Antimicrobial Activity of Endophytic Fungi Isolated From *Psidium Guineensis* Sw.

Hlaing Tha Zin[1], Yee Yee Thu[2]

Abstract

In this study, the plant samples (leaves and fruits) of *Psidium guineensis* Sw. were collected from Hlegu Township in Yangon Region. Ten endophytic strains (HTZ 1 to HTZ 10) were isolated from leaves and fruits of *Psidium guineensis* Sw. on three different media at Microbiology Laboratory, Department of Botany, Dagon University. Fungal strains HTZ 1, HTZ 3 may be identified as *Cephalosporium* spp., strains HTZ 2, HTZ 4, HTZ 6 and HTZ 8 may be identified as *Rhizoctonia* spp., strain HTZ 9 was identified as *Aspergillus* sp., strain HTZ 10 may be identified as *Phomopsis* sp. Antimicrobial activity of the fermented broths from all isolated strains was examined on five test organisms by paper disc diffusion method and fermentation was carried out for 4 to 9 days. The fermented broths of all isolated strains showed good antimicrobial activity on *Agrobacterium tumefaciens*, *Candida albicans*, *Escherichia coli* and *Malassezia furfur* from fourth day to eighth day fermentation. Crude metabolites of ten active strains were extracted with ethyl acetate, and their antimicrobial activity was also examined on five test organisms. The crude extracts of ten strains indicated excellent antimicrobial activity on *Agrobacterium tumefaciens*, *Candida albicans*, *Escherichia coli* and *Malassezia furfur* at day 7 fermentation. Therefore, the most active strains (HTZ 1 to HTZ 9) should be chosen to produce the bioactive compounds which inhibit *Agrobacterium tumefaciens* causing crown gall disease on plants, *Candida albicans* causing alimentary tract and vaginal infections, *Escherichia coli* causing urinary tract infection and *Malassezia furfur* causing dandruff and skin infections on humans.

Keywords: Antimicrobial activity, Endophytic fungi, *Psidium guineensis* Sw.

Introduction

Psidium guineensis Sw. belongs to in the family Myrtaceae and guava trees are grown in farm gardens on the Shan Plateau near Pyin Oo Lwin and elsewhere in Myanmar. *Psidium guineensis* Sw. is a guava species distributed in South America, some part of Africa and South Asia. It is a plant of interest of many researchers due to its high phenolic content, antioxidant properties and antibacterial properties of the leaf and fruit extracts.

Endophytes are microorganisms living in the internal tissue of plants without any obviate consequences to the host at least at a certain stage of their life (Petrini, 1991). The term endophyte denotes a symbiotic relationship between host and endophyte (Schulz, *et al*., 1999, Schulz and Boyle, 2006). They are comprised of diverse microbial communities including archaeal, bacterial, fungal, and protistic taxa (Hardoim, *et al*., 2015). Endophytic fungi and their host plants have an intimate relationship, and fungi can produce bioactive secondary metabolites that protect against plant pathogens and pests as a growth regulator (Chutulo and Chalannavar, 2020).

Endophytic fungi such as *Rhizoctonia* spp. were isolated from barley and wheat in Erzurum, Turkey (Demirci, 1998). Potential antimicrobial and antiproliferative activity of the crude extract of the endophytic fungus *Rhizoctonia* sp. isolated from *Annona crassiflora* (Mendonca, *et al*., 2015). Endophytic fungi, *Aspergillus* strains constitute one of the most prolific sources of secondary metabolites with diverse chemical classes and interesting biological activities (Hagag and Miada, 2022). The antimicrobial agent phomopsichalasin was isolated from an endophytic *Phomopsis* sp. fermented on shredded wheat (Horn, *et al*., 1995).

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The objectives of this research are to isolate endophytic fungal strains from *Psidium guineensis* Sw., to investigate antimicrobial activity of fermented broths of all isolated strains and to evaluate antimicrobial activity of all the extracts of isolated strains.

Materials and Methods

Collection of Plant Samples

The plant samples (leaves and fruits) of *Psidium guineensis* Sw. (Red flesh guava) were collected from Hlegu Township in Yangon Region. The collected samples were then taken for the experiments which were carried out at Microbiology Laboratory, Department of Botany, Dagon University.

Isolation of Endophytic Fungal Strains from *Psidium guineensis* Sw.

Endophytic strains were isolated from the leaves and fruits of *Psidium guineensis* Sw. (Red flesh guava) on three different media. Isolation of endophytic strains was carried out by the following procedure (Lee *et al*., 1996).

1. The plant samples were washed under running tap water for ten minutes.
2. The plant parts (leaves and fruits) were cut into about 1.5 cm - 2.0 cm pieces.
3. These pieces were sterilized by soaking in 75% alcohol for 1 to 2 minutes.
4. These parts were dried on sterilized paper and then they were placed on agar plates containing different media.
5. Then, the plates were incubated for 3-7 days at room temperature.

Composition of Culture Media (Atlas, 1993)

Medium 1

Nutrient Agar Medium (NA)

Nutrient Agar 3.9 g, Distilled Water 100 ml, pH 6.8

Medium 2

Sucrose Yeast Extract Agar Medium (SY)

Sucrose 1.0 g, Yeast Extract 0.3 g, Distilled Water 100 ml, Agar Powder 2.25 g, pH 6.8

Medium 3

Lactose Yeast Extract Agar Medium (LY)

Lactose 1.0 g, Yeast Extract 0.3 g, Distilled Water 100 ml, Agar Powder 2.25 g, pH 6.8

Antimicrobial Activity of Isolated Fungal Strains

Fermentation

Isolated ten fungal strains grown on 5 days old slant cultures were inoculated into 10 conical flasks (50 ml) containing 20 ml of sucrose/yeast extract medium in each for three days at 75 spm as seed culture. After three days, seed cultures were transferred to the ten fermentation flasks at 30°C on shaker for 10 days at 100 rpm. Every day inhibitory zones were measured to examine antimicrobial activity of isolated strains (Strobel and Sullivan, 1999).

Sucrose/Yeast Extract Medium (SY)

Sucrose 1.0 g, Yeast extract 0.3 g, NaCl 0.3 g, $CaCO_3$ 0.01 g, Distilled Water 100 ml, pH 7

Test agar plates

There are five test organisms: three bacterial test organisms (*Agrobacterium tumefaciens, Bacillus subtilis* and *Escherichia coli*) and two fungal test organisms (*Candida albicans* and *Malassezia furfur*) in Table 1. Broth culture (0.3 ml) of each test organism is added into 100 ml nutrient agar medium, and then poured into plates.

Paper disc diffusion assay

After solidification, paper discs impregnated with the fermented broth samples were applied on the test plates. These plates were incubated at 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 which inhibit the growth of test organisms (Davis and Stout, 1971).

Table 1. Test organisms and diseases

Test organisms	Diseases
Agrobacterium tumefaciens	Crown gall diseases.
Bacillus subtilis	It can cause dysentery, but at the first sign of diarrhea.
Candida albicans	Vaginal candidasis, urogenital infection, alimentary tract infection.
Escherichia coli	Cholera, diarrhea and vomiting, urinary tract infections.
Malassezia furfur	Dandruff.

Antimicrobial activity of crude extracts of fungal strains

The fungal strains were inoculated into the ten conical flasks containing SY seed medium. Each flask contained 20 ml of medium. After three days, seed cultures (1 ml of each) were transferred to the ten fermentation flasks at 30°C for 7 days on the shaker at 100 rpm. Each flask contained 20 ml of medium. At day 7 fermentation, the fermented broth of each fungal strain was extracted with ethyl acetate (10 ml) at pH 4.5. Then, the extracts were dried in the incubator at 40ºC. Then, each dried extract was added 0.5 ml of ethyl acetate and mixed thoroughly with glass rod. The extract (20 µl/disc) of each strain was applied for their antimicrobial activity.

Results

Isolation of Endophytic Fungi

Ten fungal strains were isolated from leaves and fruits of *Psidium guineensis* Sw. Eight fungal strains were isolated from leaves and two strains from fruits of *Psidium guineensis* Sw. The strains were given as temporary names HTZ 1 to HTZ 10 as shown in Figures 1.

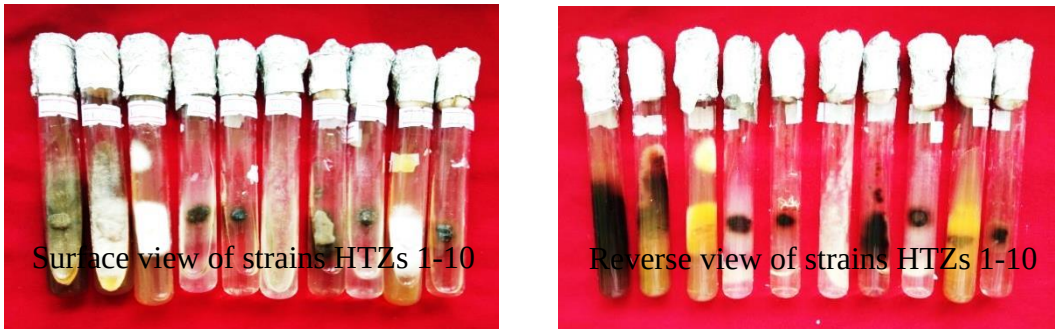


Fig. 1. Isolated fungal strains on slant cultures (HTZ 1 to HTZ 10)

Antimicrobial Activity of Isolated Fungal Strains

Antimicrobial activity of day 4 fermentation

Ten endophytic fungal strains were isolated from leaves and fruits of *Psidium guineensis* Sw. Ten isolated fungal strains indicated antimicrobial activity on four test organisms. At day 4 fermentation, strain HTZ 10 showed highly antibacterial activity whereas strains HTZs 1, 3, 4, 7 and 8 showed moderately antibacterial activity on *Agrobacterium tumefaciens*. Strains HTZs 1, 3, 4, 8 and 10 indicated moderately antifungal activity on *Malassezia furfur*. Strains HTZs 1, 2, 3, 4, 7, 8 and 10 showed weak antifungal activity on *Candida albicans.* Strains HTZs 2 and 4-10 showed weak activity against *Escherichia coli*. Only strain HTZ 5 inhibited moderately antibacterial activity on *Bacillus subtilis* but other nine strains did not show activity against *Bacillus subtilis* in Table 2.

Table 2. Inhibitory zones of day 4 fermented broths of ten strains

Strain / Test organisms	HTZ 1	HTZ 2	HTZ 3	HTZ 4	HTZ 5	HTZ 6	HTZ 7	HTZ 8	HTZ 9	HTZ 10
Agrobacterium tumefaciens	16	12	14	14	9	10	15	13	9	18
Bacillus subtilis	-	-	-	-	14	-	-	-	-	-
Candida albicans	10	9	10	11	-	-	10	9	-	10
Escherichia coli	+	10	+	10	9	9	10	10	10	10
Malassezia furfur	14	12	15	15	9	10	12	14	10	16

8-12 mm = weak activity, 13-17 mm = moderate activity, >18 mm = high activity

Antimicrobial activity of day 5 fermentation

At day 5 fermentation, strains HTZs 1 and 7 showed moderately antibacterial activity whereas the other strains showed weak activity on *A. tumefaciens*. All strains indicated weak antimicrobial activity against *M. furfur* and *E. coli*. Strains HTZs 1, 2, 3, 7, 8 and 10 showed weak antifungal activity against *C. albicans* but other strains did not show activity against *C. albicans.* Only strain HTZ 5 indicated moderately antibacterial activity on *B. subtilis* but other nine strains did not show against *B. subtilis* in Table 3.

Table 3. Inhibitory zones of day 5 fermented broths of ten strains

Strain / Test organisms	HTZ 1	HTZ 2	HTZ 3	HTZ 4	HTZ 5	HTZ 6	HTZ 7	HTZ 8	HTZ 9	HTZ 10
Agrobacterium tumefaciens	13	10	10	11	11	9	13	11	11	11
Bacillus subtilis	-	-	-	-	13	-	-	-	-	-
Candida albicans	9	8	8	-	-	-	8	9	-	9
Escherichia coli	10	11	11	8	8	8	9	9	8	8
Malassezia furfur	12	9	10	11	10	8	12	10	10	10

8-12 mm = weak activity, 13-17 mm = moderate activity, >18 mm = high activity

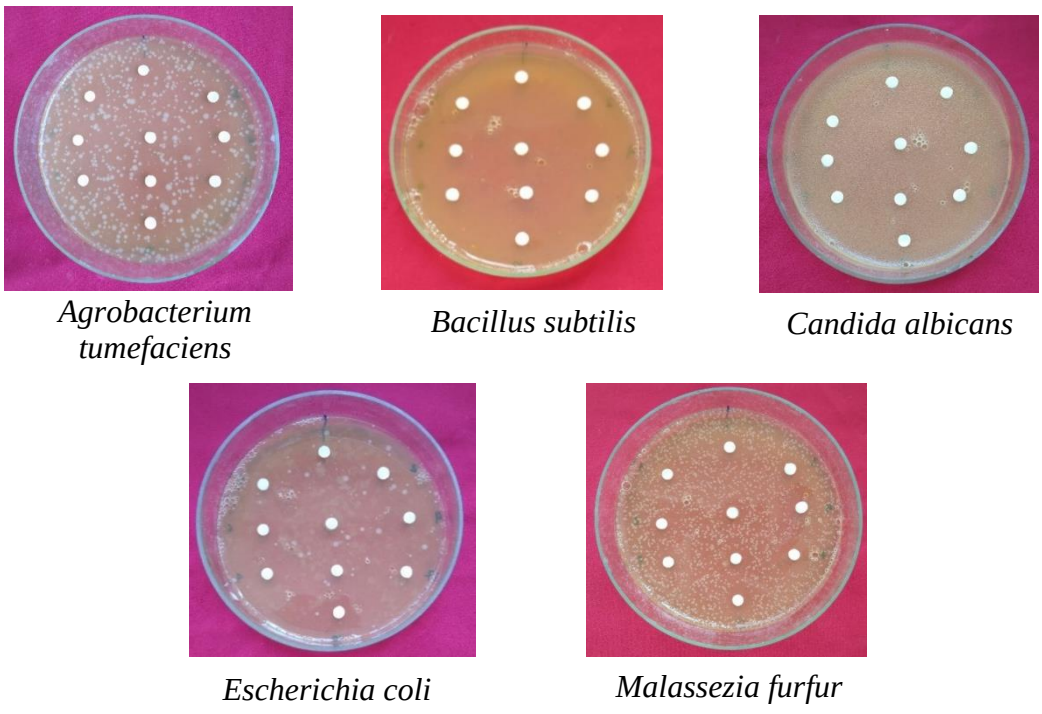
Antimicrobial activity of day 6 fermentation

At day 6 fermentation, strains HTZs 1, 2, 3 and 8 showed moderately antimicrobial activity whereas other strains showed weak activity on *A. tumefaciens*. Strains HTZs 1, 2, 4, 5, 6, 7 and 10 indicated moderately antifungal activity but strains HTZs 3 and 9 indicated weak activity on *M. furfur*. All strains showed weak antibacterial activity on *E. coli*. Strains HTZs 1, 2, 3, 4, 7, 8 and 10 showed weak activity against *C. albicans* but other strains did not show activity against *C. albicans*. Only strain HTZ 5 indicated moderately antibacterial activity on *B. subtilis* but other nine strains did not show against *B. subtilis* in Table 4 and Figure 2.

Table 4. Inhibitory zones of day 6 fermented broths of ten strains

Strain / Test organisms	HTZ 1	HTZ 2	HTZ 3	HTZ 4	HTZ 5	HTZ 6	HTZ 7	HTZ 8	HTZ 9	HTZ 10
Agrobacterium tumefaciens	13	14	14	12	11	11	12	15	11	12
Bacillus subtilis	-	-	-	-	13	-	-	-	-	-
Candida albicans	10	9	9	10	-	-	9	9	-	9
Escherichia coli	11	11	12	10	11	10	9	10	10	10
Malassezia furfur	13	13	10	15	13	13	16	13	11	13

8-12 mm = weak activity, 13-17 mm = moderate activity, >18 mm = high activity



Agrobacterium tumefaciens *Bacillus subtilis* *Candida albicans*

Escherichia coli *Malassezia furfur*

Figure. 2. Inhibitory zones of day 6 fermented broths of ten strains

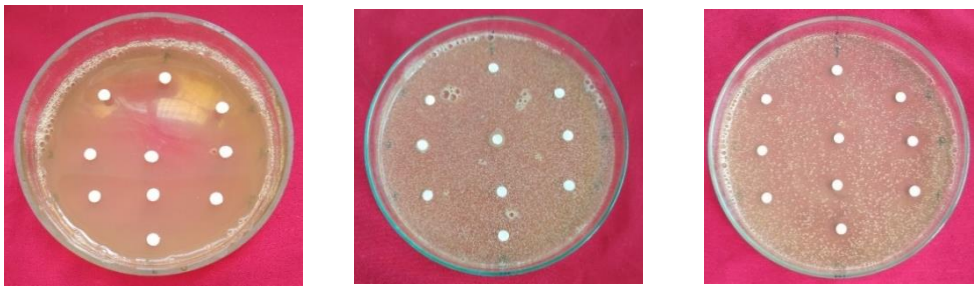
Antimicrobial activity of day 7 fermentation

At day 7 fermentation, all strains showed weak activity against *A. tumefaciens*. Strains HTZs 3 and 4 indicated highly antifungal activity, strains HTZs 1, 2 and 7 indicated moderately activity while strains HTZs 5, 6, 8, 9 and 10 indicated weak activity on *M. furfur***.** Strains HTZs 5, 7, 8, 9 and 10 showed highly antibacterial activity while all strains showed moderately antibacterial activity on *E. coli*. Strains HTZs 1, 4, 7, 8 and 10 showed moderately antifungal activity whereas other strains showed weak activity on *C. albicans*. Only strain HTZ 5 indicated moderately antibacterial activity on *B. subtilis* but other 9 strains did not show against *B. subtilis* in Table 5 and Figure 3.

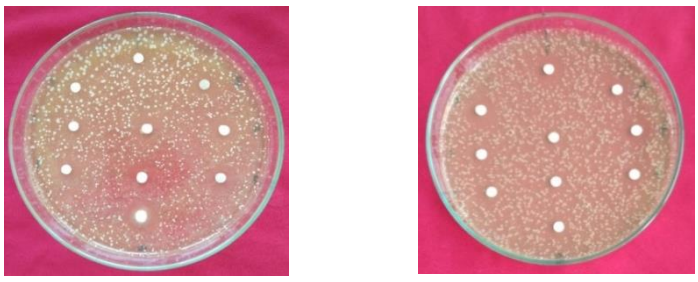
Table 5. Inhibitory zones of day 7 fermented broths of ten strains

Strain / Test organisms	HTZ 1	HTZ 2	HTZ 3	HTZ 4	HTZ 5	HTZ 6	HTZ 7	HTZ 8	HTZ 9	HTZ 10
Agrobacterium tumefaciens	12	11	11	11	10	10	12	12	11	12
Bacillus subtilis	-	-	-	-	13	-	-	-	-	-
Candida albicans	13	11	11	13	10	-	13	13	10	13
Escherichia coli	14	14	13	16	18	14	18	19	20	20
Malassezia furfur	17	15	20	18	11	12	13	11	12	12

8-12 mm = weak activity, 13-17 mm = moderate activity, >18 mm = high activity



Agrobacterium tumefaciens *Bacillus subtilis* *Candida albicans*



Escherichia coli *Malassezia furfur*

Figure 3. Inhibitory zones of day 7 fermented broths of ten strains

Antimicrobial activity of day 8 fermentation

At day 8 fermentation, all strains showed weak activity against *A. tumefaciens*. Strains HTZs 2, 3, 4, 7, 8 and 10 indicated moderately antifungal activity while other strains showed weak activity on *M. furfur*. Strains HTZs 5, 7, 8 and 9 inhibited moderately antibacterial activity while other strains showed weak activity on *E. coli*. Strains HTZs 1, 2, 3, 5, 7, 8 and 10 showed weak antifungal activity against *C. albicans* but other strains did not show activity against *C. albicans*. Only strain HTZ 5 indicated weak activity against *B. subtilis* but other 9 strains did not show against *B. subtilis* in Table 6.

Table 6. Inhibitory zones of day 8 fermented broths of ten strains

Strain / Test organisms	HTZ 1	HTZ 2	HTZ 3	HTZ 4	HTZ 5	HTZ 6	HTZ 7	HTZ 8	HTZ 9	HTZ 10
Agrobacterium tumefaciens	11	12	11	12	10	11	11	11	10	12
Bacillus subtilis	-	-	-	-	11	-	-	-	-	-
Candida albicans	8	8	9	-	8	-	8	9	-	9
Escherichia coli	12	12	10	12	14	12	13	15	13	12
Malassezia furfur	12	13	15	14	11	11	13	15	12	16

8-12 mm = weak activity, 13-17 mm = moderate activity, >18 mm = high activity

Antimicrobial Activity of Crude Extracts of Fungal Strains

Among all strains, the extracts of day 7 fermented broth of nine fungal strains (HTZs 1 to 9) showed highly antimicrobial activity but strain HTZ 10 indicated weak activity against four test organisms. The extracts of nine strains (HTZ 1- HTZ 9) indicated highly antimicrobial activity on *Agrobacterium tumefaciens* (21 mm – 30 mm), *Candida albicans* (20 mm – 30 mm), *Escherichia coli* (25 mm – 38 mm) and *Malassezia furfur* (20 mm – 30 mm). But, all strains did not show antibacterial activity on *Bacillus subtilis* in Table 7 and Figure 4.

Table 7. Antimicrobial activity of crude extracts of all strains

Strain / Test organisms	HTZ 1	HTZ 2	HTZ 3	HTZ 4	HTZ 5	HTZ 6	HTZ 7	HTZ 8	HTZ 9	HTZ 10
Agrobacterium tumefaciens	25	25	26	21	22	26	26	28	30	15
Bacillus subtilis	-	-	-	-	-	-	-	-	-	-
Candida albicans	22	23	25	20	25	24	26	30	24	12
Escherichia coli	27	30	37	25	38	30	30	28	30	15
Malassezia furfur	25	28	30	20	20	25	26	28	27	12

8-12 mm = weak activity, 13-17 mm = moderate activity, >18 mm = high activity

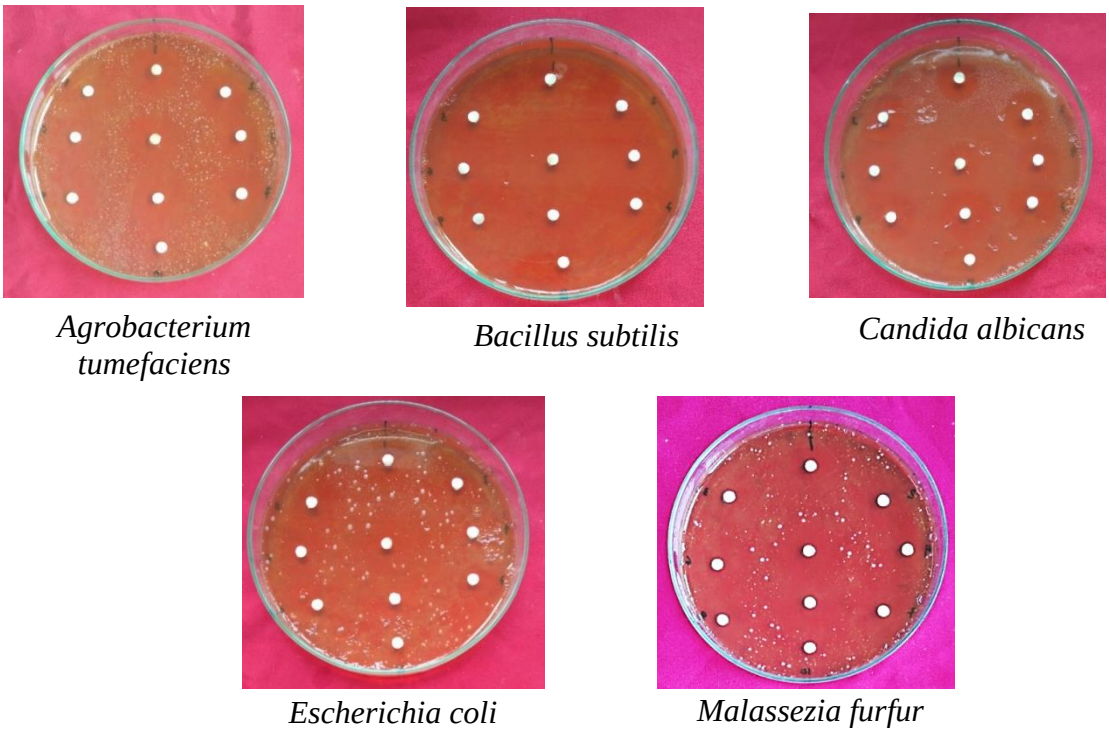


Figure 4. Antimicrobial activity of crude extracts of isolated strains

Discussion and Conclusion

In this study, ten fungal strains: two *Cephalosporium* spp., four *Rhizoctonia* spp., one *Aspergillus* sp., one *Phomopsis* sp. and two unknown species were isolated from *Psidium guineensis* Sw. The fermented broths of all isolated strains (HTZ 1 to HTZ 10) showed good activity against *Agrobacterium tumefaciens, Candida albicans, Escherichia coli* and *Malassezia furfur* from day 4 to day 8 fermentation. The ethyl acetate extracts of day 7 fermented broths of all ten strains indicated highly antimicrobial activity on four test organisms.

Hagag and Miada (2022) have isolated endophytic *Aspergillus* spp. as sources of secondary metabolites with diverse chemical classes and interesting biological activities. Yee Yee Thu *et al*., (2016) have isolated *Aspergillus* sp. from different plant species to produce the bioactive compounds. Htet Htet Zaw and Yee Yee Thu (2020) also isolated endophytic fungi *Cephalosporium* spp. from *Hesperathusa crenulate* (Roxb.) Rome. and tested antifungal activity on *C. albicans* and *M. furfur*.

Mendonca, *et al*., (2015) stated that potential antimicrobial and antiproliferative activity of the crude extract of the endophytic fungus *Rhizoctonia* sp. isolated from *Annona crassiflora*. Horn, *et al*. (1995) isolated an endophytic *Phomopsis* sp. from wheat to produce a novel antimicrobial agent. Sanjay, *et al*., (2011) isolated an endophytic *Phomopsis* sp. from *Odontoglossum* sp. (Orchidaceae) in Northern Ecuador to produce a mixture of volatile organic compounds. Yee Yee Thu, *et al*., (2019) isolated endophytic fungus *Phomopsis* sp. from the leaves of *Psidium guajava* L. to produce the antimicrobial agents.

In conclusion, endophytic fungi have been known as excellent source of antimicrobial agents. Nowadays the discovery of biological agents that possess selective toxicity against human and plant pathogens is very important. The most active strains should be chosen to produce the bioactive compounds which inhibit *Agrobacterium*

tumefaciens causing crown gall disease on plants, *Candida albicans* causing alimentary tract and vaginal infections, *Escherichia coli* causing urinary tract infection and *Malassezia furfur* causing dandruff and skin infections on humans. Since ten endophytic fungal strains were isolated and they possessed good antimicrobial activity in this research, these findings could be very helpful to man and plants.

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