BIS-NAPHTHO-γ-PYRONES FROM ENDOPHYTIC FUNGUS ISOLATED FROM THE LEAF OF *Andrographis paniculata* (Burn.f.) Wall. ex Nees AND THEIR BIOACTIVITIES

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Abstract

In this research work, one of the Myanmar medicinal plants, *Andrographis paniculata* (Burn.f.)Wall. ex Nees was selected for chemical investigation of its endophytic fungus. The endophytic fungi from the leaf of the selected medicinal plant were cultivated on water-agar medium under sterile condition and isolation of pure culture was carried out. Selection of the target fungus was done based on chemical screening (spot pattern on TLC). The selected fungus was found to be *Alternaria* sp. according to morphological studies. After selecting the target fungus, up-scaled fermentation for pure isolated target fungus was performed on M_2 medium. After two weeks, the well-grown culture broths were exhaustively extracted with ethyl acetate. Then, the ethyl acetate extract was evaporated to dryness under reduced pressure to obtain brown crude extracts. The resulting microbial extracts were chromatographed to isolate the pure metabolites. The two pure yellow pigment organic compounds bis-naphtho-g-pyrones derivatives and ferulic acid were isolated from the extracts by using various chromatographic techniques. Furthermore, the structure of isolated compounds were deduced by NMR and mass studies.In addition, antimicrobial activities, cytotoxicity assay and the evaluation of antioxidant activitiy for the two pyrone derivatives compounds were also performed.

Keywords: *Andrographi spaniculata* (Burn.f.) Wall. ex Nees, Endophytic fungi, WA (water agar), M_2 (Malt extract, Glucose, Yeast extract), *Alternaria* sp., bis-naphtho-γ-pyrones

Introduction

People depend on plants because they provide compounds essential for human existence. Plants produce primary compounds such as sugars and proteins that are used in a plant's basic metabolism and form the base of our food web. Plants also produce an array of chemicals that are known as secondary compounds because they are not usually integral to basic

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metabolism (Simpson and Ogorzaly, 1995). Medicinal plants and their endophytes are important resources for discovery of natural products (Huang *et al*., 2007). Endophytes are microorganisms that reside asymptomatically in the tissues of higher plants and are a promising source of novel organic natural metabolites exhibiting a variety of biological activities (Pimentel *et al*., 2011). Endophytic fungi are the main sources for the production of secondary metabolites. They live asymptomatically inside the tissues of higher plant (Bano*et al*., 2016). They have the ability to produce same or similar bioactive compounds as those originated from their host plant. Some endophytic fungi are rare; many of the biological active substances extracted from endophytic fungi are reported to be novel. Therefore, it is important to explore endophytic fungi in the medicinal plants (Singh *et al*., 2015).

In this research work, one Myanmar traditional medicinal plant, *Andrographi spaniculata* (Burn.f.)Wall. ex Nees (Kress *et al.* 2003) was selected for chemical analysis. *Andrographi spaniculata* is a herbaceous plant, commonly known as "King of Bitter", in the family Acanthaceae. It is widely distributed in Myanmar. It is locally known as Say-khar-gyi. The bioactive chemical constituents isolated from the endophytic fungus of the leaves of *A. paniculata* have been little explored. So, this drew our attention to investigate the chemical constituents of endophytic fungus isolated from the leaves of *A. paniculata.*

Materials and Methods

Sample Collection

The sample was collected form Monywa Township, Sagaing Region.

General Experimental Procedures

FT IR (Fourier Transform Infrared) spectrophotometer (Shimadzu, Japan), optical rotation was measured on a Perkin-Elmer polarimeter (model 241) (Perkin-Elmer, San Jose, CA, USA). NMR (Nuclear Magnetic Resonance) spectra were measured on a Varian Inova 600 (599.740 MHz) and a Varian Unity 300 (300.145 MHz) spectrometer. ESIMS were measured on a Quattro Triple Quadruple mass spectrometer with a Finnigan TSQ 7000 with nano-ESI API ion source. Column chromatography was carried out on MN silica gel 60, 0.05-0.2 mm; TLC (Thin Layer Chromatography) was

performed on Polygram SIL G/UV$_{254}$. All silica gel materials were purchased from Macherey-Nagel, Düren, Germany. Size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex; Amersham Biosciences, Freiburg, Germany, purchased from Sigma-Aldrich Chemie, Steinheim, Germany). Micropipette (1000 mL), brown bottles, electric balance, shaker and UV-spectrophotometer (UV-7504, KWF, China) were also used.

Microbiological Materials

Autoclave: Abstell Hearson, Autoclave volume 119 L, working temperature 121 °C, working pressure 1.2 kg/cm^2. Esco Horizontal Laminar-Flow-Cabinet: EQU/ 03-EHC, Esco Micro Pte-Ltd, Singapore. Petridishes: 90 mm diameter, 16 mm height, China.

Nutrients

M$_2$ medium
Malt extract   10 g
Glucose   4 g
Yeast extract   4 g
Distilled water   1 L
The pH was adjusted to 7.8 using 2 N NaOH.

Water agar medium

Agar   20 g
Distilled water   1 L

All cultures media were autoclaved at 1.2 bar and 120 °C. Sterilization time for 1 L culture: 15 min.

Procedure
Preparation of M$_2$ Medium and WA (Water Agar) Medium

M$_2$ medium is the most widely used medium for growing fungi and bacteria which attack living plants or decay dead plant matter. Malt extract 10 g were mixed with 4 g of glucose and 4 g of yeast extract. Distilled water was added such that the total volume of the suspension is one liter. Then, the medium was sterilized by autoclaving at 121 °C for 15 min. After sterilization, chloramphenicol (50 mg/L) was added to the medium to suppress

bacterial contamination. Then, the medium was poured into petridish (Win, 2009).

Water agar medium is used to isolate individual fungal colonies. It was prepared by 20 g agar in 1 L distilled water. The procedure for sterilization and addition of antibiotic was the same as for M_2 medium.

Isolation of Endophytic Fungi

The fresh leaves were thoroughly washed in running tap water to remove dust and debris, and then air-dried on sterile filter paper and cut into small pieces using a blade. Sterile conditions were maintained for the isolation of endophytes and all the work was performed in a laminar flow hood to avoid contamination. Surface sterilization of the samples was achieved with 95 % EtOH for 30 s, 10 % sodium hypochlorite for 10 min, 70 % EtOH for 2 min, and then dried aseptically. The inner tissues were placed on isolation media (water agar; WA) in petridishes supplemented with 50 mg/L of chloramphenicol to suppress bacterial growth, and incubated at 25 °C until the outgrowth of endophytes was discerned. Individual fungal colonies were picked and transferred onto sterile WA medium and periodically checked for purity. A total of 7 strains were isolated and each strain was inoculated on 200 mL M_2 medium for pre screening (chemical screening) (Figure 1). The target fungus was selected for further studies due to its spot pattern on TLC (De, 1997; Larran, 2002; Radu and Kqueen, 2002).

Selected fungus

Figure 1: Isolated fungi from *Andrographi spaniculata* (Burn.f.) Wall.exNees on M_2 medium

Up-scaled Fermentation for the Selected Fungus

The well grown agar cultures of the selected strain were used to inoculate 50 flasks of 1 L Erlenmeyer flasks containing 400 mL M_2 medium supplemented with antibiotic.

After two weeks, they were harvested and extracted with ethyl acetate. Then, the ethyl acetate was evaporated to dryness under reduced pressure. The resulting crude extract (11.25 g) was chromatographed to isolate the metabolites. Before harvesting, 1.5 mL of MeOH was added to each flask to kill fungus and left for 24 h.

Isolation of Metabolites from the Selected Fungus

EtOAc extracts were dissolved in the mixture of n-hexane and EtOAc and silica gel was added to that mixture and evaporated the solvents. The obtained crude extracts were homogenous adsorbed on silica gel.

Then, the crude extracts were subjected to silica gel using stepwise gradient of *n*-hexane:ethyl acetate. Totally 567 fractions were obtained. Each fraction was checked by TLC. Eleven combined fractions were obtained. Fraction VI was subjected to Sephadex LH-20 using MeOH to isolate TMH-3 (R_f = 0.31, 1:1 (*n*-hexane : ethyl acetate)) as pale yellow compound. It showed UV absorption band at 254 nm. After purification of fraction IX on Sephadex LH-20 using methanol and RP-18 (Reverse phase) using methanol and water, TMH-1 (R_f= 0.50, 2:1 (*n*-hexane : ethyl acetate)) and TMH-2 (R_f= 0.53, 1:2 (*n*-hexane : ethyl acetate)) were isolated as yellow compounds. They showed UV absorption band at 254 nm. The yield percents of pure compounds (TMH-1), (TMH-2) and (TMH-3) were found to be 0.15% (16.5 mg), 0.09 % (10.5 mg) and 0.04% (4.7 mg) upon ethyl acetate extracts (11.25 g) respectively.

Structure Elucidation of the Isolated Compounds

The structure elucidation of isolated compounds was determined by spectroscopic methods such as FT IR, ^{1}H NMR, ^{13}C NMR, DEPT, DQF-COSY, HMQC, HMBC, NOESY and ESI MS spectrometry respectively.

Screening of Antimicrobial Activities of Pure Compounds (TMH-1 and TMH-2)

Antimicrobial activities tests for pure compounds (TMH-1 and TMH-2) were performed at PRD (Pharmaceutical Research Department), Insein, Yangon.

Determination of Cytotoxicity of Pure Compounds (TMH-1 and TMH-2) by Brine Shrimp Lethality Bioassay

Pure compounds (TMH-1) and (TMH-2) were investigated by brine shrimp lethality bioassay according to the procedure described by Dockery and Tomkins, (2000).

Determination of Antioxidant Activity of Pure Compound (TMH-1) by DPPH Free Radical Scavenging Assay

The free radical scavenging activity of pure compound (TMH-1) was measured by using DPPH free radical scavenging assay (Marinovaand Batchvarov, 2011).

Results and Discussion

Structure Elucidation of Pure Compound (TMH-1)

In the aromatic region of ^{1}H NMR spectrum (Figure 6 (a)), two singlets at *d* 7.35 and *d* 7.23 ppm were ascribed to two aromatic methine protons. In the HMBC spectrum (Figure 8 (a)), these methine protons at *d* 7.35 and *d* 7.23 ppm showed *b* -correlation with sp^2 quaternary carbon at *d* 112.1 ppm.

Moreover, in the HMBC spectrum (Figure 8 (a)), sp^2 methine proton at *d* 7.23 ppm showed *a*-coupling with one sp^2 quaternary carbon at *d* 161.5 ppm and *b*-coupling with sp^2 quaternary carbon at *d* 119.0 ppm and sp^2 methine carbon at δ 103.1 ppm and small correlation with sp^2 quaternary carbon at *d* 159.4 ppm. In addition, one sp^2 methine proton at *d* 7.35 ppm showed α-correlation with one sp^2 quaternary carbon at *d* 152.1 ppm which is probably attached to oxygen. Moreover, the proton at *d* 7.35 ppm showed *b*-correlations with sp^2 quaternary carbon at *d* 105.5 ppm and sp^2 methine carbon at δ103.2 ppm. Therefore, fragment (a) could be confirmed (Figure 2).

Fragment (a)

Figure 2:(→) HMBC correlation in fragment (a)

In the HMBC spectrum (Figure 8 (a)), there was the observation of *b*-coupling between singlet methoxy protons at *d* 3.80 ppm and aromatic sp^2 quaternary carbon at *d* 161.5 ppm and also the occurrence of *b*-coupling between singlet methoxy protons at *d* 3.45 ppm and aromatic sp^2 quaternary carbon at *d* 159.4 ppm.

Furthermore, in the HMBC spectrum (Figure 8 (a)), one sp^2methine proton at *d* 7.35 ppm showed *g*-correlations with carbonyl carbon at *d* 186.2 ppm and sp^2 quaternary carbon at *d* 154.7 ppm. The latter could be connected to hydroxyl group.

In the HMBC spectrum (Figure 8 (a)), the methine proton at δ6.15 showed α-coupling with one sp^2 quaternary carbon at δ 170.6 ppm and one carbonyl carbon at δ 186.2 ppm. Furthermore, it showed *b*-coupling with one sp^2 quaternary carbon at δ 105.5 ppm and methyl carbon at δ 20.6 ppm respectively. Moreover, methyl singlet at δ2.43 ppm showed *a*-coupling with one sp^2 quaternary carbon at δ 170.6 ppm and *b* -coupling with one sp^2methine carbon at δ107.9 ppm which implies the partial structure (I).

In the NOESY spectrum (Figure 9 (a)), there is a spatial correlation between sp^2methine proton at δ7.23 ppm and methoxy protons at δ3.80 ppm. Moreover, methine proton at δ6.15 ppm showed NOESY with methyl protons at δ2.43 ppm. Therefore, partial structure (I) could be confirmed (Figure 3).
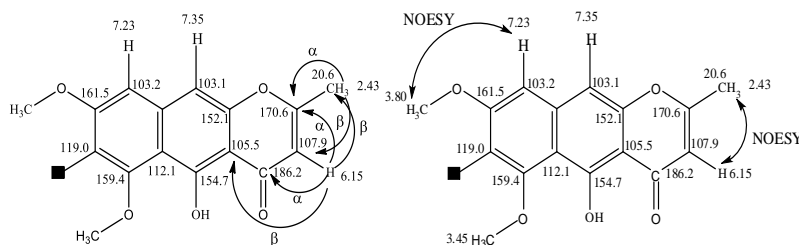
Partial Structure (I)

Figure 3: (→) HMBC correlation and (↔) H-H COSY correlation in Partial Structure (I)

In the aromatic region of ^{1}H NMR spectrum (Figure 6 (a)), one methine doublet at δ 6.50 ppm (J = 2.20 Hz) showed meta coupling with the doublet methine proton at δ 6.25 ppm (J = 2.15 Hz). Moreover, DQF-COSY spectrum (Figure 8 (b)) displayed the correlation between two methine protons at δ 6.50 and δ 6.25 ppm as expected in fragment (b) (Figure 4).

In the HMBC spectrum (Figure 8 (a)), one sp^2 methine proton at δ 6.50 ppm showed a-coupling with two sp^2 quaternary carbons at δ 163.3 and 162.3 ppm and b-coupling with one sp^2 quaternary carbon at δ 109.4 ppm and one sp^2 methine carbon at δ 97.5 ppm.

Moreover, one sp^2 methine proton at δ 6.25 ppm showed α-coupling with one sp^2 quaternary carbon at δ 163.3 ppm and β-coupling with sp^2 methine carbon at δ 98.3 ppm and sp^2 quaternary carbon at δ 109.4 ppm. From these data, the carbon atoms in the benzene ring could be assigned.

In the HMBC spectrum (Figure 8 (a)), there was the observation of b-correlation between singlet methoxy protons at δ 3.60 ppm and aromatic sp^2 quaternary carbon at δ 163.3 ppm. Moreover, b-correlation between singlet methoxy protons at δ 3.96 ppm and aromatic sp^2 quaternary carbon at δ 162.3 ppm was also detected.
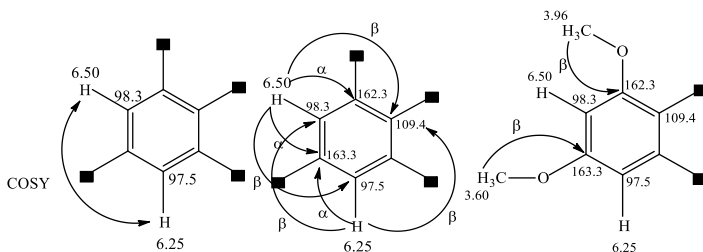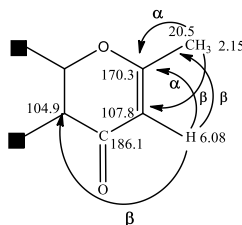
Fragment (b)

Figure 4: (→) HMBC correlation and (↔) H-H COSY correlation in fragment (b)

In the HMBC spectrum (Figure 8 (a)), sp^2methine proton at δ 6.08 ppm showed α-coupling with one sp^2 quaternary carbon at δ 170.3 ppm and b-coupling with one sp^2 quaternary carbon at δ 104.9 ppm and methyl carbon at δ 20.5 ppm respectively. In addition, the methyl singlet at δ 2.15 ppm showed α-coupling with one sp^2 quaternary carbon at δ 170.3 ppm and b-coupling with one sp^2 methine carbon at δ 107.8 ppm.

All HMBC correlations and chemical shifts of carbons showed similar structural feature of g-pyrone ring as in partial structure (I). Therefore, fragment (c) could be elucidated.

Fragment (c)

Fragments (b) and (c) could be connected by using HMBC spectrum. According to the spectrum, one sp^2methine proton at δ6.25 ppm showed b-coupling with one sp^2 quaternary carbon at δ 107.0 ppmand g-coupling with one sp^2 quaternary carbon at δ163.2 ppm and. Therefore, partial structure (II) could be elucidated.

In the NOESY spectrum (Figure 9 (a)), there is a spatial correlation between sp^2methine proton at δ6.50 ppm and methoxy protons at δ 3.96 ppm.

Moreover, methine proton at δ 6.25 ppm showed NOESY correlation with methoxy protons at δ 3.60 ppm, methine proton at δ 6.08 ppm also showed NOESY correlation with methyl protons at δ 2.15 ppm. Therefore, partial structure (II) could be confirmed (Figure 5).
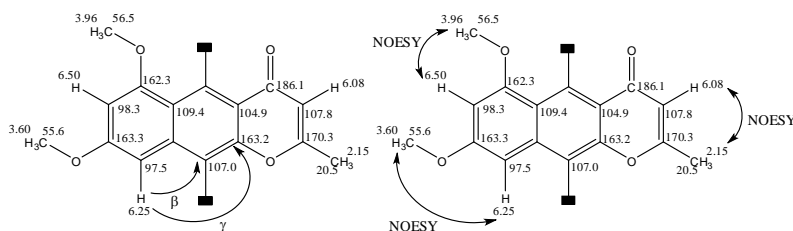

Partial Structure (II)

Figure 5: ($\rightarrow$) HMBC correlation and ($\leftrightarrow$) H-H COSY correlation in Partial Structure (II)

The connections between the partial structures (I) and (II) were inferred mainly on the basis of NOESY spectrum (Figure 9 (a)). Therefore, complete structure of pure compound (TMH-1) could be elucidated (Figures 6-9). The partial molecular formula of pure compound (TMH-1) was assigned as $C_{32}H_{25}O_9$. Molecular formula of pure compound (TMH-1) was $C_{32}H_{26}O_{10}$. Therefore, the remaining molecular formula must be one hydroxyl group (-OH).

By logically, the chemical shift of C-5', C-9a and C-9'a could be assigned as δ 162.2, 142.1 and 142.4 ppm.
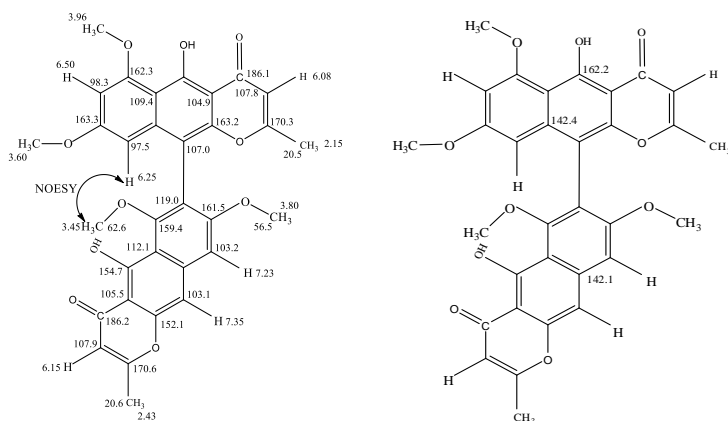
Complete Structure of Pure compound (TMH-1)

The high resolution (+) ESI MS gave pseudomolecular ion peak at *m/z* 593.1407 [M + Na]$^+$, and *m/z* 571.1590 [M + H]$^+$ (Figure 9 (b)). Thus, the molecular mass was deduced as 570 Daltons. The molecular formula was corresponded to $C_{32}H_{26}O_{10}$. The hydrogen deficiency index was 20.
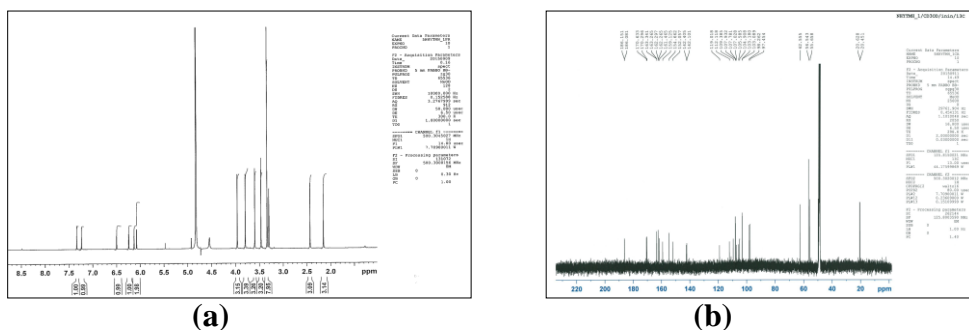

(a)   **(b)**
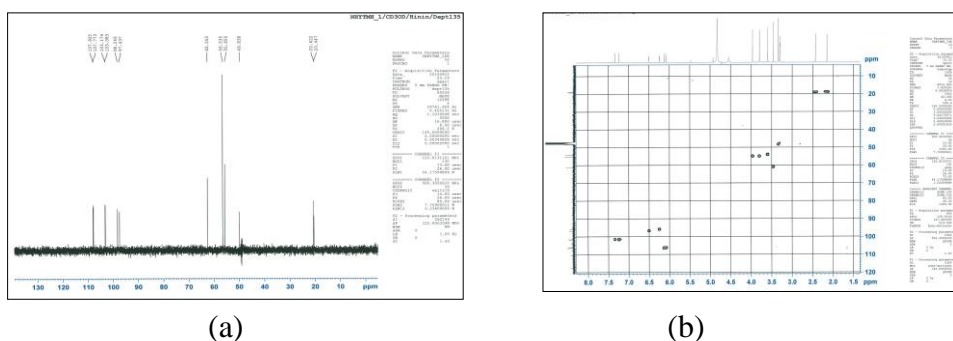
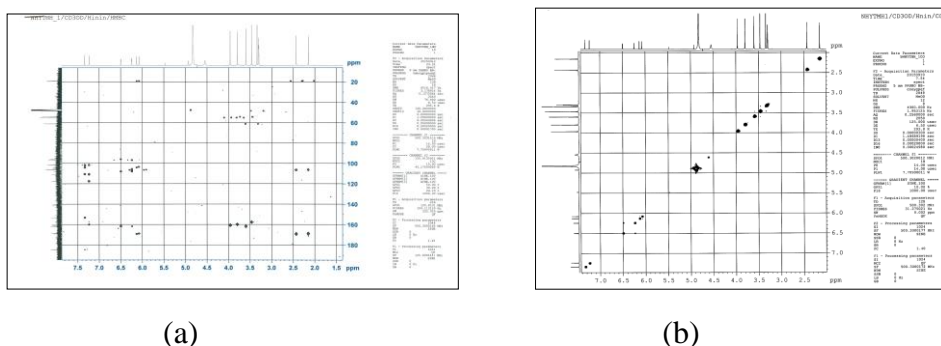**Figure 6:** (a) ^{1}H NMR and (b) ^{13}C NMR spectra of TMH-1

(a)   (b)

Figure 7: (a) DEPT and (b) HMQC spectra of TMH-1

(a)   (b)

Figure 8: (a) HMBC and (b) DQF-COSY spectra of TMH-1

(a)   (b)
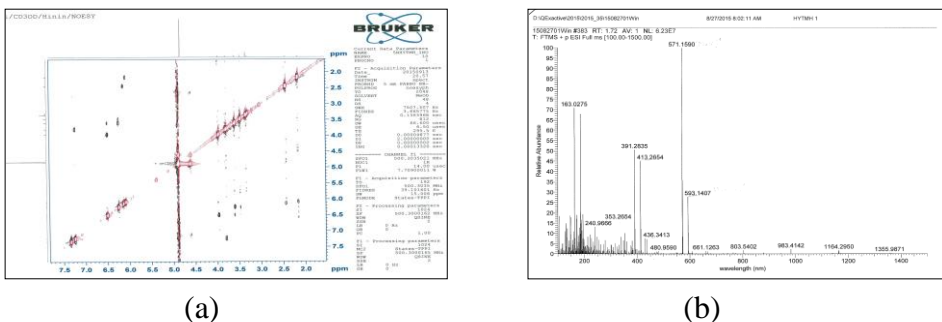
Figure 9: (a) NOESY and (b) ESI-MS spectra of TMH-1

Structure Elucidation of Pure Compound (TMH-2)

Compounds TMH-1 and TMH-2 were not easily differentiated when they were analyzed by other spectroscopic methods such as FT IR, ESI MS and ^{1}H NMR which indicated that they had very similar molecular structures. The mass spectral data, obtained by electrospary ionization (ESI) in the positive ion mode, suggested them to be isomers of $C_{32}H_{26}O_{10}$ [M+H]$^+$ at *m/z* 571. Pure compound TMH-2 showed almost the same NMR data discussed above, but this isomer contains a different partial structure (I) (Figures 10-12).
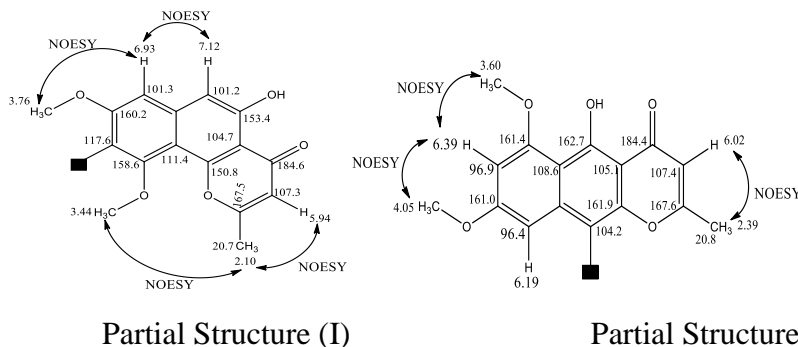
Partial Structure (I)   Partial Structure (II)

The connections between the partial structures (I) and (II) were inferred mainly on the basis of NOESY spectrum (Figure 13 (a)). By logically, the chemical shift of C-9a' and 6a could be assigned as δ 140.5 and 140.7 ppm.
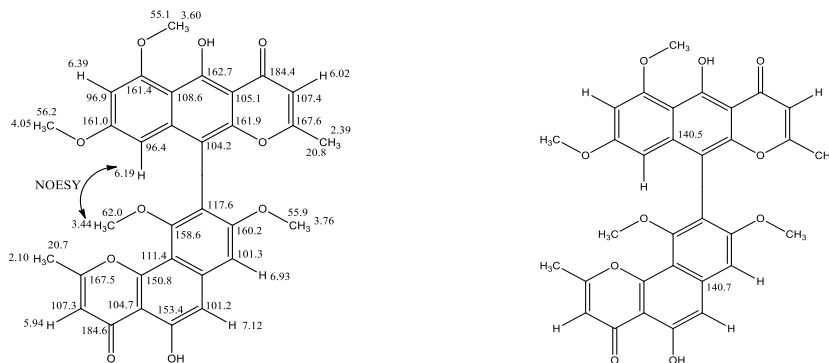
Complete Structure of Pure compound (TMH-2)

The complete isolated pure organic compounds (TMH-1) and (TMH-2) could be expressed as dimericnaphtho-g-pyrone derivatives. The chemical name of pure compounds (TMH-1) and (TMH-2) could be assigned as 5, 5'-dihydroxy-6, 6', 8, 8'-tetramethoxy-2, 2'-dimethyl-4H, 4'H-7,10'-bibenzo [g] chromene-4, 4'-dione and 5,5'-dihydroxy-6', 8, 8', 10'-tetramethoxy-2, 2'-dimethyl-4H, 4'H-9,10'-bibenzo[h, g]chromene-4, 4'-dione re
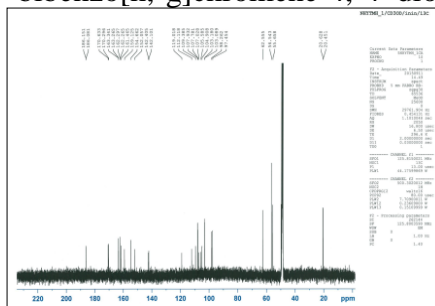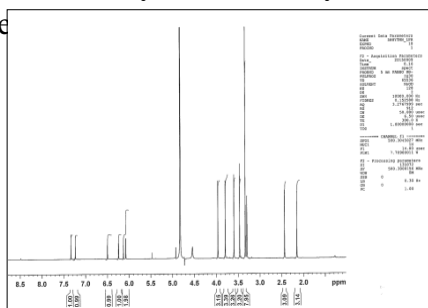
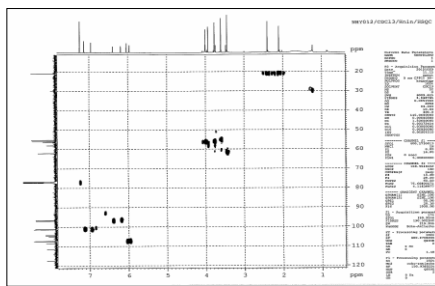| (a) | (b) |

Figure 10: (a) ^{1}H NMR and (b) ^{13}C NMR spectra of TMH-2

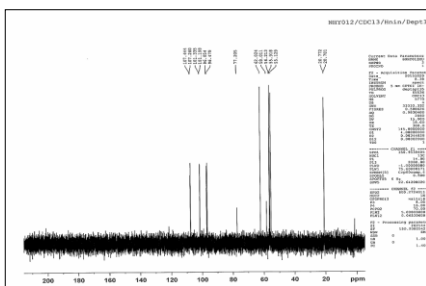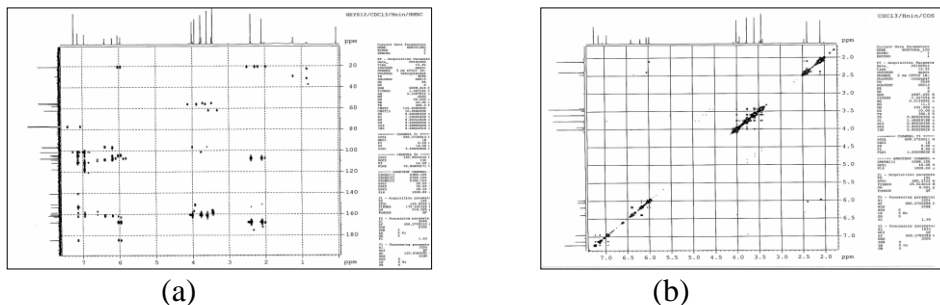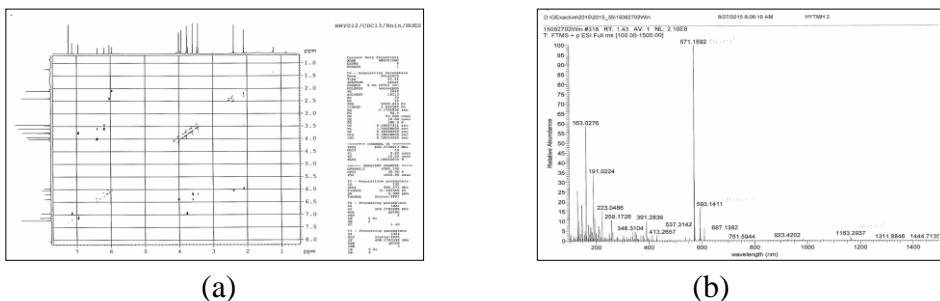**Figure 11:** (a) DEPT and (b) HMQC spectra of TMH-2

(a)   (b)

Figure 12: (a) MHBC and (b) DQF-COSY spectra of TMH-2

(a)   (b)

Figure 13: (a) NOESY and (b) ESI-MS spectra of TMH-2

Structure Elucidation of Pure Compound (TMH-3)

The ^{1}HNMR spectrum (Figure 16 (a)) showed two doublets at δ 7.49 (J = 15.8 Hz) and δ6.32 ppm (J = 15.8 Hz) for a trans disubstituted α, b-unsaturated carbonyl group of ester, acid or amide. In DQF-COSY spectrum (Figure 18 (a)), the methine proton at d 6.32 ppm which is attached to carbon at d 118.8 ppm showed correlation with another methine proton at d 7.49 ppm (Figure 14).
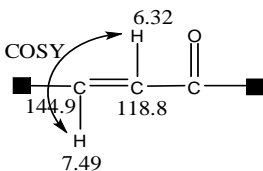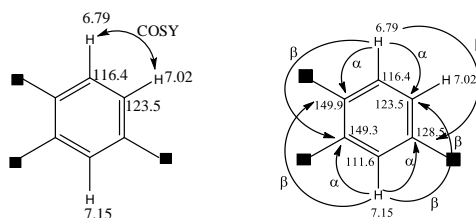

Fragment (a)

Figure 14:(↔) H-H COSY correlation in fragment (a)

In the aromatic region of ^{1}HNMR spectrum (Figure 16 (a)), doublet of doublet at *d* 7.02 ppm (*J* = 8.1, 1.7 Hz) showed ortho coupling to the proton at *d* 6.79 (*J* = 8.2 Hz) and meta coupling with the proton at *d* 7.15 ppm (*J* = 1.7 Hz). The pattern in the aromatic region was the ABX system of a 1, 2, 4-trisubstituted benzene ring. Moreover, DQF-COSY spectrum (Figure 18 (a)) displayed the correlation between two methine protons *d* 6.79 and *d* 7.02 ppm.

In the HMBC spectrum (Figure 17 (b)), sp^2 methine proton at δ 6.79 showed α-coupling with sp^2 quaternary carbon at δ 149.9 ppm and methine carbon at δ 123.5 ppm and *b*-correlations with two sp^2 quaternary carbons at δ 149.3 and δ 128.5 ppm. Moreover, sp^2 methine proton at δ 7.15 showed α-coupling with sp^2 quaternary carbon at δ 149.9 ppm and methine carbon at δ 123.5 ppm and *b*-correlations with two sp^2 quaternary carbons at δ 149.3 and δ 128.5 ppm (Figure 15).


Fragment (b)

Figure 15:($\leftrightarrow$) H-H COSY correlation and ($\rightarrow$) HMBC correlations in fragment (b)

Moreover, there was the observation of *b*-correlation between singlet methoxy protons at δ 3.88 ppm and aromatic sp^2 quaternary carbon at δ 149.3 ppm which indicated fragment (b).

Fragments (a) and (b) could be connected by further HMBC correlation. One sp^2methine proton at *d* 7.49 ppm showed *b*-coupling with two sp^2methine carbons at *d* 123.5, *d* 111.6 ppm and carbonyl carbon at *d* 173.1 ppm. Furthermore, the proton at *d* 6.32 ppm indicated *b*-correlation with one sp^2quaternary carbon at *d* 128.5 ppm. The attachment of remaining one – OH group to one downfield quaternary carbon at *d* 173.0 ppm accomplished the complete structure of pure compound (TMH-3). The chemical name of

isolated pure compound (TMH-3) is 2(E)-3-(4-hydroxy-3-methoxy phenyl)-2-propenoic acid. So, pure compound (TMH-3) was assigned as ferulic acid.
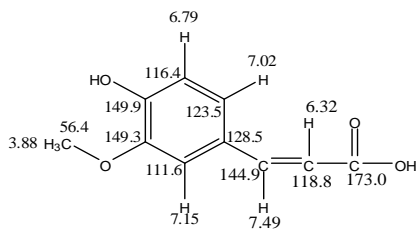
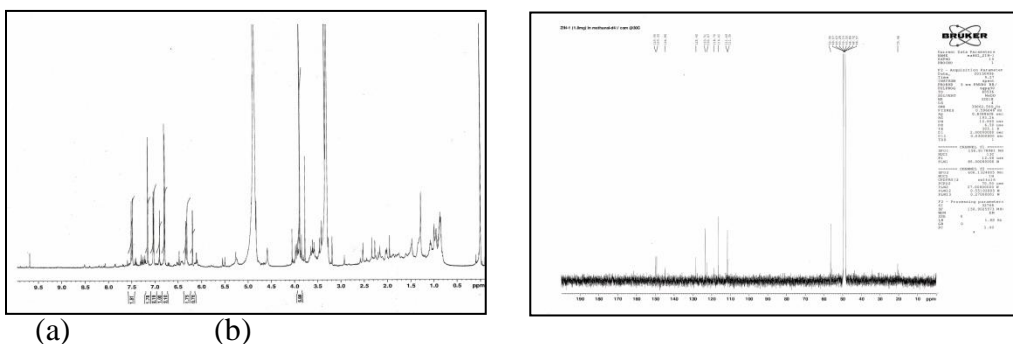Complete Structure of Pure compound (TMH-3) (Ferulic acid)

Figure 16: (a) ^{1}H NMR and (b) ^{13}C NMR spectra of TMH-3

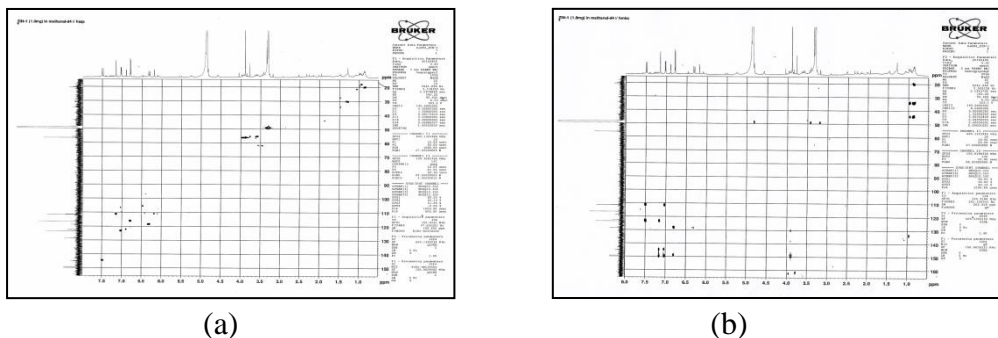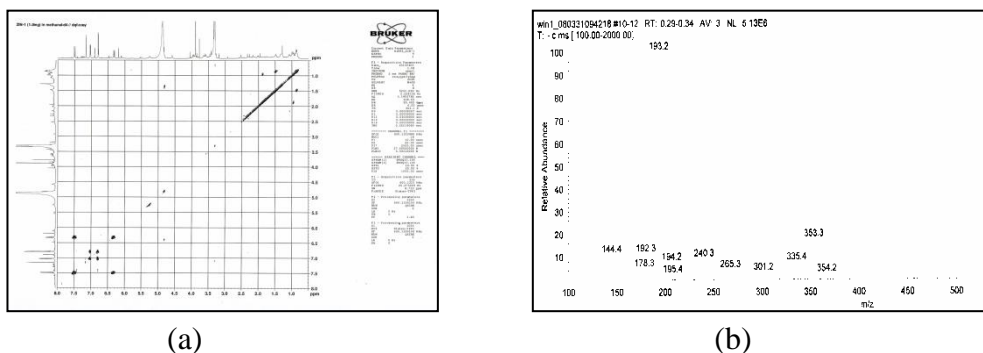**Figure 17:** (a) MHQC and (b) HMBC spectra of TMH-3

| (a) | (b) |

Figure 18: (a) DQF-COSY and (b) (-) ESI spectra of TMH-3

Antimicrobial Activities of Pure Compounds (TMH-1 and TMH-2)

Due to the lack of appropriate test system or limitation of resources, the compounds (TMH-1 and TMH-2) could be tested against only six different microorganisms. The results are shown in Table 1.

Table 1: Results of Antimicrobial Activities of Pure Compounds (TMH-1 and TMH-2)

No.	Types of microorganisms	Diameter of Inhibition Zone (mm)	
		TMH-1	TMH-2
1.	*Bacillus subtilis*	11	11
2.	*Staphylococcus aureus*	-	12
3.	*Pseudomononas aeruginosa*	12	11
4.	*Bacillus pumilus*	11	11
5.	*Candida albicans*	12	11
6.	*E.coli*	12	12

Agar well ~ 10 mm

10 mm ~ 14 mm (+) weak activity

15 mm ~ 19 mm (++) medium activity

20 mm above (+++)   high activity

Cytotoxicity of the Isolated Compounds (TMH-1 and TMH-2)

Pure compounds (TMH-1) and (TMH-2) were screened for cytotoxicity using brine-shrimp bioassay. The assay was based on the ability

of pure compounds to kill laboratory cultured brine shrimp. The results are shown in Table 2.

Table 2: Results of Brine Shrimp Lethality Bioassay

Test materials	% Mortality under the concentration studied (mg/mL)				LD_{50} (μg/mL)	Toxicity
(w/v)	0.1	1	10	100	0.26	Toxic
TMH-1	47.22	63.33	80	87.22		
(w/v)	0.3	3	30	300	0.38	Toxic
TMH-2	49.49	67.76	63.91	73.29		
(w/v)	1	10	100	1000	1.5	Toxic
$K_2Cr_2O_7$	48.63	73.13	74.67	100		
(w/v)	1	10	100	1000	50>1000	Non-toxic
Caffeine	0	0	9.58	12.73		

The LD_{50}-values of pure compounds (TMH-1) and (TMH-2) were 0.26 and 0.38 μg/mL which were considered toxic. Reference standard potassium dichromate showed LD_{50}-value (1.5 μg/mL). No mortality was found in negative control (caffeine) group.

Antioxidant Activity of the Isolated Compound (TMH-1)

The radical scavenging activity of pure compound (TMH-1) was determined by using DPPH assay method. The result is shown in Table 3.

Table 3: Mean % Inhibition of Pure Compound (TMH-1) in Different Concentrations and Its IC_{50} Value

Sample (μg/mL)	Mean % Inhibition in different concentrations						IC_{50} (μg/mL)
TMH-1	**0.78**	**1.56**	**3.13**	**6.25**	**12.50**	**25.0**	1.06
	47.77	54.02	77.23	80.80	84.97	85.57	

Table 4: Mean % Inhibition of Standard Gallic Acid in Different Concentrations and Its IC$_{50}$ Value

Sample (µg/mL)	Mean % Inhibition in different concentrations						IC$_{50}$ (µg/mL)
	0.31	**0.63**	**1.25**	**2.50**	**5.00**	**10.00**	
Gallic acid	27.06	33.12	34.42	41.13	49.35	97.40	5.07

From these results, the lower IC$_{50}$ value indicates the higher antioxidant activity. The pure compound (TMH-1) exhibited lower IC$_{50}$ value (1.06 µg/mL) than the standard gallic acid (5.07µg/mL). Therefore, the pure compound (TMH-1) responds higher antioxidant activity than the standard gallic acid.

Conclusion

In this research work, one of the medicinal plants, *Andrographis paniculata* (Burn.f.)Wall. exNees which was collected from Monywa Township, Sagaing Region, was selected for chemical investigation of its endophytic fungus due to its interesting medicinal uses.

Endophytes from *A. paniculata* were cultivated on water-agar medium. Selection of the target fungus from pure isolated cultures was performed based on chemical screening. The fermentation on large scale for the selected fungus was performed on M$_2$ medium. The well-grown solid cultures were extracted with EtOAc. In addition, pure compounds (TMH-1) and (TMH-2) were isolated as yellow pigments from extracts by applying modern separation techniques such as thin layer and column chromatography.

Structure elucidation of pure compounds (TMH-1) and (TMH-2) were done by using different types of NMR experiments such as ^{1}H NMR, ^{13}C NMR, DEPT, DQF-COSY, HMQC, HMBC, NOESY and ESI MS respectively. The pure compounds (TMH-1) and (TMH-2) were elucidated as bis-naphtho-g-pyrones derivatives**.** Together with pure compounds, other secondary metabolite namely, ferulic acid was isolated from the extracts. According to the results of antimicrobial activity, the isolated pure compound (TMH-1) showed weak activity on five selected organisms such as *Bacillus subtilis, Pseudomononas aeruginosa, Bacillus pumilus, Candida albicans* and *E.coli* and (TMH-2) showed weak activity on all tested organisms. In brine shrimp cytotoxicity test, the LD$_{50}$-value of pure compounds (TMH-1) and

(TMH-2) were found to be 0.26 and 0.38 µg/mL respectively. Then, the antioxidant activity of pure compound (TMH-1) was evaluated by using DPPH radical scavenging assay. Pure compound (TMH-1) showed the antioxidant activity with IC_{50} values of 1.06µg/mL.

Acknowledgements

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