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[Home](#) [About](#) [Login](#) [Register](#) [Search](#) [Current](#) [Archives](#) [Announcements](#) [Statistics](#) [Indexing & Abstracting](#) [Journal History](#) [Contact](#)
[Home](#) > [Archives](#) > **Vol 13, No 3 (2013)**

Vol 13, No 3 (2013)

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Table of Contents

Articles

- | | |
|--|---------|
| <p>QUINOLIN-6-AMINES: SYNTHESIS AND BIOLOGICAL EVALUATION
 Hament Panwar, Nidhi Chaudhary, Ranjana Dubey, Tilak Ram
 10.22146/ijc.21274 Abstract views : 526 PDF views : 515</p> | 185-192 |
| <p>SECONDARY METABOLITE FROM ENDOPHYTIC FUNGI <i>Chochlibus lunatus</i> OF THE RHIZOME OF TUNJUK LANGIT (<i>Helmynthostachys zaylanica</i>)
 Fitrya Fitrya, Muharni Muharni
 10.22146/ijc.21275 Abstract views : 425 PDF views : 482</p> | 193-198 |
| <p>ISOLATION AND PRESENCE OF ANTIMALARIAL ACTIVITIES OF MARINE SPONGE <i>Xestospongia</i> sp.
 Murtihapsari Murtihapsari, Apriani Sulu Parubak, Bertha Mangallo, Wiwied Ekasari, Puji Budi Asih, Ayu Indah Lestari
 10.22146/ijc.21276 Abstract views : 658 PDF views : 782</p> | 199-204 |
| <p>FLAVANONES FROM THE WOOD OF <i>Morus nigra</i> WITH CYTOTOXIC ACTIVITY
 Ferlinahayati Ferlinahayati, Yana Maolana Syah, Lia Dewi Juliawaty, Euis Holisotan Hakim
 10.22146/ijc.21277 Abstract views : 488 PDF views : 549</p> | 205-208 |
| <p>NEW PYRAN OF AN ENDOPHYTIC FUNGUS <i>Fusarium</i> sp. ISOLATED FROM THE LEAVES OF BROLOWALI (<i>Tinaspora crispata</i>)
 Elfita Elfita, Munawar Munawar, Muharni Muharni, Suprayetno Suprayetno
 10.22146/ijc.21278 Abstract views : 479 PDF views : 484</p> | 209-215 |
| <p>A NEW CYTOTOXIC DOLABELLANE FROM THE INDONESIAN SOFT CORAL <i>Anthelia</i> sp.
 Anggia Murni, Novriyandi Hanif, Junichi Tanaka</p> | 216-220 |

ARTICLE IN PRESS

List of the accepted articles for future issues.

[Focus & Scope](#)
[Author Guidelines](#)
[Author Fees](#)
[Online Submission](#)
[Publication Ethics](#)
[Plagiarism Policy](#)
[Editorial Board](#)
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Search Scope

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[▶ By Author](#)
[▶ By Title](#)
[▶ Other Journals](#)

INFORMATION




[▶ For Readers](#)
[▶ For Authors](#)
[▶ For Librarians](#)

KEYWORDS

[HPLC QSAR TIO2](#)

 10.22146/ijc.21279  Abstract views : 657  views : 520	
POLYMERIC BIOMATERIALS FILM BASED ON POLY(VINYL ALCOHOL) AND FISH SCALE COLLAGEN BY REPETITIVE FREEZE-THAW CYCLES FOLLOWED BY GAMMA IRRADIATION	221-228
<i>Dian Pribadi Perkasa, Erizal Erizal, Basril Abbas</i>	
 10.22146/ijc.21280  Abstract views : 548  views : 1021	
PRODUCTION AND CHARACTERIZATION OF BIOSURFACTANT BY <i>Pseudomonas fluorescens</i> USING CASSAVA FLOUR WASTEWATER AS MEDIA	229-235
<i>Venty Suryanti, Soerya Dewi Marliyana, Desi Suci Handayani, Desi Ratnaningrum</i>	
 10.22146/ijc.21281  Abstract views : 630  views : 893	
SIMULTANEOUS DETERMINATION OF CADMIUM, COPPER AND LEAD IN SEA WATER BY ADSORPTIVE STRIPPING VOLTAMMETRY IN THE PRESENCE OF CALCON AS A COMPLEXING AGENT	236-241
<i>Deswati Deswati, Hamzar Suyani, Safni Safni, Umiati Loekman, Hilfi Pardi</i>	
 10.22146/ijc.21282  Abstract views : 598  views : 735	
FORMULATION AND <i>IN VITRO</i> STUDY OF PROPRANOLOL HYDROCHLORIDE CONTROLLED RELEASE FROM CARBOXYMETHYL CHITOSAN-BASED MATRIX TABLETS	242-247
<i>Hernawan Hernawan, Septi Nurhayati, Khoirun Nisa, A.W. Indrianingsih, Cici Darsih, Muhammad Kismurtono</i>	
 10.22146/ijc.21283  Abstract views : 765  views : 975	
COMPARATIVE EVALUATION OF CONVENTIONAL VERSUS RAPID METHODS FOR AMPLIFIABLE GENOMIC DNA ISOLATION OF CULTURED <i>Azospirillum</i> sp. JG3	248-253
<i>Stalis Norma Ethica, Dilin Rahayu Nataningtyas, Puji Lestari, Istini Istini, Endang Semiarti, Jaka Widada, Tri Joko Raharjo</i>	
 10.22146/ijc.21284  Abstract views : 719  views : 813	
EXTENDING THE LIFE TIME OF POLYMER INCLUSION MEMBRANE CONTAINING COPOLY(EUGENOL-DVB) AS CARRIER FOR PHENOL TRANSPORT	254-261
<i>Agung Abadi Kiswandono, Dwi Siswanta, Nurul Hidayat Aprilita, Sri Juara Santosa, Takashi Hayashita</i>	
 10.22146/ijc.21285  Abstract views : 514  views : 555	
THE INFLUENCE OF PVA-cl.CITRIC ACID/CHITOSAN MEMBRANE HYDROPHILICITY ON THE TRANSPORT OF CREATININE AND UREA	262-270
<i>Retno Ariadi Lusiana, Dwi Siswanta, Mudasir Mudasir, Takashi Hayashita</i>	
 10.22146/ijc.21286  Abstract views : 802  views : 783	
CHARACTERISTIC OF AIRBORNE PARTICULATE MATTER SAMPLES COLLECTED FROM TWO SEMI INDUSTRIAL SITES IN BANDUNG, INDONESIA	271-277
<i>Diah Dwiana Lestiani, Muhayatun Santoso, Syukria Kurniawati, Andreas Markwitz</i>	
 10.22146/ijc.21287  Abstract views : 712  views : 693	
CONVERSION OF THE LOW QUALITY INDONESIAN NATURALLY-OCCURRING MINERALS INTO SELECTIVE TYPE OF ZEOLITES BY SEED-ASSISTED SYNTHESIS METHOD	278-282
<i>Rino R. Mukti, Shofarul Wustoni, Agus Wahyudi, Ismunandar Ismunandar</i>	
 10.22146/ijc.21288  Abstract views : 741  views : 650	

Note

PyPLIF-ASSISTED REDOCKING INDOMETHACIN-(R)-ALPHA-ETHYL-ETHANOLAMIDE INTO CYCLOOXYGENASE-1	283-286
<i>Muhammad Radifar, Nunung Yuniarti, Enade Perdana Istyastono</i>	
 10.22146/ijc.21289  Abstract views : 564  views : 646	

adsorption

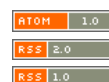
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 characterization chitosan
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CURRENT ISSUE



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

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ABSTRACT

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

ABSTRAK

Tumbuhan *Helmyntostachys 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 *Helmyntostachys 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: *Helmyntostachys 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 *Helmyntostachys 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

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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 (Czapez 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 (^1H) and 125 MHz (^{13}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 (^1H and ^{13}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 λ_{max} 273 nm indicating $\pi \rightarrow \pi^*$ transition. The IR spectrum (KBr) showed typical absorptions of C-H aliphatic (2958.6, 2922.0 cm^{-1}), carbonyl (1728.1 cm^{-1}), C=C conjugated (1579.6-1463.9 cm^{-1}) and C-O stretch (1272.9 cm^{-1}).

^1H -NMR spectrum of isolated compound (CHCl_3 -*d*1) showed two signals for sp^2 proton at δH 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 δH 4.22 ppm (2H, *m*) was attributed to methylene proton sp^3 attached to electronegative atom, so that the signal appeared at deshielding area. Signal at δH 1.68 ppm (1H, *m*) according to methyne sp^3 was coupled with four other protons so that the signal appeared as multiplet (Fig. 2).

^1H -NMR spectrum showed signals for proton characteristic of methyl group at δH 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 δH 1.26 ppm (14H, *s*) was attributed to four methyl group and two methine group.

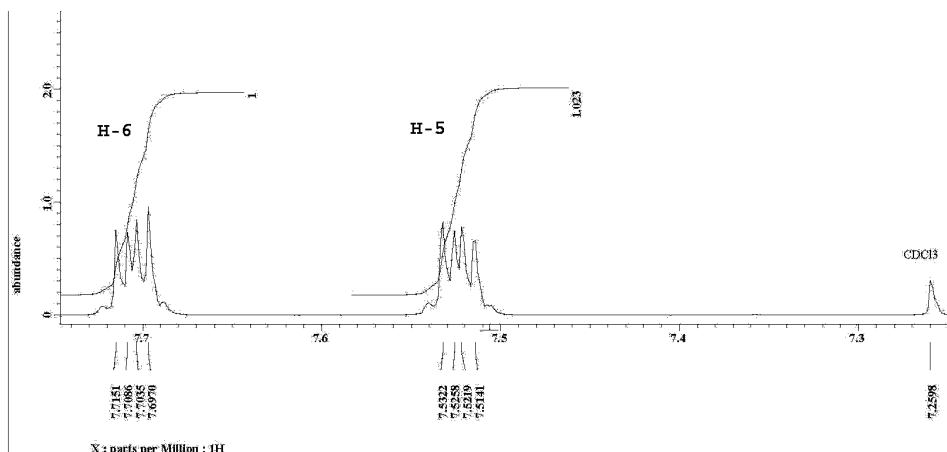


Fig 1. ¹H-NMR at δH 7.2-7.7 ppm of compound 1

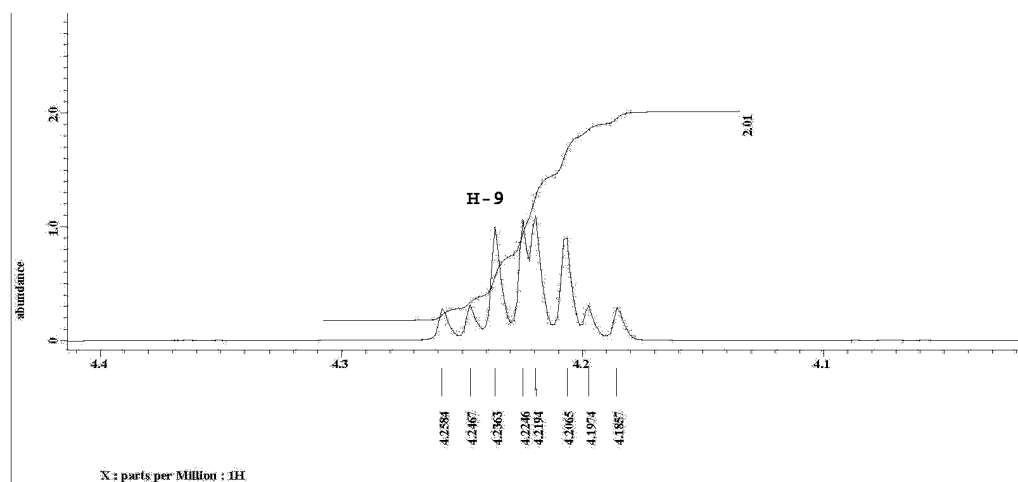


Fig 2. ¹H-NMR at δH 4.1-4.4 ppm of compound 1

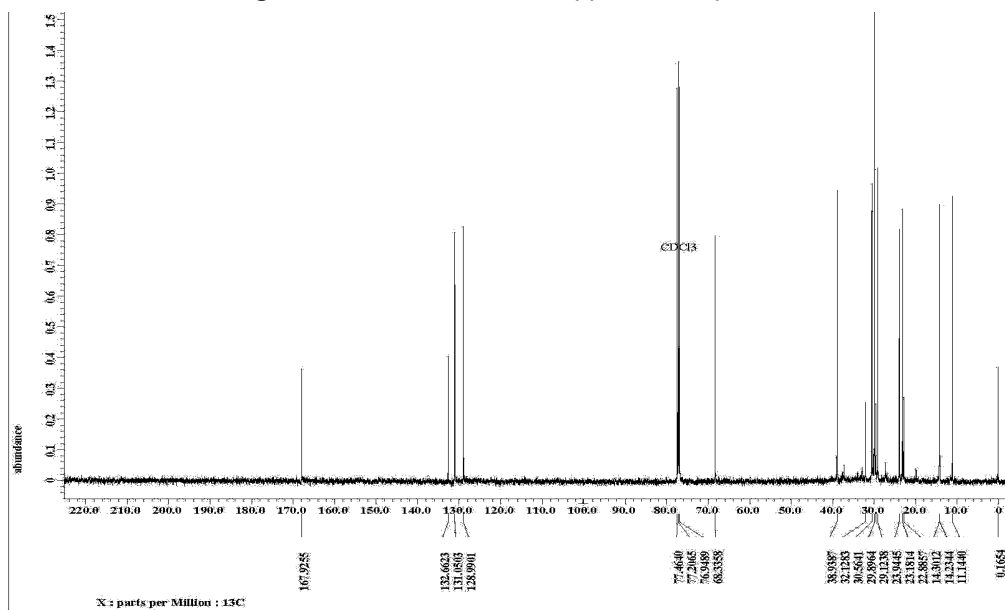


Fig 3. ¹³C-NMR spectrum of compound 1

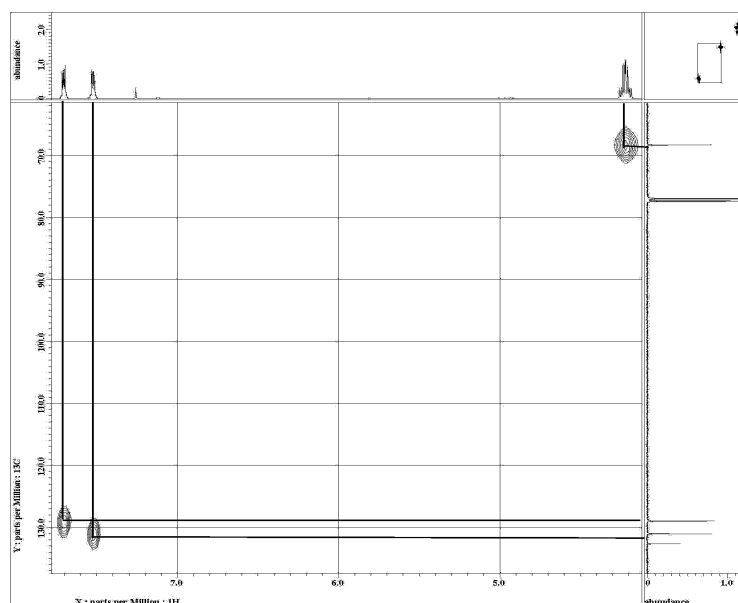


Fig 4. HMBC spectrum of compound 1 at δ H 4-8 ppm and δ C 70-130 ppm

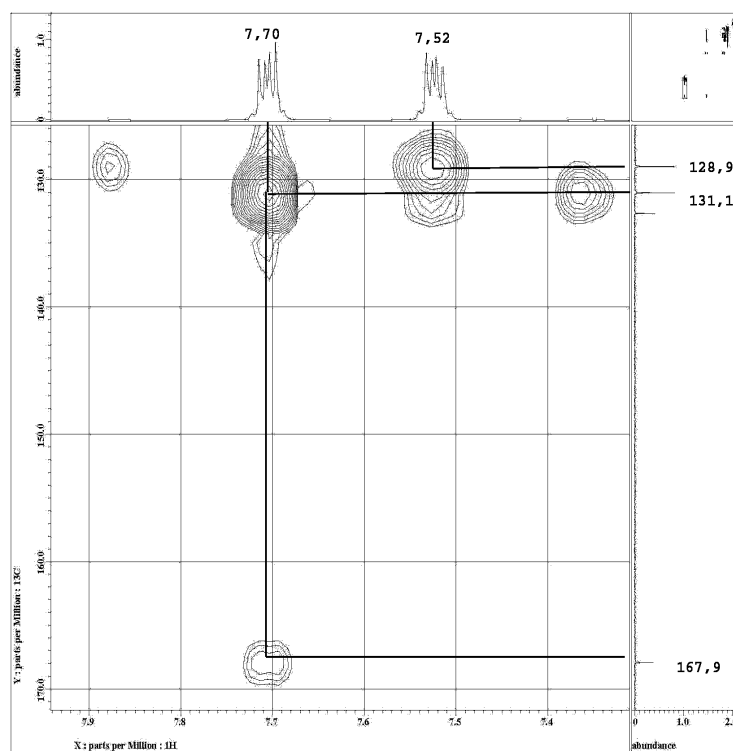


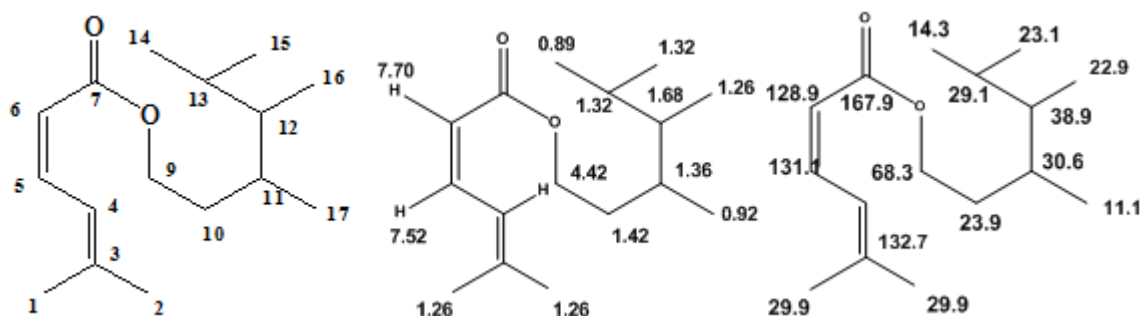
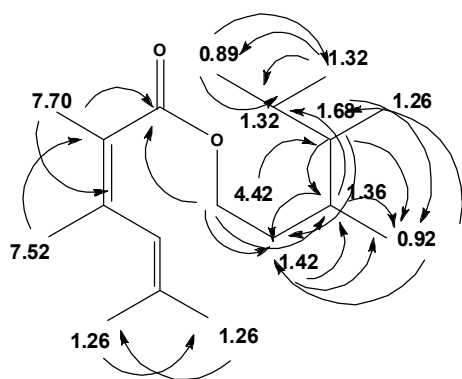
Fig 5. HMBC spectrum of compound 1 at δ H 7.4 -7.9 ppm and δ C 130-170 ppm

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

Furthermore, signal at δ C 68.3 ppm is specific for carbon attached to oxygen and 12 signals at δ C 11-40 ppm were from 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 ^1H and ^{13}C -NMR spectrum of isolated compound (chloroform- d_1)

Carbon Position	δH (ppm) Compound 1	δC (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 δH 7.70 ppm attached to carbon at δC 128.9 ppm. HMBC spectrum showed that carbon at δC 128.9 ppm is correlated with carbon at δC 167.9 and 131.1 ppm. Therefore, the proton at δH 7.70 ppm is located closed to carbonyl group (Fig. 4). Proton at δH 7.52 ppm (1H, *dd*, 3.2; 5.5) attached to carbon at δC 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 δC 68.3 ppm and HMBC spectrum showed correlation with carbon at δC 167.9; 23.9; 30.6; and 38.9 ppm. Proton at δH 1.68 (1H, *m*) was attached with carbon at δC 38.9 and correlated with carbon at δC 23.9; 30.6; and 11.1 ppm. Proton at δH 1.42 ppm (1H, *m*) was attached with carbon at δC 23.9 and correlated with carbon at δC 30.6 and 11.1 ppm. Based on these data indicated that both of protons are located closed to carbon at δC 30.6 ppm. HMQC spectrum showed that carbon was attached to proton at δH 1.36 (1H, *m*). Proton at δH 1.32 ppm (1H, *m*) was attached with carbon at δC 29.1 and 23.1 and correlated with carbon at δC 14.3; 23.1 and 29.1 ppm, respectively. Based on these data indicated that proton at δH 1.32 ppm was resulted from methine group and methyl group.

Furthermore, HMQC spectrum showed that proton at δH 0.89 (3H, *d*, $J=5.8$) and 0.92 ppm (3H, *d*, $J=7.15$) was attached to carbon at δC 14.3 and 11.1 ppm and correlated with carbon at δC 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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