FERMENTATION STUDIES OF *RHIZOCTONIA* SP. ISOLATED FROM THE LEAVES OF *COMBRETUM INDICUM* (L.) R. A. DEFILIPPS.

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Abstract

An endophytic fungi *Rhizoctonia* sp. was isolated from the leaves of *Combretum indicum* (L.) R. A. DeFillips. In the present study, fermentation (one day old) of age of inoculum was the best for fermentation. In the size of inoculum optimization, fermentation (2%) showed the best antimicrobial activity on six test organisms and fermentation (pH 5) of pH utilization was the best for the production of bioactive compounds according to the results of inhibitory zones of their antimicrobial activity against six test organisms. In the study of extraction of metabolites by using different solvents system, the extracts with methanol, ethanol, ethyl acetate and n-hexane showed the high activity on six test organisms: *Bacillus pumilus*, *Bacillus subtilis*, *Candida albicans*, *Micrococcus luteus*, *Pseudomonas* sp. and *Staphylococcus aureus*. Among them, ethyl acetate extract showed the best antimicrobial activity.

Keywords: Endophytes, *Rhizoctonia* sp., antimicrobial activity

Introduction

Endophytes are an endosymbiotic group of microorganisms, often bacteria or fungi that colonize the intercellular or intracellular locations of plants (Pimentel *et al*., 2011; Singh and Dubey, 2015). Medicinal plants are known to harbor endophytic microorganisms, which are found to play an important role in the production of pharmaceutically important compounds. Endophytic fungi are generally considered superior because of their ubiquitous and diverse nature. They produce many secondary metabolites greater than other endophytic microorganisms (Zhang *et al*., 2006).

The aims of this study were to evaluate fermentation studies of isolated fungal strain *Rhizoctonia* sp. and to conduct extraction of metabolites using five different solvents.

Materials and methods

Collection of plant samples

The leaves of *Combretum indicum* (L.) R. A. DeFilipps were collected from the University of Yangon Campus. The collected plant material was recorded by photographs and identified by Wu and Raven, 2007. Myanmar name was recorded by Kress *et al*. 2003.

Isolation of endophytic Fungi (Suto,1999)

The leaves of plant were washed in running tap water for five minutes. The leaves were cut into small pieces (3cm). These parts were sterilized by soaking in 75% ethanol for 15 seconds. Then, these parts cut into smaller pieces (1cm) and dried on sterilized paper. After that, they were placed on agar plates containing sucrose-yeast extract medium (SY medium) and nutrient agar medium (NA medium) supplemented with chloramphenicol (100µg / L) to inhibit bacterial growth. These plates were incubated at room temperature for 3-7 days and transferred to new plates. Then, isolated fungal strains were transferred into slant culture of test tubes.

Agar well diffusion method

The agar plates containing test organisms were punched to make the wells (8 mm in diameter) using sterile cork borer and filled with the stock solution and then these plates were

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incubated at room temperature for 24 hours. After incubation, the diameters of the growth inhibition zones surrounding the wells were measured in mm. These zones indicate the presence of antimicrobial activities which inhibit the growth of test organisms selectively. (Collins, 1965).

Fermentation studies

(i) Age of inoculum of *Rhizoctonia* sp.

One day old, two days old and three days old of seed cultures were transferred into 50 ml fermentation flasks containing 25 ml of nutrient agar (NA) medium. They were incubated for seven days. Then, these fermented broths were checked for their inhibitory activities by agar well diffusion method. (Strobel and Sullivan, 1999).

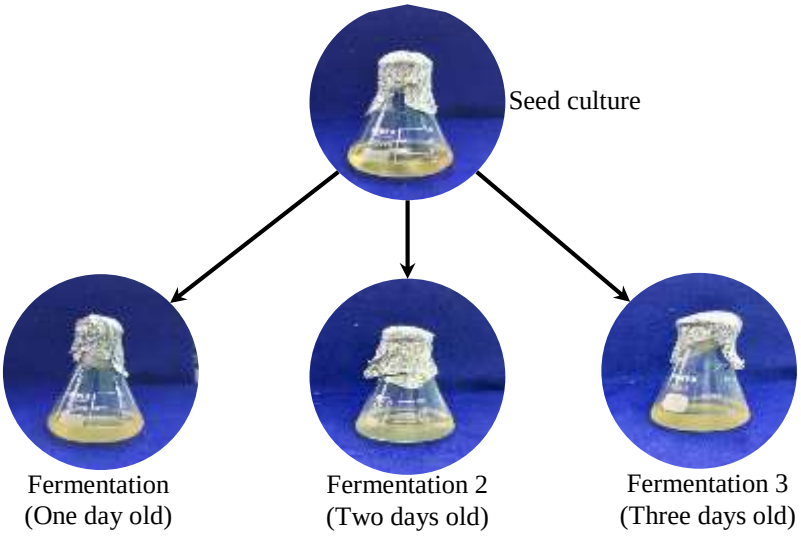


Figure 1. Seed culture and fermentation for age of inoculum

(ii) Size of inoculum of *Rhizoctonia* sp.

The proper cultivation and transfer (size of inoculum) are essential to produce bioactive metabolites. A piece from fungal plate culture of *Rhizoctonia* sp. was inoculated into 125 ml of conical flasks containing 100 ml of nutrient agar (NA) medium. The flasks were incubated at room temperature for one day. After one day, the seed cultures (1.0%, 1.5%, 2.0%, 2.5% and 3.0%) were transferred into five conical flasks (125 ml) containing 100 ml of fermentation medium as shown in Figure 2. The fermentation was carried out for seven days. (Monaghan *et al*., 1999)

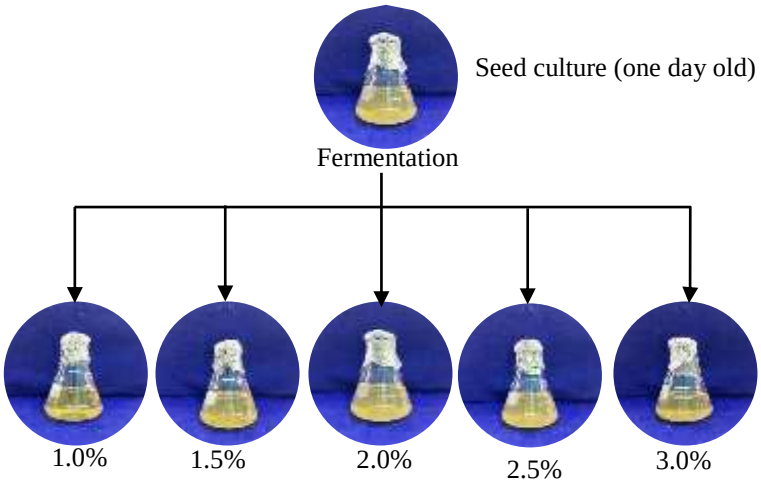


Figure 2. Seed culture and fermentation for size of inoculum

(iii) pH utilization of *Rhizoctonia* sp.

For the seed culture, a piece from fungal plate culture of *Rhizoctonia* sp. was inoculated into 125 ml of conical flask containing 100 ml of fermentation medium (Nutrient agar medium) and then flasks were incubated at room temperature for one day. Five 125 ml conical flasks containing 100 ml fermentation medium were adjusted at pH 4, 5, 6, 7, 8 and autoclaved. The seed culture (2%) was transferred to each fermentation flask with pH 4 to 8 and fermentation was carried out for one day. After one day, five fermentation flasks were checked their antimicrobial activity. (Monaghan *et al*., 1999).

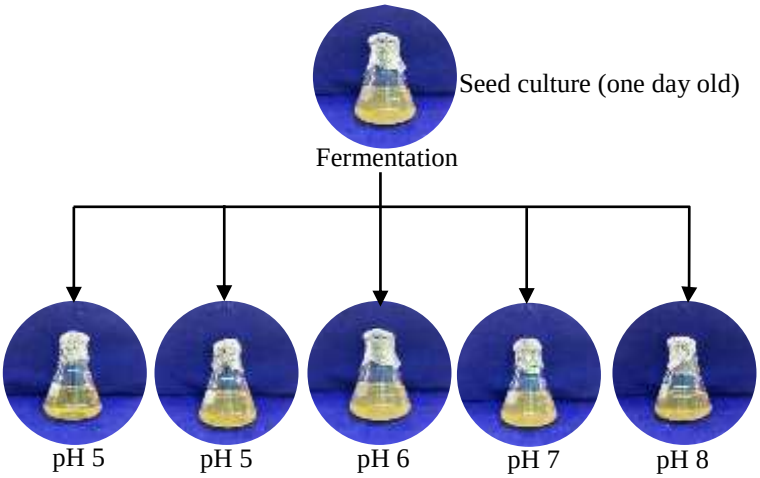


Figure 3. Seed culture and fermentation for pH of inoculum

Extraction and antimicrobial activity of bioactive compounds from *Rhizoctonia* sp.

A piece from fungal plate culture of *Rhizoctonia* sp. was cultured in 300 ml Erlenmeyer flasks containing 150 ml of fermentation medium (NA medium) and incubated at room temperature, 180 rpm for one day. Then, the media was harvested by centrifugation at 5000 rpm for 20 min. The supernatant was transferred and equal volume of five different solvents (ethanol, methanol, ethyl acetate, n-hexane and petroleum ether) were separately added to the filtrate. The mixture was shaken for 30 min and placed in water bath at 50°C to evaporate the aqueous and organic layer of crude extract and obtain a gummy crude extract. The crude extract was re-dissolved in a small volume of each solvent (1mg/1ml) and screened for their antimicrobial activity on six test organisms. The plates were incubated at room temperature for 24 hours and checked for their inhibitory zones. (Ahmed, 2007)

Results

Scientific classification

Scientific name:	***Combretum indicum*** (L.) R.A. DeFilipps
Synonym:	*Quisqualis indica* L.
Common Names:	Dawe-hmaing-nwe, Mawk-nang-nang, Rangoon creeper
Family:	Combretaceae

Outstanding characters

Lianas to 8 m tall. Branchlets brownish yellow pubescent. **Petiole** 5-9 mm, densely brown pilose when young; **leaf blade** mostly oblong-elliptic or elliptic, 5-18 × 2.5-7 cm, abaxially sometimes brown pilose, adaxially glabrous except slightly brown pilose on midvein, finely white verruculose, rarely tomentose on both surfaces, base obtuse, apex acuminate to shortly caudate;

lateral veins in 7 or 8 pairs. **Inflorescences** lax; bracts deciduous, filiform-linear to ovate, 3-12 mm, brown pilose. **Flowers** fragrant. **Calyx** tube 5-9 cm, yellow pilose; lobes deltoid, 2-3 mm, apex acute or shortly acuminate but not cuspidate. **Petals** opening white, later turning yellowish abaxially and reddish adaxially, obovate to oblanceolate, 10-24 × 4-10 mm, apex rounded to obtuse. **Fruit** red when young, greenish black or brown when ripe, fusiform, or narrowly ovoid, sharply 5-ridged, 2.7-4 × 1.2-2.3 cm, glabrous, apex mucronate.

Figure 4. Habit of *Combretum indicum* (L.) R. A. DeFilipps

Antimicrobial activity of isolated endophytic fungus *Rhizoctonia* sp.

In this study, isolated endophytic fungi *Rhizoctonia* sp. showed the best antimicrobial activities against *Agrobacterium tumefaciens, Bacillus pumilus, Candida albicans, Escherichia coli, Pseudomonas* sp. and *Staphylococcus aureus.*

Morphological and microscopical characters of isolated fungus *Rhizoctonia* sp.

In morphological character, the surface colour of *Rhizoctonia* sp. was gray, its reverse colour is black and the margin was undulate. The microscopical character was that cell of mycelium usually long, septa of branches usually set off from the main hyphae; asexual fruit bodies and conidia absent; sporodochium-like bodies and chlamydospore-like cells in chains. Therefore, according to Barnett, 1998 description, this fungus may be *Rhizoctonia* sp.

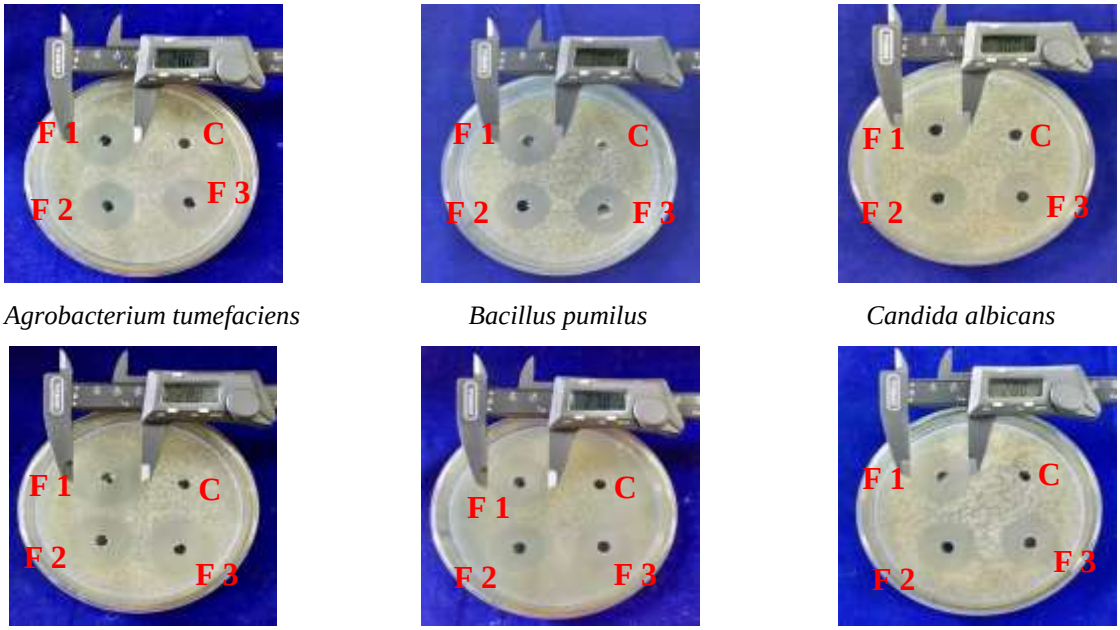
Fermentation studies of isolated fungus *Rhizoctonia* sp.

(i) Age of inoculum

In age of inoculum, fermentation 1 (One day old) showed the better activity than fermentation 2 and fermentation 3. It showed the highest activity on *Candida albicans, Micrococcus luteus* and *Staphylococcus aureus* at 1st and 2nd days. It also showed the result with high inhibition rate at 1st day on *Pseudomonas* sp., 4th and 5th days on *Agrobacterium tumifaciens* and 3rd day on *Bacillus pumilus.*

Table 1. Effect of age of inoculum on antimicrobial activity against six test organisms

Days / Test organisms	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
Agrobacterium tumefaciens	17	22	27	30	30	29	28
Bacillus pumilus	23	31	32	30	30	30	30
Candida albicans	32	32	30	30	30	30	27
Micrococcus luteus	30	30	28	27	26	24	24
***Pseudomonas* sp.**	31	30	28	28	27	27	27
Staphylococcus aureus	30	30	29	28	28	28	27



Agrobacterium tumefaciens *Bacillus pumilus* *Candida albicans*

Micrococcus luteus *Pseudomonas sp.* *Staphylococcus aureus*

Figure 5. Effect of age of inoculum on antimicrobial activity against six test organisms

(F1=Fermentation 1; one day old, F2= Fermentation 2; two day old, F3=Fermentation 3; three day old)

(ii) Size of inoculum

In the study of size of inoculum optimization, among the fermentation (1.0%, 1.5%, 2%, 2.5% and 3.0%), 2% of fermentation was suitable to produce the bioactive compound. It showed the highest antimicrobial activity on *Bacillus pumilus*, *Bacillus subtilis*, *Candida albicans*, *Micrococcus luteus*, *Pseudomonas sp*., *Staphylococcus aureus*.

Table 2. Effect of size of inoculum on antimicrobial activity against six test organisms

Days / Test organisms	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
Bacillus pumilus	29	27	27	27	24	22	19
Bacillus subtilis	29	29	28	27	27	26	26
Candida albicans	30	27	25	25	21	18	13
Micrococcus luteus	32	30	30	28	28	25	25
***Pseudomonas* sp.**	31	30	28	28	25	20	20
Staphylococcus aureus	29	28	28	28	26	26	25

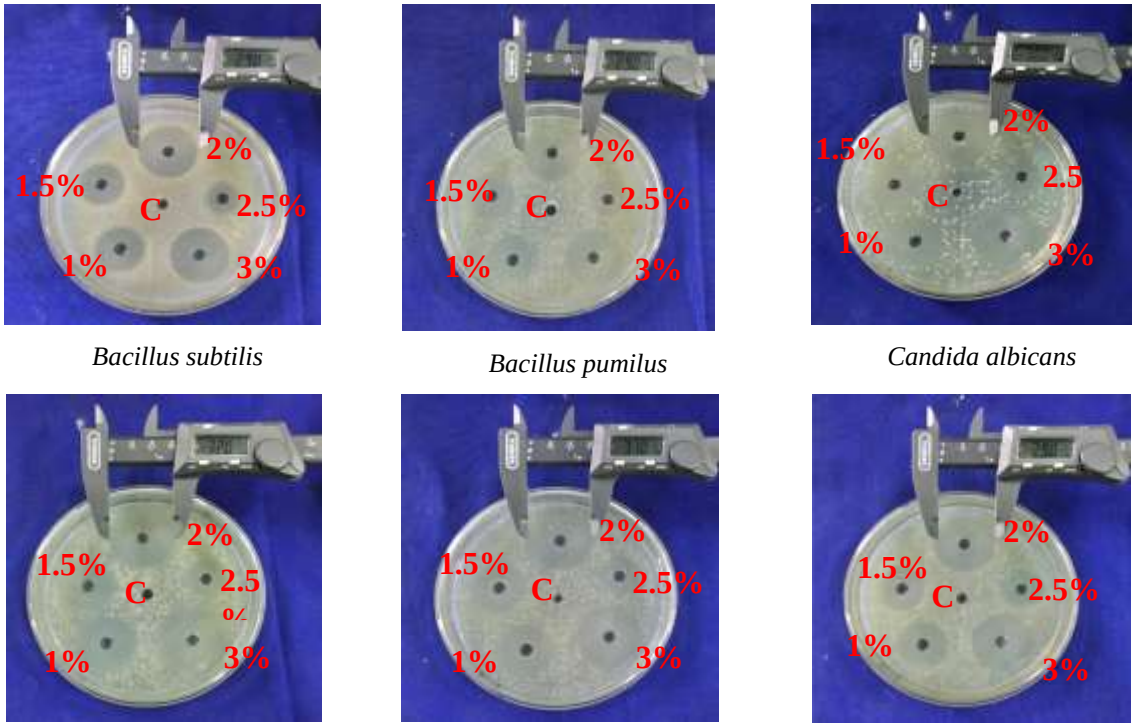


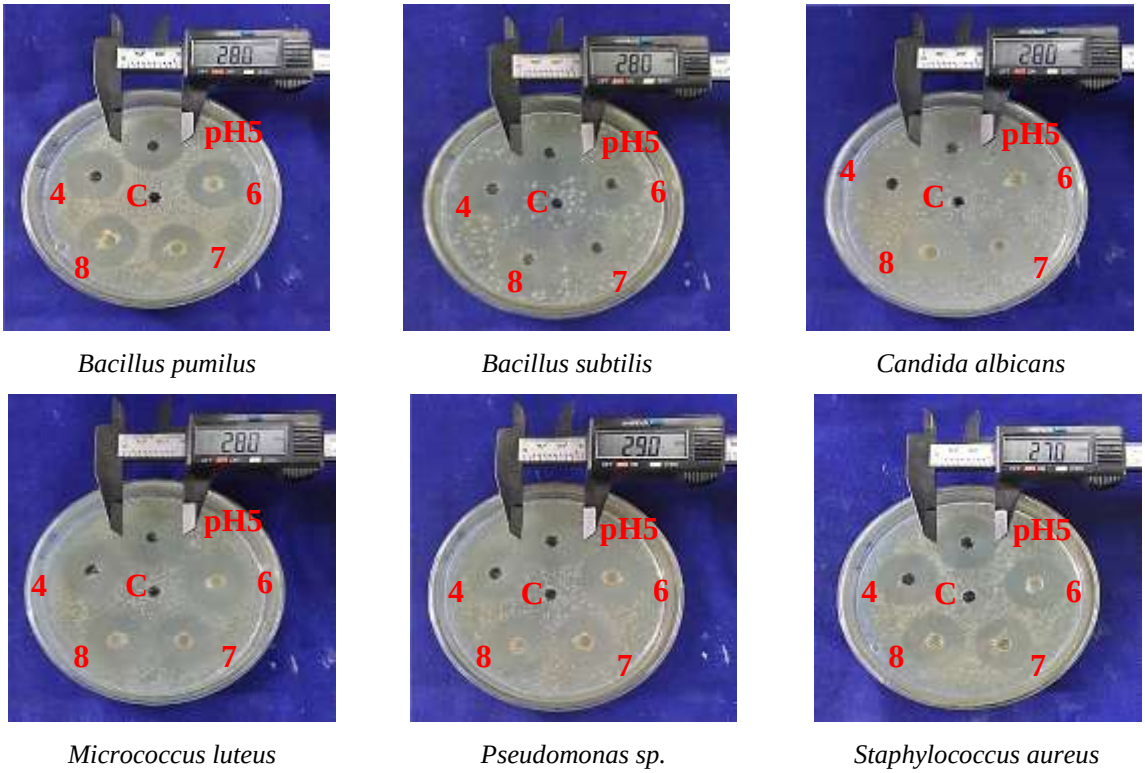
Figure 6. Effect of size of inoculum on antimicrobial activity against six text organisms

(iii) Effect of different pH

Among pH 4, 5, 6, 7 and 8 of fermented broth of *Rhizoctonia* sp., pH 5 was the best for extraction of the bioactive compounds from fermented broth according to the result of inhibitory zones against six test organisms. Fermentation with pH 5 showed the highest antimicrobial activity on *Bacillus pumilus*, *Candida albicans* and *Pseudomonas* sp. at 4th day. The best antimicrobial activity against *Bacillus subtilis* was found in pH 5 at 2nd, 3rd and 4th days. It also showed the result with high inhibition rate at 3rd and 4th days on *Micrococcus luteus* and at 4th and 5th days on *Staphylococcus aureus*.

Table 3. Effect of pH of inoculum on antimicrobial activity against six test organisms

Days / Test organisms	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
Bacillus pumilus	25	25	27	28	25	25	25
Bacillus subtilis	26	28	28	28	27	26	26
Candida albicans	22	25	27	28	26	25	25
Micrococcus luteus	25	26	28	28	26	26	25
***Pseudomonas* sp.**	24	24	28	29	27	27	27
Staphylococcus aureus	23	24	26	27	27	25	25



Bacillus pumilus *Bacillus subtilis* *Candida albicans*

Micrococcus luteus *Pseudomonas sp.* *Staphylococcus aureus*

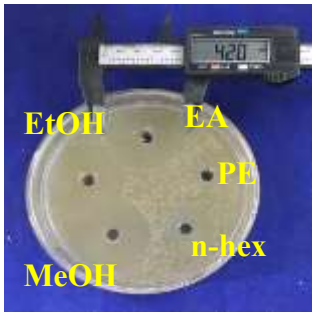
Figure 7. Effect of pH of inoculum on antimicrobial activity against six test organisms

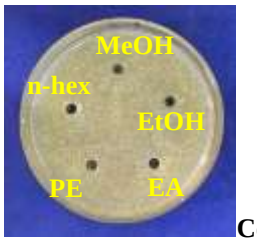
Extraction and antimicrobial activity of bioactive compounds from *Rhizoctonia* sp.

In the study of extraction of metabolites by using different solvent system, the extracts with methanol, ethanol, ethyl acetate, petroleum ether and n-hexane showed the highest activity on six test organisms: *Bacillus pumilus*, *Bacillus subtilis*, *Candida albicans*, *Micrococcus luteus*, *Pseudomonas* sp. and *Staphylococcus aureus*. Among them, ethyl acetate extract showed the best activity on six test organisms. Therefore, ethyl acetate was the best to produce bioactive metabolites for *Rhizoctonia* sp. (Ahmed, 2007)

Table 4. Antimicrobial activity of different extracted metabolites produced by *Rhizoctonia* sp.

Solvents / Test organisms	Methanol	Ethanol	Ethyl acetate	Petroleum ether	n-hexane
Bacillus pumilus	40.0	40.0	42.0	-	26.0
Bacillus subtilis	41.0	40.0	42.0	20.0	22.0
Candida albicans	40.0	41.0	43.0	26.0	20.0
Micrococcus luteus	40.0	40.0	40.0	30.0	26.0
***Pseudomonas* sp.**	40.0	40.0	44.0	-	24.0
Staphylococcus aureus	42.0	40.0	43.0	-	20.0



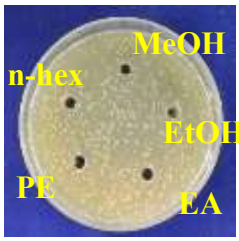


Control

Bacillus pumilus

Control

Bacillus subtilis

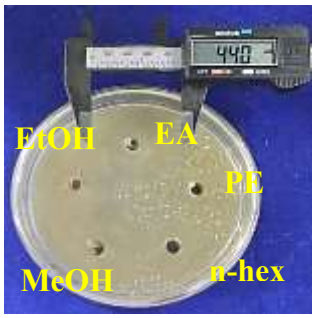


Control

Candida albicans

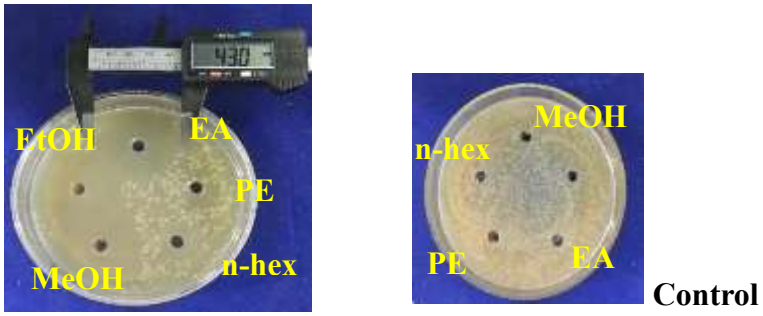
Control

Micrococcus luteus



Control

Pseudomonas sp.



Staphylococcus aureus

Figure 8. Effect of solvent on extraction of metabolites on six test organisms

Discussion and conclusions

In the present study, *Rhizoctonia* sp. was isolated from the leaves of *Combretum indicum* (L.) R. A. DeFilipps. In the study of fermentation, fermentation (one day old) of age of inoculum was the best for fermentation. In the size of inoculum optimization, fermentation (2%) showed the best antimicrobial activity on six test organisms and fermentation (pH 5) of pH utilization was the best to produce bioactive compounds. The antimicrobial activity of different extraction of the metabolites was carried out by using different solvent system: methanol, ethanol, ethyl acetate, petroleum ether and n-hexane. Among them, the extracts with ethyl acetate showed the high inhibition activity on *Bacillus subtilis*, *Bacillus pumilus*, *Candida albicans*, *Micrococcus luteus*, *Pseudomonas* sp. and *Staphylococcus aureus*.

In Myanmar, Aye Pe (2001), Ni Ni Win (2011), Kyawt Kyawt Aung (2014), Kyi Kyi Khine (2014), Phoo Wint Yee Thaw (2015), Soe Soe Yu Hnin (2018), Hnin Wit Mhon (2018), and Kay Thwe Lwin (2021) have isolated many endophytic fungal (including *Rhizoctonia* sp.) and bacterial strains from different plant species to isolate the bioactive compounds and had good antimicrobial activity on *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Malassezia furfur*, *Salmonella typhi* and *Staphylococcus aureus*.

Yu Myat Maw (2016) found that *Rhizoctonia* sp. isolated from the leaves of *Ipomoea* sp. showed highest activity at pH 5 on *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Malassezia furfur*, *Salmonella typhi* and *Staphylococcus aureus*. Shah *et al*., (2017) proved that the antimicrobial activities of N-hexane, chloroform, ethanol and aqueous extracts of leaves, flowers, roots, and stems of *Quisqualis indica* against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Bascillus subtilis*.

Dutta *et al*., (2019) studied that the aqueous and ethyl acetate extracts of leaves, stems and flowers of *Quisqualis indica* Linn. showed antimicrobial activities against *Staphylococcus aureus* and *Escherichia coli.* Kay Tha Ye Soe Win (2015) investigated that the ethanol, ethyl acetate and aqueous extract of leaves of *Quisqualis indica* exhibited antibacterial activity against *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Malassezia furfur*.

Kamber *et al*., (2014) found that the inhibitory effect of leaf and flower extracts of *Combretum indicum* were effective against *Staphylococcus aureus*. Zahidul *et al*., (2017) investigated that the antimicrobial activity of different extract (petroleum ether, methanol and aqueous) of *Quisqualis indica* leaves against different types of bacterial strains both gram positive and gram negative bacteria.

In conclusion, *Rhizoctonia* sp. indicated the highest antimicrobial activity on test organisms. The best fermentation condition was 2% of one day old seed culture and pH 5 to produce bioactive metabolites. *Rhizoctonia* sp. showed the highest antimicrobial activity on

different test organisms and the good fermentation results and indicated the highest inhibition activity in the extraction of secondary metabolites. Therefore, *Rhizoctonia* sp. should be chosen for further investigations.

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