indicated very high activity against six test organisms except *C. albicans* and *X. oryzae* on medium 2 at 3rd day fermentation. Strains HH1, 9and 14 showed very high activity against eight test organisms on medium 1 whereas strain HH 8 and 15 indicated very high activity against eight test organisms on medium 2 at 4th day fermentation.

Strains HH 2 indicated very high activity against eight test organisms on medium 1 while strain HH 8 also exhibited very high activity against eight test organisms on medium 2 at 5th day fermentation. Strains HH 2, 9 and 13 exhibited very high activity against eight test organisms on medium 1 whereas strains HH 1, 4, 8, 12, 13 and 15 showed very high activity against eight test organisms on medium 2at 6th day fermentation.

Strains HH 1, 2 and 9 exhibited very high activity against six test organisms except *Asp. flavus* and *C. albicans* on medium 1 at 7th day fermentation. Strains HH 8, 11, 12, 13 and 15 showed very high activity against six test organisms except *Asp. flavus* and *C. albicans* on medium 2 at 7th day fermentation. Strains HH 1, 2, 6, 8, 9, 13 and 15 indicated very high activity against eight test organisms on medium 1 and strain HH1, 2, 3, 5, 6, 8, 9, 12, 13 and 15 also exhibited very high activity againsteight test organisms on medium 2 at 8th day fermentation. Strains HH 1, 2, 6, 8, 9 and 13 indicated very high activity against eight test organismson medium 1 while also strain HH1, 2, 3, 5, 6, 8, 9, 12 and 15exhibited very high activity againsteight test organisms on medium 2at 9th day fermentation.

Strains HH1, 2, 3, 5, 6, 8, 9, 10 and 13 indicated very high activity against eight test organismson medium 1 and also strain HH1, 2, 4, 5, 6, 8 and 9exhibited very high activity against eight test organisms on medium 2 at 10th day fermentation. Strains HH 2 indicated very high activity against eight test organismson medium 1 and also strain HH1, 2, 3, 4, 5, 6, 8 and 9 exhibited

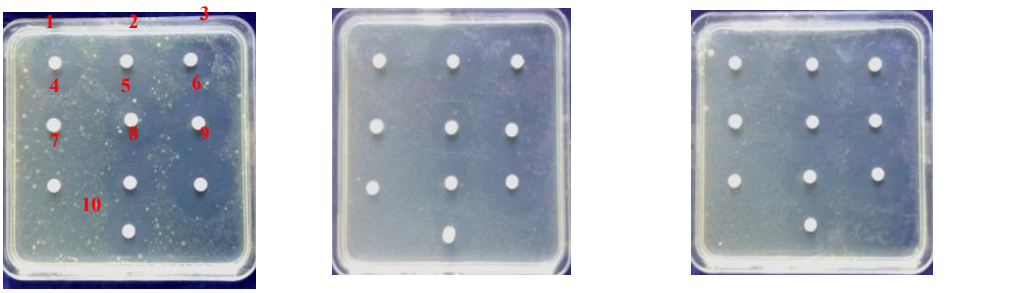
very high activity against eight test organisms on medium 2 at 11 day fermentation.

Strains HH 2 and 13 indicated very high activity against six test organisms except *Asp. flavus* and *C. albicans* on medium 1 and also strain HH2 and 8 exhibited very high activity against eight test organisms on medium 2 at 12 day fermentation.

Strains HH2 indicated very high activity against *Agro. tumefaciens, B. subtilis, S. typhi* and *X. oryzae* on medium 1 and also strain HH8 exhibited very high activity against even test organisms except *C. albicans* on medium 2 at 13 day fermentation as shown in Figures 20-23.

Agrobacterium tumefaciens *Xanthomonas oryzae* *Malassezia furfur*

Figure 20. Inhibitory zones of fermented broth (3 days old) of isolated strains from *Hesperethusa crenulata* sample 1



Salmonella typhi *Xanthomonas oryzae* *Aspergillus flavus*

Figure21. Inhibitory zones of fermented broth (8 days old) of isolated strains from *Hesperethusa crenulata* sample 1

Aspergillus flavus *Xanthanmonas oryzae* *Malassezia furfur*

Figure 22. Inhibitory zones of fermented broth (3 days old) of isolated strains from *Hesperethusa crenulata* sample 2

Salmonella typhi *Micrococcus luteus* *Malassezia furfur*

Figure 23. Inhibitory zones of fermented broth (8 days old) of isolated strains from *Hesperethusa crenulata* sample 2

Discussion and Conclusion

In the present study, sixteen endophytic fungal strains (ten strains from the sample 1 and six strains from the sample 2) were isolated from the leaves, barks, stems and roots of *Hesperethusa crenulata* (Roxb.) Roem. All strains initially showed different antimicrobial activity on *Agrobacterium tumefaciens, Aspergillus flavus, Bacillus subtilis, Candida albicans, Malassezia furfur, Micrococcus luteus, Salmonella typhus* and *Xanthomonas oryzae.* Zhao, *et al.* (2010) isolated endophytic fungi from their host plants for producing the bioactive compounds.

In this study, fourteen strains of all isolated strains indicated their antimicrobial activity from 3 day to 13 days on eight test organisms. However,

3 to 6 days of fermented broths in medium 1 and 2 of strain HH 7 showed their antimicrobial activity. Similarly 3 to 6 days of fermented broths in medium 1 and 2 of strain HH 14 exhibited its antimicrobial activity. Majority of isolated strains were showed their antimicrobial activity until the 13 day fermented broth. Among sixteen isolated fungi, strains HH 1, 2, 6, 8, 9, 11, 12 and 13 strains indicated very high antimicrobial activity on eight test organisms.

Strains HH 1, 2, 9 and 13 were identified as *Cephalosporium* sp. and they showed very high antimicrobial activity on eight test organisms.These findings are agreement with the statements of Crawford *et al* (1952), Selim *et al* (2011) and Prathyusha (2014).Crawford *et al* (1952) has reported that *Cephalosporium* sp. has antibacterial activity against Gram-positive bacteria. Selim *et al*. (2011) stated that endophytic *Cephalosporium* sp. has antimicrobial activity against different pathogenic bacteria and yeasts. Prathyusha (2014) has reported that *Cephalosporium* sp. has antimicrobial (antibacterial and antifungal) activity.

Strain HH12 was identified as *Rhizoctonia* sp. and it exhibited antimicrobial activity on eight test organisms.This finding is agreement with the statements of Prathvi *et al.* (2013) and Andrea *et al.* (2015). Prathvi *et al.* (2013) stated that endophytic *Rhizoctonia* sp. has antimicrobial activity against *Bacillus subtilis* and *Candida albicans.* Andrea *et al.* (2015) has reported that endophytic *Rhizoctonia sp.* has antimicrobial activity especially on *Candida albicans*.

In conclusion, the majority of bioactive fungal strains used in this study were found to inhibit harmful diseases causing agents such as bacteria and fungi. This funding is very beneficial to help humans' health since the bioactive compounds could be produced from the active fungal strains to protect some microbial diseases.

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