Active Crystal Compound of Endophytic *Periconia* sp. Isolated from *Coccinia indica* Wight. & Arn.

Kyawt Kyawt Aung[1], Mon Mon Thu[2] and Yee Yee Thu[3]

Abstract

In this research paper, a fungal strain *Periconia* sp. was isolated from *Coccinia indica* Wight. & Arn. (Kim-mone). For extraction of the bioactive compound, 6 L fermentation of this fungal strain was carried out, and after fermentation the filtrate was applied on XAD-2 resin and extracted with methanol at Microbiology Laboratory, Department of Botany, University of Yangon. The methanol extract was eluted on silica gel and Sephadex LH-20 columns with various solvent systems for isolation of the bioactive compound. The three compounds including one crystal compound were isolated from six liters fermentation of this strain. In this paper the crystal compound was mentioned. This crystal compound was characterized by UV, FT-IR, 1D-NMR (^{1}H-NMR & ^{13}C-NMR) and 2D-NMR spectra at Chemistry Department, Ramkhamhaeng University, Bangkok. This active compound was identified as "chloramphenicol" and it is a useful antibiotic. Antimicrobial activity of this compound was examined by paper disc diffusion assay at Microbiology Laboratory, Department of Botany, University of Yangon. This compound showed very strong antimicrobial against *Aspergillus flavus*, *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Malassezia furfur*, *Micrococcus luteus*, *Salmonella typhi* and *Staphylococcus aureus.* Its minimum inhibitory concentration (MIC) was 0.25 μg *in vitro*.

Key words: Chloramphenicol, *Coccinia indica* Wight. & Arn., *Periconia* sp.

Introduction

Endophytes are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial arenas (Tan, *et al.* 2001). The mechanisms through which endophytes exist and respond to their surroundings must be better understood in order to be more predictive about which higher plants seek, study, and spend time isolating microfloral components. This may facilitate the product discovery processes (Wang *et al*., 2010).

Endophytic fungi have been isolated novel secondary metabolites, some of which have beneficial biological activities. Endophytic fungi are an important and novel resource of natural bioactive compounds. An endophyte is acquired in pure culture. Its ability to be grown is investigated in a numerous of different media and growth conditions (Parkinson, 1994).

Many endophytic fungi elaborate antibiotic compounds in culture that are active against human and plant pathogens (Drefuss and Chaplea, 1994). An identification of organisms is a critical step to understand and analyse the biological processes. Endophytic fungi have proven to be a rich resource of biologically active secondary metabolities, and several novel bioactive substances have been isolated from various strains recently (Strobel and Daisy, 2003).

In this research, an endophytic fungus *Periconia* sp. was isolated from *Coccinia indica* Wight. & Arn. The purpose of this study was to isolate and purify the bioactive compounds from endophytic fungus, to characterize structure elucidation of

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the isolated compound by spectroscopic methods and to evaluate antimicrobial activity of the isolated compound.

Materials and Methods

Isolation of an Endophytic Fungus

The *Coccinia indica* Wight. & Arn. plant sample was collected in the medicinal garden of Defence Services Medical Academy (DSMA), Mingaladon Township, Yangon, Yangon Reagion. Screening of endophytic fungus was carried out with the following scheme: (1) Plant parts were washed in running tap water for 15 min. (2) Plant parts were cut into about small pieces. (3) The surfaces of cut-plant pieces were sterilized by soaking it in 75% ethanol for 2 min. (4) Sterile surfaces were soaked in 5.3% sodium hypocloride for 5 min. (5) Cut-plant pieces were washed out sodium hypocloride by soaking in 75% ethanol for 0.5 min. (6) They were dried and cut into smaller pieces, and placed on agar plates and then incubated for 3 days to 3 weeks. The isolated fungus was transferred into test tubes containing sucrose/yeast extract medium and incubated for 2-5 days (Lee *et al*., 1996).

Fermentation of *Periconia* sp.

A piece of fungus from the slant culture of *Periconia* sp. was inoculated into 300 ml of conical flask containing 180 ml of sucrose/yeast extract seed medium. The flask was incubated at 28^{o}C for three days. The three days old seed culture (180 ml) was transferred into the six flasks of 2 L conical flask containing 1 L of the fermentation medium. The flasks were incubated at 100 rpm for three days at room temperature (Monaghan *et al*., 1999).

Extraction of Bioactive Compound from *Periconia* sp.

After fermentation, the fermented broth was applied on an Amberlite XAD-2 resin column. The column was washed with water followed by three liters of methanol. The methanol extract was evaporated by incubator at 45°C. This extract was tested antimicrobial activity on *Aspergillus flavus, Candida albicans, Escherichia coli* and *Malassazia furfur* (Strobel & Sullivan, 1999).

Isolation and Purification of Bioactive Compounds

The bioactive compounds from *Periconia* sp. were isolated and purified by using various solvent systems on silica gel column. The column was eluted with chloroform and chloroform-methanol mixture (10:0, 9:1, 8:2 and 7:3) and then 10 ml of each fraction was collected. The column size was 2.5 x 25 cm and flow rate was 2 ml per minute. The crystal fractions were carefully washed with dichloromethane in four times (Grabley *et al*., 1999).

Identification of Isolated Compound from *Periconia* sp.

The UV spectrum of the isolated compound was determined in methanol and recorded on PerkinElmer (Lambda 25) UV/VIS spectrometer. The infra-red spectrums of the isolated by SHIMADZU, and the isolated compound were prepared as a 1% KBr pellet form Infra- red spectroscopy at the Universities Research Centre, Yangon.

The ^{1}H- and ^{13}C-NMR and ^{1}H-^{1}H NMR (COSY) spectra of the isolated compound from *Phomopsis* sp. were recorded at Department of Chemistry, Ramkhamhaeng University, Thailand. All data of the isolated compound were compared to Advanced Chemistry Development ACD Labs (Robert and Francis, 2014).

Antimicrobial Activities of Isolated Compound from *Periconia* sp.

The isolated compound was tested their antimicrobial activities on various test organisms by paper disc diffusion assay. MIC values (2.5 µl, 3.5 µl, 4.5 µl of 1.0 mg/mL) of the compound were utilized on eleven test organisms. The paper disc size is 6.0 milimeter (Davis and Stout, 1971).

Results

Antimicrobial Activity of Extracts of *Periconia* sp.

The methanolic extract showed higher antimicrobial activity than acetone extract on *Aspergillus flavus*, *Candida albicans*, *Escherichia coli* and *Malassezia furfur* in Table 1.

Table 1. Inhibitory zones (mm) of the extracts

	A. flatus	*C. albicans*	*E. coli*	*M. furfur*
Acetone extract	11	12	12	18
Methanol extract	15	16	17	22

10- to 12 mm = weak activity, 13 mm – 17 mm = high activity, >18 mm = very high

Isolation and Purification of Bioactive Compound from *Periconia* sp.

Among the fractions from crude extract column, the fractions 6 to 10 were crystals and washed with dichloromethane. Then, these fractions were purecrystals according to their spots on TLC plates under UV 254 nm. The fractions 6 to 10 were the same R_f value and colour on TLC plate so that these fractions were combined as seen in Figure 1.

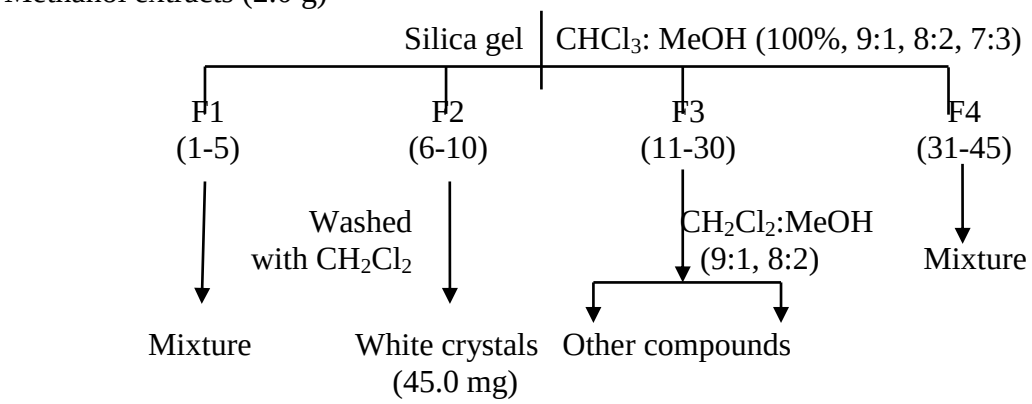

Figure 1. Isolation procedure of the bioactive compounds

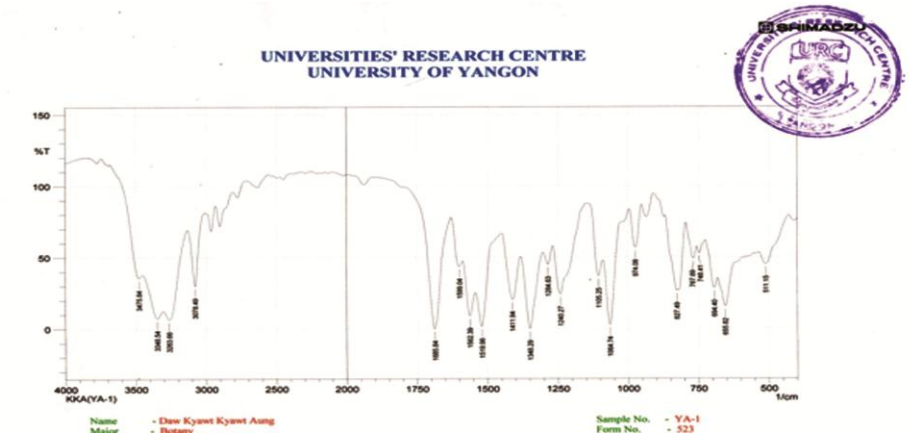

Figure 2. FT-IR spectrum of the isolated compound

Table 2. FT-IR spectral data assignment of the isolated compound

Wave number (cm^{-1})		**Assignment**
Observed	Literature	
3475, 3263, 3078 3348	3472 3350	O-H stretching vibration (alcohol and phenol) NH stretching vibration
2980,2900	2975	C-H stretching vibration of –CH_3 and –CH_2-, CH groups
1685	1685	-C=O- stretching vibration
1599,1562, 1519	1590	C=C stretching vibration of aromatic ring character
1411	1410	C-H bending vibration of -CH_2- groups
1348	1345	CH_3 bending vibration
1064	1062	-C-O-C stretching vibration of cyclic ether
974, 827	1972	C-H & C-O bending vibration
694,655	690	-C=C-H bending vibration

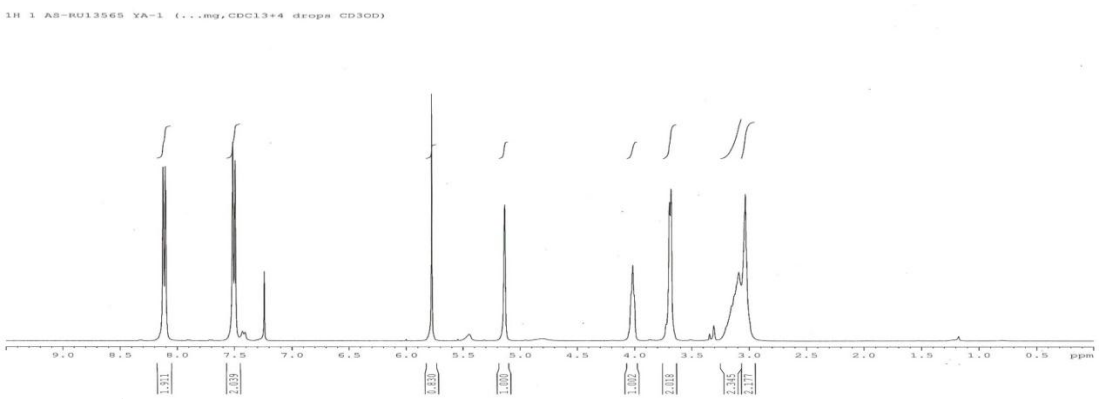

Figure 3. ^{1}H-NMR spectrum of the isolated compound

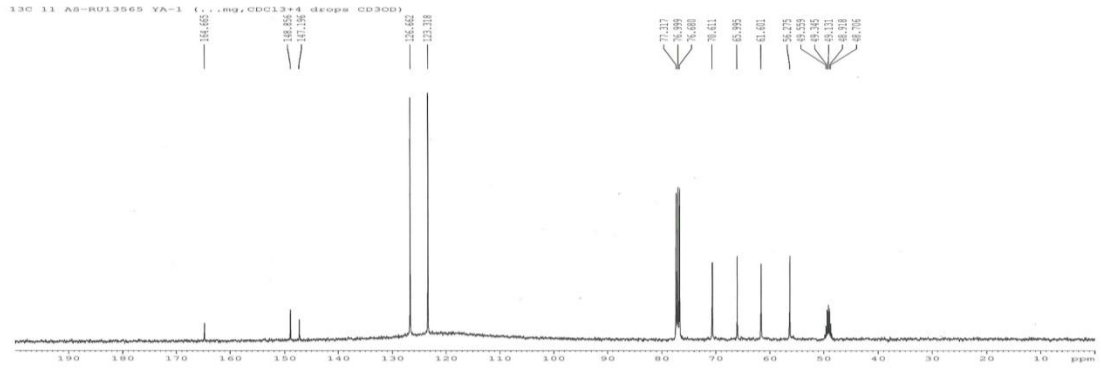

Figure 4. ^{13}C-NMR spectrum of the isolated compound

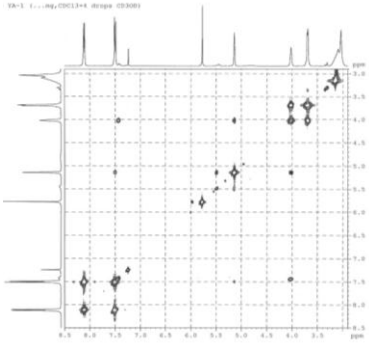

Figure 5. ^{1}H-^{1}H-NMR (COSY) spectrum of the isolated compound

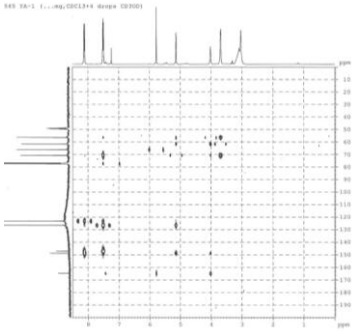

Figure 6. ^{1}H-^{13}C-NMR (HMBC) spectrum of the isolated compound

Table 3. NMR data of the compound

No.	(δ_C) ppm	(δ_H) ppm	^{1}H-^{1}H COSY	HMBC
1	123.32	8.12 (*d*) 2H	7.49	8.12, 7.49
2	126.66	7.49 (d) 2H	8.12, 5.14, 4.02	7.49, 5.14
3	65.99	5.78 (*s*) 1H	-	-
4	70.61	5.14 (*s*) 1H	7.49, 4.02	8.12, 7.49
5	-	4.02 (*t*) OH	7.49, 5.14, 3.70	-
6	56.28	3.70 (*d*) 1H	4.02	7.49
7	61.60	3.09 (*s*) 1H	-	-
8	61.60	3.04 (*s*) 1H	-	-
9	164.66 (C=O)	-		5.78, 4.02
10	148.86	-		8.12, 5.14, 4.02
11	147.19	-		7.49

Identification of Isolated Compound

The isolated compound was VU active under 254 nm and gave purple spot on TLC plate with vanillin/sulphuric acid spray reagent. Its R_f value was 0.89 (chloroform). This substance was good soluble in acetone or methanol. Its nature was needle-shaped crystals. It was identified by its characteristic UV absorption λ_{max} at

206 and 273. The UV absorption at 206 and 273 nm was due to the presence of double bond conjugated system.

In IR spectrum, O-H stretching vibration (alcohol and phenol groups) showed at 3475, 3348, 3263 and 3078 cm^{-1}. C-H stretching vibrations of methyl and methylene group were found at 2980 and 2900 cm^{-1}. Its IR spectrum showed the absorption for ketone group C=O at 1685 cm^{-1}, and for olefinic groups at 1599, 1562 and 1519 cm^{-1}. C-H bending vibration of methylene group was found at 1411cm^{-1} and at 1348 cm^{-1} was for CH_3 bending vibration. The band at 1046 cm^{-1} was due to the presence of C-O-C stretching vibration of cyclic ether. The bands at 974 and 827 cm^{-1} were attributed C-H and C-O bending vibration. In addition, the bands for C=C-H bending vibration were observed at 694 and 655cm^{-1} as seen in Table 2 and Figure 2.

According to its ^{1}H-NMR spectrum, aromatic protons (Ar-H) were found as double (*d*) at δ 8.12 and 7.49 ppm. Methine protons (CH) were indicated as singlet (*s*) at δ 5.78, 5.14, 3.09, 3.04 ppm, as doublet (*d*) at 3.70 ppm. Hydroxyl groups (-OH) were substituted as triplet (*t*) at δ 4.02 ppm in this compound in Table 3 and Figure 3.

Its ^{13}C-NMR spectrum indicated eleven carbon signals for nine methines at δ 123.32, 126.66, 65.99, 70.61, 56.28 and 61.60 ppm; and three quaternary carbon atoms at δ 164.66, 148.86 and 147.19 ppm, including the one carbonyl group (C=O) in Table 3 and Figure 4. The relationships of protons and carbons of the isolated compound (2D-NMR) were mentioned in Table 3 and Figures 5 and 6. According to its UV data, FT-IR spectral data, 1D-NMR and 2D-NMR spectral data, the isolated compound was identified as chloramphenicol. Its molecular formula was $C_{11}H_{12}$ $Cl_2N_2O_5$ and its molecular weight was 323 as shown in Figure 7.

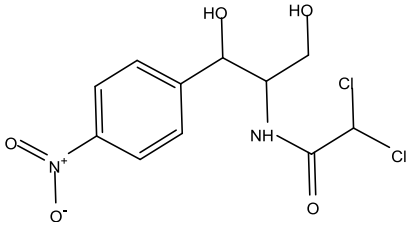

Figure 7. Chloramphenicol

Antimicrobial activities of the isolated compound

Among isolated compounds, the compound showed very strong antimicrobial activities on *A. flavus*, *B. subtilis*, *C. albicans*, *E. coli*, *M. luteus*, *S. typhi*, *S. aureus* and *M. furfur*. The values of the isolated compound were 2.5 µg, 3.5µg and 4.5µg of 1 mg/mL as shown in Table 4 and Figure 8.

Table 4. Antimicrobial activity of the isolated compound

Test organisms	Inhibitory zones (mm) 2.5 µg/disc	Inhibitory zones (mm) 3.5 µg /disc	Inhibitory zones (mm) 4.5 µg /disc
Aspergillus flavus	26	28	28
Bacillus subtilis	20	28	28
Cadida albicans	16	25	28
Escherichia coli	26	30	32
Micrococcus luteus	26	30	30
Staphylococcus aureus	15	16	22
Salmonella typhi	25	28	30
Malassezia furfur	26	28	32

10 mm-12 mm = weak activity, 13 mm – 17 mm = high activity, >18 mm = very high activity

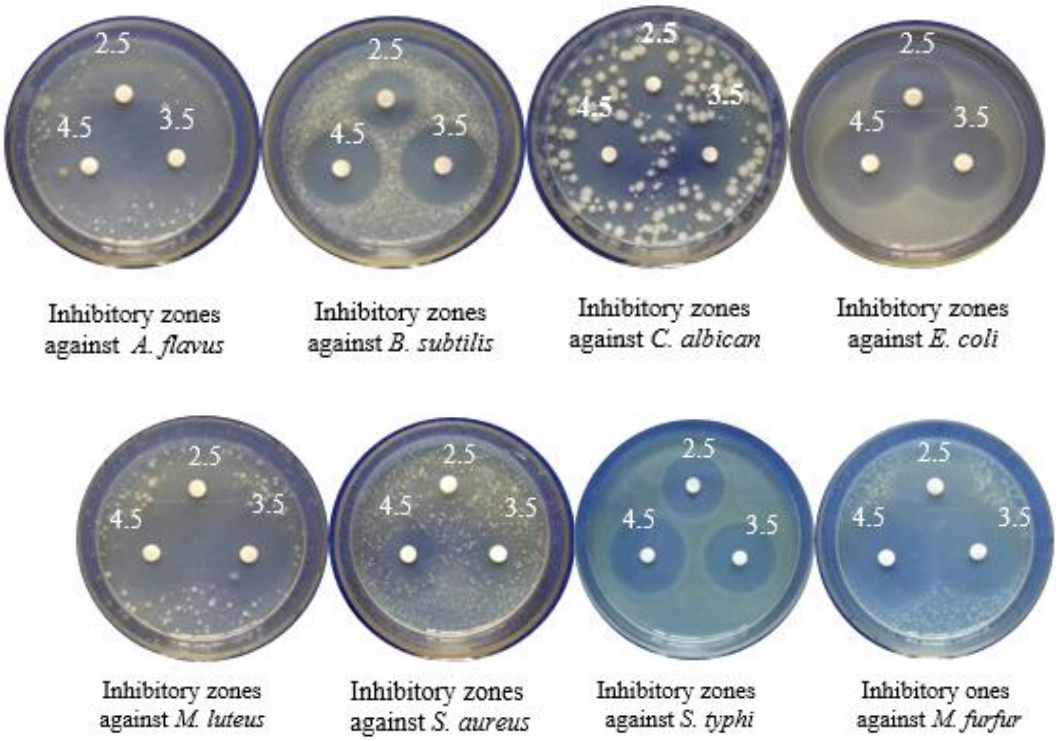

Figure 8. Antimicrobial activity of the isolated compound

Discussion and Conclusion

In this research work, an endophytic fungus *Periconia* sp. was isolated from the leaves of *Coccinia indica* Wight.& Arn. (Kim-mone). In order to produce the bioactive compounds from *Periconia* sp., the three day old of seed culture was transferred into the fermentation flasks. Fermentation flasks (6 L) of shaker culture were incubated for three days at room temperature and extracted with methanol on XAD 2 resin. The silica gel column was eluted with various solvent systems for the isolation and purification of the bioactive compounds from the crude extract. The white needle-shaped crystal compound 'chloramphenicol' was isolated from *Periconia* sp.

In 2006, Yee Yee Thu has isolated endophytic fungi from the leaves of *Coccinia indica* Wight. & Arn. (Kim-mone). The endophytic fungus *Periconia* sp. isolated from the medicinal plant *Annona muricata*, showed anti-tumor activites in preliminary *in vitro* assays. As part of their search for anti-tumor bioactive metabolites from endophytic fungus *Periconia* sp., ethylacetate extract of the fermentation broth the fungus *Periconia* sp. was investigated, which led to two new terpenes named periconone A and 2-caren-4,8-olide (Zhang *et al*., 2011).

Chloramphenicol was first isolated from *Streptomyces venezuelae* in 1947 and in 1949 a team of scientists who published their identification of the chemical structure and their synthesis. They reported it as the first antibiotic to be made instead of extracted from a micro-organism (Rebstock *et al*., 1949; Pongs, 1979). The endophytic fungus *Periconia* sp. produced an active compound under liquid culture (John *et al*., 1999).

The isolated compound showed very strong antimicrobial activities on *A. flavous, B. subtilis, C. albicans, E. coli, M. luteus, S. typhi, S. aureus* and *M. furfur* in 2.5µg, 3.5µg, 4.5µg *in vitro*. Carone *et al.* (2014) stated that chloramphenicol has a broad spectrum of activity and has been effective in treating ocular infections such as conjunctivitis, blepharitis, etc. caused by *Staphylococcus aureus, Streptococcus pneumoniae* and *Escherichia coli*.

Chloramphenicol is still used occasionally in topical preparations (ointments and eye drops) for the treatment of bacterial conjunctivitis (*Lancaster, 1998*). Oily chloramphenicol (or chloramphenicol oil suspension) is a long-acting preparation of chloramphenicol first introduced by Roussel in 1954; marketed as Tifomycine. It was originally used as a treatment for typhoid (Lewis, 1998). Chloramphenicol is available as a generic worldwide under many brand names in Eastern Europe and Russia, including chlornitromycin, levomycetin and chloromycetin.

In conclusion, nowadays life-threatening fungal and bacterial diseases are strongly increasing. So, it is essential to produce antibiotics that can fight serious diseases. In this research, the bioactive compound isolated from *Periconia* sp. showed highly antimicrobial activity *A. flavus, B. subtilis, C. albicans, E. coli, M. luteus, S. typhi, S. aureus* and *M. furfur* which can cause serious diseases on humans. Therefore, this bioactive compound could effectively be applied to cure microbial infections and thus be produced as "antibiotics" at the pharmaceutical industry. It is very helpful and beneficial to human beings.

Acknowledgments

The authors are grateful to Professor Dr. Apichart Suksamrarn, Department of Chemistry, Ramkhamhaeng University, Bangkok, Thailand for NMR spectra. We wish greatly to mention our sincere thanks to Pro-Rectors from Dagon University, and Professors from Department of Botany, Dagon University for their kind efforts to do 3rd Myanmar Korea Conference on Useful Plants 2020 sponsored by Chonbuk National University, Republic of Korea.

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