

## SECONDARY METABOLITE FROM ENDOPHYTIC FUNGI *Chochlibolus lunatus* OF THE RHIZOME OF TUNJUK LANGIT (*Helmythostachys zaylanica*)

Fitrya<sup>1,\*</sup> and Muharni<sup>2</sup>

<sup>1</sup>Department of Pharmacy, University of Sriwijaya, Jl. Raya Palembang-Prabumulih Km 32, Indralaya, OI, South Sumatera, Indonesia

<sup>2</sup>Department of Chemistry, University of Sriwijaya, Jl. Raya Palembang-Prabumulih Km 32, Indralaya, OI, South Sumatera, Indonesia

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### ABSTRACT

*Helmythostachys zaylanica* which is locally known as tunjuk langit is included *Ophioglossaceae* genus. The rhizome of tunjuk langit is traditionally used as anticancer. *Chochlibolus lunatus* was isolated from the tissues of tunjuk langit rhizome. Fungal strain was identified based on the characteristics of its colony and cell morphology. *Chochlibolus lunatus* was cultured in Potato Dextrose Broth (PDB) medium and it was then extracted using ethylacetate (EtOAc). A secondary metabolite compound was isolated from EtOAc extract. This isolation was done by chromatography technique and the structure of this compound was determined by mean of UV, IR and NMR spectroscopic technique. Isolated compound is like oil. Based on spectra analyses, we concluded that this isolated compound was 11,12,13-trimethylheksyl-2-methylhexa-2,4-dienoat.

**Keywords:** *Helmythostachys zaylanica*; *Chochlibolus lunatus*; 11,12,13-trimethylhexyl-2-methylhexa-2,4-dienoat

### ABSTRAK

Tumbuhan *Helmythostachys zaylanica* yang dikenal dengan nama tunjuk langit termasuk kedalam genus *Ophioglossaceae*. Akar tumbuhan tunjuk langit secara tradisional digunakan sebagai obat antikanker. Jamur endofitik *Chochlibolus lunatus* telah diisolasi dari jaringan akar *Helmythostachys zaylanica*. Strain jamur ini diidentifikasi berdasarkan koloni dan morfologi sel. *Chochlibolus lunatus* dikultur dalam medium PDB kemudian diekstraksi dengan etilasetat. Dari ekstrak etilasetat telah diisolasi satu senyawa metabolit sekunder. Isolasi dilakukan dengan teknik kromatografi dan penentuan struktur molekul dilakukan dengan metode spektroskopi UV, IR dan NMR. Senyawa hasil isolasi berupa oil. Berdasarkan analisa data spektrum disimpulkan bahwa senyawa hasil isolasi adalah 11,12,13-trimetilheksil-2-metilheksa-2,4-dienoat.

**Kata Kunci:** *Helmythostachys zaylanica*; *Chochlibolus lunatus*; 11,12,13-trimetil-2-metilheksa-2,4-dienoat

### INTRODUCTION

Ophioglossaceae genus is well known to be rich in flavonoid compounds [1]. One species of this genus is *Helmythostachys zaylanica* which is locally known as Tunjuk langit [2]. Chen, et al. and Murakami, et al. reported some flavonoid compounds from the rhizome of *H. zaylanica* [3-4]. The research of the bioactivity of *H. zaylanica* showed that the rhizome of *H. zaylanica* has aphrodisiac properties, hepatoprotective effect, antioxidant activity, neuroprotective activity and antiinflammatory activity [5-9]. In previous research, we discovered two flavonoids with antioxidant activity from the rhizome of *H. zaylanica*, one of them reported as flavonoid ugonin J [10].

The use of bioactive compound from natural plants as a drug source is frequently limited by low percentage

of compound content. To solve this problem, there are some methods to produce the bioactive compound, such as tissue culture, enzyme discovery, and gen transplantation. However, these methods have insignificant opportunity and high difficulty [11]. Fortunately, there is an appropriate technology, i.e. Endophytic, to overcome these problems. This technology makes use of endophytic microbe which is specifically applied for certain plant [12]. This microbe life is as mutualism symbiosis with host plant and produces secondary metabolites together. We produced secondary metabolites by cultivation of microbe without extraction of the host plant [13-14]. This method has superiority in time and cost. In this paper we describe the isolation and determination of endophytic fungi from the rhizome of *H. zaylanica*. Exploration and elucidation of the structure of

\* Corresponding author. Tel/Fax : +62-82179056752  
Email address : fitrya\_apt@yahoo.com

secondary metabolite isolated from endophytic fungus were also carried out.

## EXPERIMENTAL SECTION

### Materials

The rhizome of *H. zaylanica* was collected from Indralaya, Ogan Ilir, South Sumatera. This specimen was identified at Herbarium Bogoriense, Bogor. Materials for this research were N-hexane, ethylacetate, ethanol, silica gel 60 G (70-230 Mesh, Merck), TLC plate with silica gel 60 GF254, 0.25, 20x20 cm (Merck), potato dextrose broth (PDB), potato dextrose agar (PDA), medium for cultivation and isolation endophytic fungi, a series medium for physiologic assay or microbial identification (Czapek Dox Agar (CDA), Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA).

### Instrumentation

Instrumentation used in this study included distillation apparatus, rotary evaporator, counter colony, water bath, autoclave, microscope, incubator, NMR JEOL JNM ECA-500 spectrometer 500 MHz ( $^1\text{H}$ ) and 125 MHz ( $^{13}\text{C}$ ). UV and IR spectra were measured by using spectrophotometers (i.e., Beckman DU-700 and Shimadzu FTIR 8400) and UV lamp.

### Procedure

Isolation of endophytic fungus was done by using a method described by Debbab et al. [15]. The rhizome was rinsed in sterilized water. Surface sterilization was done by immersing the rhizome in 70% ethanol and then aseptically cleaved into small segment. The material was placed on a petri dish containing PDA medium. After several days hyphae growing from the plant material were transferred to other plates which are then incubated for 10 days and periodically checked for culture purity. Fungi produced from incubation were screened to select potential secondary metabolites. The potential fungi were cultivated with optimum condition in PDB medium to produce secondary metabolites. The fungal strain was identified based on the characteristics of their colony and morphology [16].

The secondary metabolite was produced by incubating *Chochlibolus lunatus* fungus in 4 L liquid medium of PDB till optimum condition (2 months). Separation of filtrate from biomass was done by filtering. Filtrate containing secondary metabolite was extracted using n-hexane (2 x 4 L) and ethylacetate (EtOAc) (2 x 4 L). The quality of organic solvents used in this study is technical grade. Therefore, we needed to

improve the quality by means of distillation. The extract was concentrated by using evaporator.

The concentrated EtOAc extract of endophytic fungi *Chochlibolus lunatus* was subjected to chromatographed on column with gradient system (n-hexane:EtOAc 9:1; 7:3; 3:7; 1:9, EtOAc 100%) to afford 5 fractions F1 – F5.

### Structure elucidation

The structure was elucidated using UV, IR, NMR 1-D ( $^1\text{H}$  and  $^{13}\text{C}$ -NMR) and NMR 2-D (HMQC and HMBC) spectroscopic data.

## RESULT AND DISCUSSION

Four endophytic fungus species isolated from the rhizome of *H. zaylanica* are FA1-FA4. These fungi were cultivated in liquid medium PDB for two months and then filtered. TLC of EtOAc extract showed that FA1 species was potential to produce a secondary metabolite. Based on the colony and morphology characteristics, this fungus was identified as *Chochlibolus lunatus*.

*Chochlibolus lunatus* was recultivated in 4 L PDB medium for two months. Supernatant was extracted with n-hexane and ethyl acetate. N-hexane and ethyl acetate were evaporated to produce 2 g n-hexane and 2.5 g EtOAc extracts. TLC analysis showed that n-hexane extract was not potential to produce secondary metabolite. Purification of fraction F1 gave a pure compound (**1**) which was like oil. Structure elucidation was done by spectroscopic technique. The UV spectrum (1 mg/10 mL MeOH) showed maximum absorption at  $\lambda_{\text{max}}$  273 nm indicating  $\pi \rightarrow \pi^*$  transition. The IR spectrum (KBr) showed typical absorptions of C-H aliphatic (2958.6, 2922.0  $\text{cm}^{-1}$ ), carbonyl (1728.1  $\text{cm}^{-1}$ ), C=C conjugated (1579.6-1463.9  $\text{cm}^{-1}$ ) and C-O stretch (1272.9  $\text{cm}^{-1}$ ).

$^1\text{H}$ -NMR spectrum of isolated compound ( $\text{CHCl}_3$ -*d*1) showed two signals for  $\text{sp}^2$  proton at  $\delta\text{H}$  7.70 ppm (1H, *dd*,  $J=3.2$  and 5.8) and 7.52 ppm (1H, *dd*,  $J=3.2$  and 5.8) which were vicinally coupled each other (Fig. 1). Signal at  $\delta\text{H}$  4.22 ppm (2H, *m*) was attributed to methylene proton  $\text{sp}^3$  attached to electronegative atom, so that the signal appeared at deshielding area. Signal at  $\delta\text{H}$  1.68 ppm (1H, *m*) according to methyne  $\text{sp}^3$  was coupled with four other protons so that the signal appeared as multiplet (Fig. 2).

$^1\text{H}$ -NMR spectrum showed signals for proton characteristic of methyl group at  $\delta\text{H}$  0.89 ppm (3H, *d*,  $J=5.7$ ) and 0.92 ppm (3H, *d*,  $J=7.5$ ). These signals were vicinally coupled each other. Furthermore, signal at  $\delta\text{H}$  1.26 ppm (14H, *s*) was attributed to four methyl group and two methine group.

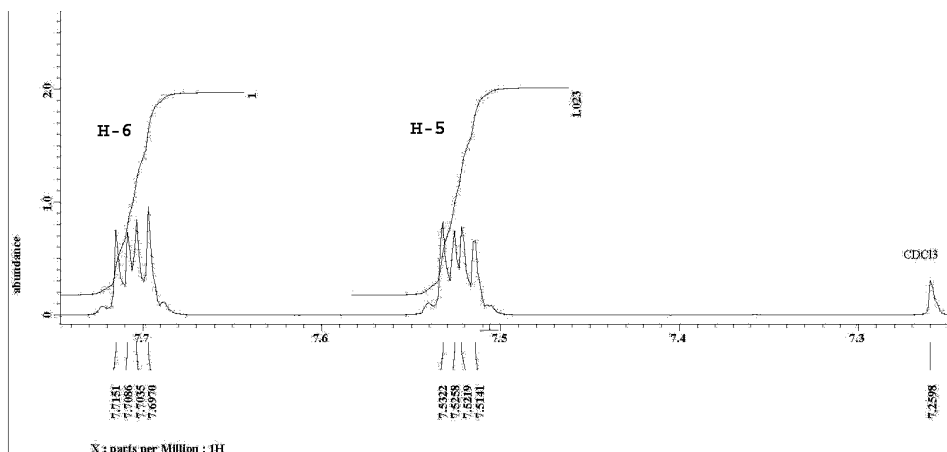


Fig 1. <sup>1</sup>H-NMR at δH 7.2-7.7 ppm of compound 1

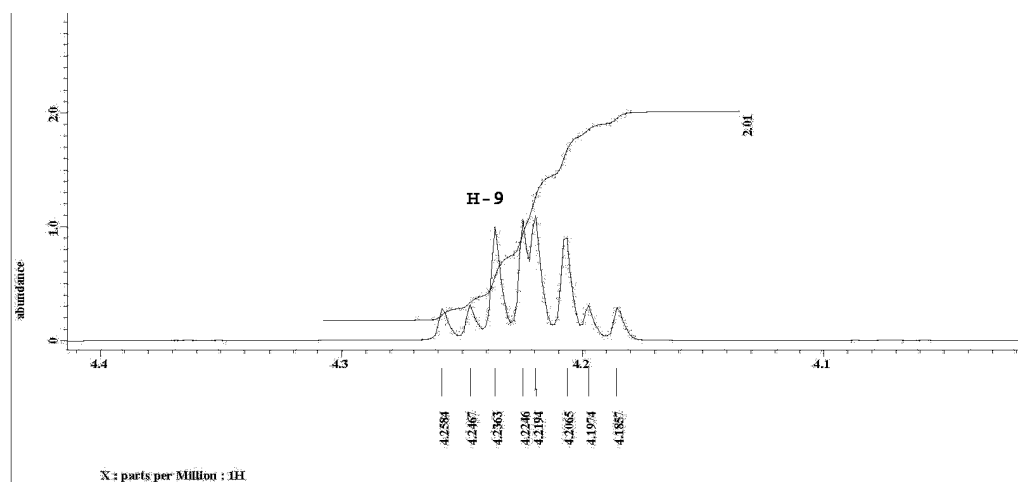


Fig 2. <sup>1</sup>H-NMR at δH 4.1-4.4 ppm of compound 1

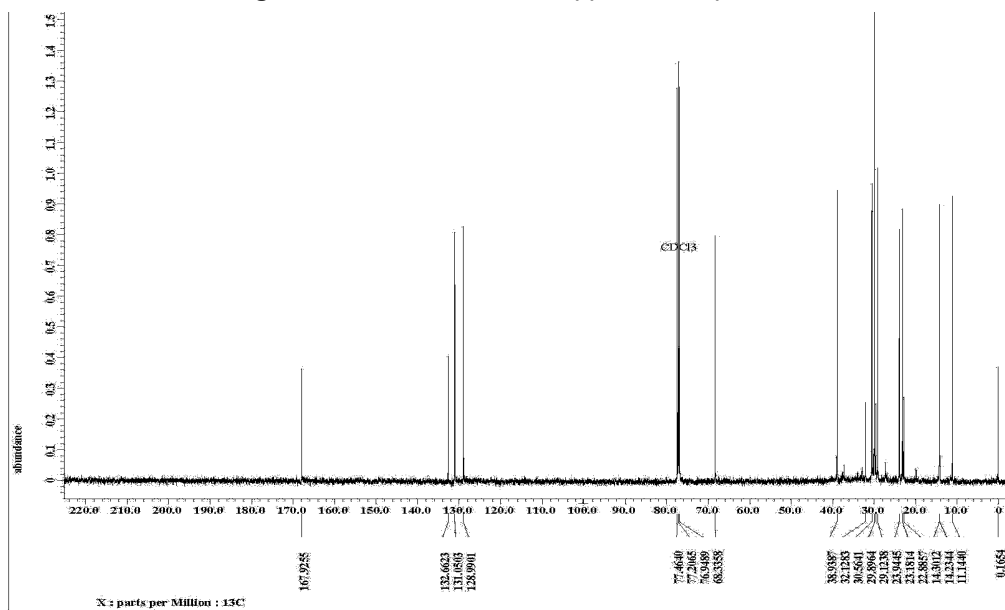


Fig 3. <sup>13</sup>C-NMR spectrum of compound 1

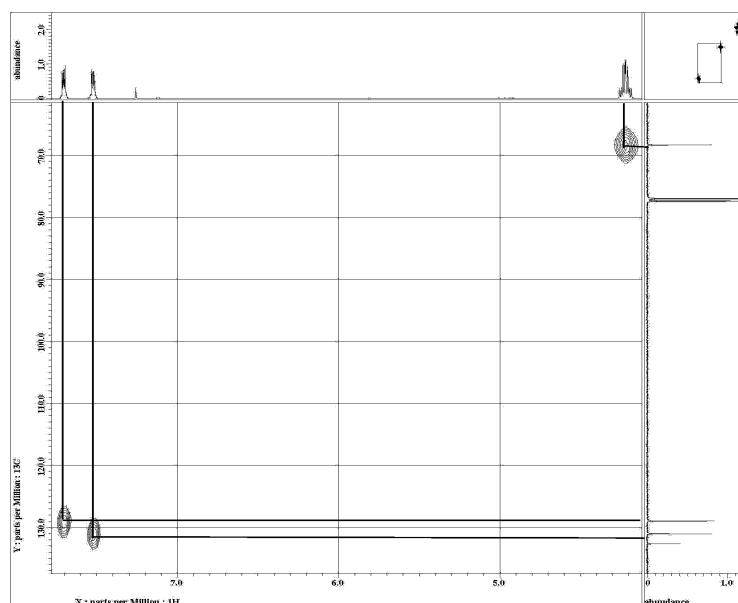


Fig 4. HMBC spectrum of compound 1 at  $\delta$ H 4-8 ppm and  $\delta$ C 70-130 ppm

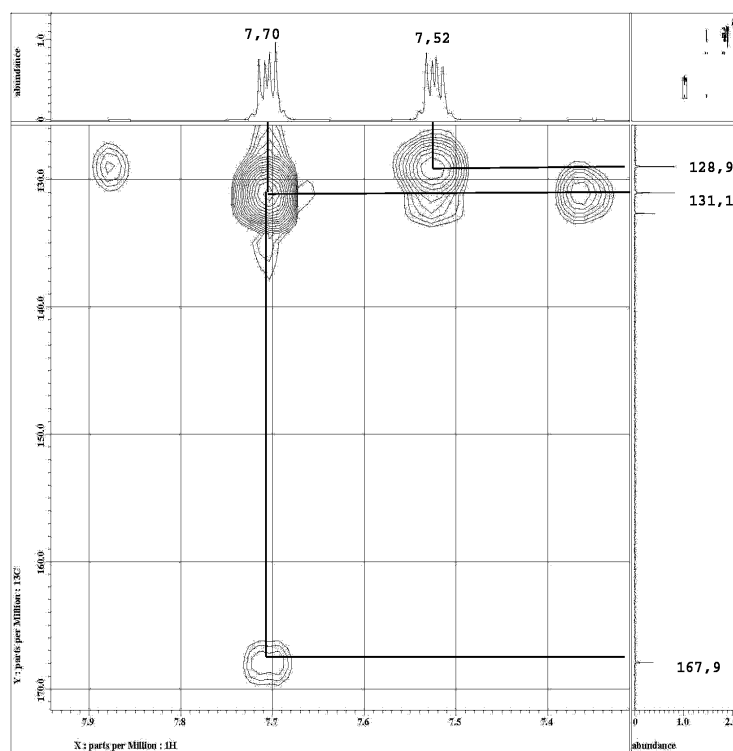


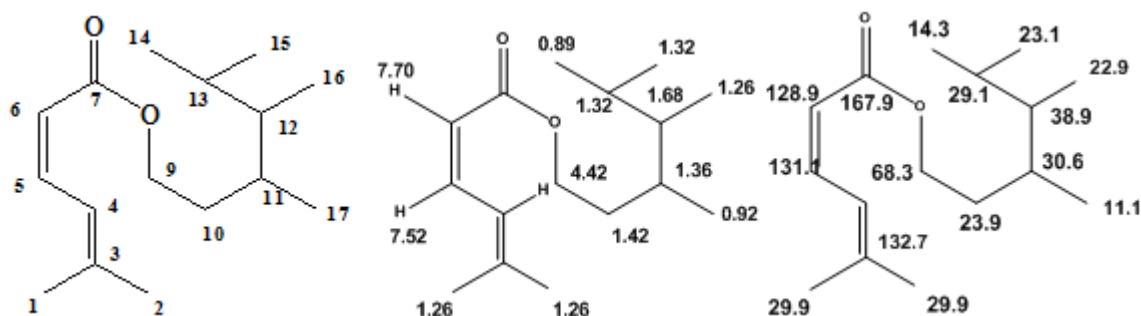
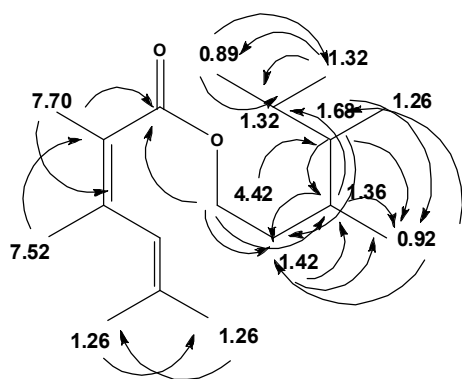
Fig 5. HMBC spectrum of compound 1 at  $\delta$ H 7.4 -7.9 ppm and  $\delta$ C 130-170 ppm

$^{13}\text{C}$ -NMR spectrum (chloroform- $d_1$ ) showed 17 carbon signals. Signal at  $\delta$ C 167.9 ppm attributed for carbonyl group is specific for carbonyl of ester or carboxylate.  $^{13}\text{C}$ -NMR showed three signals for  $\text{sp}^2$  carbon at  $\delta$ C 132.7; 131.1; and 128.9 ppm resulted from double bond having quarterner carbon (Fig. 3).

Furthermore, signal at  $\delta$ C 68.3 ppm is specific for carbon attached to oxygen and 12 signals at  $\delta$ C 11-40 ppm were from  $\text{sp}^3$  carbon of aliphatic groups. Based on these data suggested that isolated compound was unsaturated aliphatic compound.

**Table 1.** Proton and carbon chemical shift of  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectrum of isolated compound (chloroform- $d_1$ )

Carbon Position	$\delta\text{H}$ (ppm) Compound 1	$\delta\text{C}$ (ppm) Compound 1	HMBC
1	1.26 (3H, s)	29.9	C-2
2	1.26 (3H, s)	29.9	C-1
3		132.7	
4			
5	7.52 (1H, <i>dd</i> , $J=3.25$ ; 5.8)	131.1	C-6
6	7.70 (1H, <i>dd</i> , $J=3.25$ ; 5.8)	128.9	C-5, C-7
7		167.9	
9	4.22 (2H, <i>m</i> )	68.3	C-7, C-10, C-11, C-12
10	1.42 (2H, <i>m</i> )	23.9	C-17, C-11
11	1.36 (1H, <i>m</i> )	30.6	C-17, C-10, C-13
12	1.68 (1H, <i>m</i> )	38.9	C-17, C-11, C-10
13	1.32 (1H, <i>m</i> )	29.1	C-14, C-15
14	0.89 (3H, <i>d</i> , $J=5.8$ )	14.3	C-15, C-13
15	1.32 (3H, <i>m</i> )	23.1	C-14, C-13
16	1.26 (3H, s)	22.9	
17	0.92 (3H, <i>d</i> , $J=7.15$ )	11.1	C-10, C-12

**Fig 6.** Structure of isolated compound (A= structure numbering, B= chemical shift of proton and C= chemical shift of carbon)**Fig 7.** HMBC correlation of compound 1

Analysis of HMQC spectrum showed proton at  $\delta\text{H}$  7.70 ppm attached to carbon at  $\delta\text{C}$  128.9 ppm. HMBC spectrum showed that carbon at  $\delta\text{C}$  128.9 ppm is correlated with carbon at  $\delta\text{C}$  167.9 and 131.1 ppm. Therefore, the proton at  $\delta\text{H}$  7.70 ppm is located closed to carbonyl group (Fig. 4). Proton at  $\delta\text{H}$  7.52 ppm (1H, *dd*, 3.2; 5.5) attached to carbon at  $\delta\text{C}$  128.9 ppm. Based on HMBC correlation and its coupling constant ( $J=3.2$ )

indicated that these protons are closed each other or these protons are attached to double bond (Fig. 5)

Proton at 4.22 (2H, *m*) was attached with carbon at  $\delta\text{C}$  68.3 ppm and HMBC spectrum showed correlation with carbon at  $\delta\text{C}$  167.9; 23.9; 30.6; and 38.9 ppm. Proton at  $\delta\text{H}$  1.68 (1H, *m*) was attached with carbon at  $\delta\text{C}$  38.9 and correlated with carbon at  $\delta\text{C}$  23.9; 30.6; and 11.1 ppm. Proton at  $\delta\text{H}$  1.42 ppm (1H, *m*) was attached with carbon at  $\delta\text{C}$  23.9 and correlated with carbon at  $\delta\text{C}$  30.6 and 11.1 ppm. Based on these data indicated that both of protons are located closed to carbon at  $\delta\text{C}$  30.6 ppm. HMQC spectrum showed that carbon was attached to proton at  $\delta\text{H}$  1.36 (1H, *m*). Proton at  $\delta\text{H}$  1.32 ppm (1H, *m*) was attached with carbon at  $\delta\text{C}$  29.1 and 23.1 and correlated with carbon at  $\delta\text{C}$  14.3; 23.1 and 29.1 ppm, respectively. Based on these data indicated that proton at  $\delta\text{H}$  1.32 ppm was resulted from methine group and methyl group.

Furthermore, HMQC spectrum showed that proton at  $\delta\text{H}$  0.89 (3H, *d*,  $J=5.8$ ) and 0.92 ppm (3H, *d*,  $J=7.15$ ) was attached to carbon at  $\delta\text{C}$  14.3 and 11.1 ppm and correlated with carbon at  $\delta\text{C}$  29.1; 23.1; 23.9

and 38.9 ppm respectively. Spectroscopic data 1D and 2D showed at Table 1.

Based on NMR spectrum analysis, isolated compound is 11,12,13-trimethylhexyl-2-methylhexa-2,4-dienoate and has structure as shown in Fig. 6. Correlation between proton and carbon in HMBC is shown in Fig. 7.

## CONCLUSION

The endophytic fungus *Chochlibus lunatus* was isolated from the tissues of tunjuk langit rhizome. The fungal strain was identified by characteristics of their colony and morphology. A secondary metabolite has been isolated from ethylacetate extract of *Chochlibus lunatus*. Based on spectroscopic data analysis we concluded that isolated compound is 11,12,13-trimethylhexyl-2-methylhexa-2,4-dienoate.

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