

The Effect of Caramelization and Carbonization Temperatures toward Structural Properties of Mesoporous Carbon from Fructose with Zinc Borosilicate Activator	253-261
Tutik Setianingsih, Indriana Kartini, Yateman Arryanto	
💿 <i>10.22146/ijc.21236 </i> Abstract views : 587 🔤 views : 1698	
Hydrogel Based on Crosslinked Methylcellulose Prepared by Electron Beam Irradiation for Wound Dressing Application	262-268
Ambyah Suliwarno	
🍄 10.22146/ijc.21237 📶 Abstract views : 469 📟 views : 590	
Oxidation and Acetylation of Ursolic and Oleanolic Acids Isolated from <i>Fragraea fragrans</i> fruits; Antiproliferation of P388 Leukemia Cells	269-276
Dasril Basir, Julinar Julinar, Eva Agustriana, Budi Untari	
😳 <i>10.22146/ijc.21238 </i> Abstract views : 704 🔤 views : 1684	
Cytotoxic Isobractatin (Prenylated Xanthone) Epimer Mixture of Garcinia eugenifolia	277-282
Sri Hartati, I Ketut Triyono, Sri Handayani	
😳 <i>10.22146/ijc.21239 </i> Abstract views : 659 🔤 views : 662	
Synthesis and Thermal-Stability Study of Polybutylene Itaconate Modified with Divinyl Benzene and Glycerol	283-289
Atmanto Heru Wibowo, Ninis Makhnunah, Deny Irawati, Candra Purnawan, Nanik Dwi Nurhayati, Henning Storz	
😳 <i>10.22146/ijc.21240 í ii</i> Abstract views : 532 🔤 views : 537	
Di-(2-ethylhexyl)phthalate and Pyranon Derivated from Endophytic fungi <i>Penicillium</i> sp the Leave of Kunyit Putih (<i>Curcuma zedoaria</i>)	290-296
Muharni Muharni, Fitrya Fitrya, Milanti Okta Ruliza, Dwi Anjar Susanti, Elfita Elfita	
🔨 <i>10.22146/ijc.21241 </i> Abstract views : 546 🔤 views : 983	
Major Anthocyanin Pigments in the <i>Ficus padana</i> Fruits: HPLC-DAD-ESI-MS Identification and Antioxidant Activity	297-303
Daimon Syukri, Djaswir Darwis, Adlis Santoni	
💿 <i>10.22146/ijc.21242 </i> Abstract views : 760 🔤 views : 995	
Isolation of Bioactive Compounds from Aspergillus terreus LS07	304-310
Rizna Triana Dewi, Sanro Tachibana, Puspa Dewi, L.B.S. Kardono, Muhammad Ilyas	
🚭 10.22146/ijc.21243 🎢 Abstract views : 657 🔤 views : 828	
Short Communication	
Synthesis and Characterization of [Fe(picolinate) ₃][MnNi(oxalate) ₃].CH ₃ OH Polymeric Complex	311-314
Fahimah Martak, Djulia Onggo, Ismunandar Ismunandar, Agung Nugroho	

🔨 10.22146/ijc.21244 📶 Abstract views : 432 | 🔤 views : 646

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DI-(2-ETHYLHEXYL)PHTHALATE AND PYRANON DERIVATED FROM ENDOPHYTIC FUNGI Penicillium sp THE LEAVE OF KUNYIT PUTIH (Curcuma zedoaria)

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ABSTRACT

Two compounds from cultivation of the endophytic fungi <u>Penicillium sp</u> of leaves of kunyit putih (<u>Curcuma</u> <u>zedoaria</u> have been isolated. The endophytic fungus was cultivated on 5 L of Potatos Dextrose Broth (PDB) medium at room temperature (no shaking) for 3 weeks. The cultures were extracted with ethyl acetate to afford 3.0 g of residue after removal of the solvent under reduced pressure. The extract was separated and purified by silica gel column chromatography (CC) and afforded two pure compounds as colorless oily liquid (compound 1) and yellow crystal (compound 2). The structure of these compounds were characterized by detailed UV, IR, and NMR spectroscopic analysis and compound 1 as well as comparison with the reported data. Base on spectra analysis the compound 1 was determined as Di-(2-ethylhexyl)phthalate and compound 2 as 5-(4'-ethoxy-2'-hydroxy-5'-methyl-2',3'-dihydrofuran-3'-il (hydroxy) methyl-4-isopropyl-3-methyl-2-pyran-2-on). Compound 1 is not new compound, but it is new for endophytic fungus from <u>C. zeodoria</u> and compound 2 is new compound.

Keywords: endophytic fungi; Penicillium sp; Curcuma zedoaria

ABSTRAK

Telah dilakukan isolasi dua senyawa dari kultifat jamur endofitik <u>Penicillium sp</u> dari daun kunyit putih (<u>Curcuma</u> <u>zedoaria</u>. Jamur endofit dikultur dalam 5 L medium Potatos Dextrose Broth (PDB) pada suhu kamar (keadaan statis) selama 3 minggu. Kultur kemudian diekstraksi dengan etil asetat dan dipekatkan dengan rotary evaporator sehingga didapatkan ekstrak pekat etil asetat 3,0 g. Ekstrak dipisahkan dan dimurnikan dengan kromatografi kolom menggunakan fasa diam silika gel dan didapatkan dua senyawa murni berupa cairan minyak bening (senyawa 1) dan kristal kuning (senyawa 2). Struktur senyawa hasil isolasi ditentukan berdasarkan analisis data spektroskopi UV, IR, dan NMR, dan senyawa 1 juga dikonfirmasi dengan membandingkan data yang telah dilaporkan. Berdasarkan analisis data spektroskopi disimpulkan senyawa 1 adalah Di-(2-ethylhexyl)phthalate dan senyawa 2 adalah 5-(4'etoksi-2'-hidroksi-5'-metil-2',3'-dihidrofuran-3'-il (hidroksi) metil-4-isopropil-3-metil-2-piran-2-on). Senyawa 1 bukan merupakan senyawa baru, tetapi untuk pertama kalinya ditemukan pada jamur endofitik pada <u>C. zeodoria</u> dan senyawa 2 merupakan senyawa baru.

Kata Kunci: jamur endofitik; Penicillium sp; Curcuma zedoaria

INTRODUCTION

Endophytic microorganisms that redise in the tissues of living plants and may produce secondary metabolites of biologically active [1]. Novel antibiotics, antimycotics, immunosuppressants, anticancer compound are only a few examples of what has been found after the isolation, culture and purification and characterization of some choice endophytes in the recent past. Isolation of their bioactive secondary metabolites of endophytic fungus from plant could be selected mainly something on ethonobotanical history [2].

Curcuma zedoaria, a medicinal tuber plant belonging to the family Zingiberaceae has been used in the traditional system of medicine [3]. These plants were used for curing stomach diseases, toothache, blood stagnation, leucoderma, tuberculosis, enlargement of spleen, and for promoting menstruation in traditional medicine in Asia [4]. Antiinflammatory activity [5], antiulcer activity [6], and antimicrobial effect [7], of this plant rhizome have been reported.

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In our research of endophytic fungus, many bioactive compounds and new compounds were isolated [8-9]. In this paper we reported the isolation and structural identification one known compound namely Di-(2-ethylhexyl)phthalate (1) and one new compound as 5-(4'-ethoxy-2'-hydroxy-5'-methyl-2',3'-dihydrofuran-3'-il (hydroxy) methyl-4-isopropyl-3-methyl-2-pyran-2-on) (2) of Penicillium sp from the leaves of C. zedoaria. Penicillium species isolated as endophytic usually be found in plants zingiberaceae [10] and meliaceae family, although in marine organisms, three meroterpenes preaustinoids, A, B, A1, A2, and B1 have been reported to be isolated from Penicillium sp associated with the Melia azedarach [11]. Penicillium commune from the semi-mangrove plant Hibiscus tiliaceus, have been isolated one new compound 1-O-(2,4-dihydroxy-6-

methylbenzoyl)-glycerol along with thirteen known products including 1-O-acetylglycerol, N-acetyl tryptophan, 3-indolylacetic acid methyl ester, 1-(2,4dihydroxy-3,5-dimethylphenyl)ethanone, 2-(2,5-dihydroxy phenyl)acetid acid, (4R,5S)-5-hydroxyhexan-4-olide, thymidine, uracil, thymine, ergosterol, β -sitosterol, β -daucosterol, and ergosta-7,22-dien-3 β ,5 α ,6 β -triol [12].

EXPERIMENTAL SECTION

Materials

The leaves of kunyit putih were collected on May 2013 from the Indralaya, Ogan Ilir, South Sumatra. Material for isolation endophytic fungi: ethanol 70%, NaOCI, chloramphenicol, potato dextrose broth (PDB), potato dextrose agar (PDA), silica gel 60 (70-230 mesh), thin layer chromatography (TLC) from Merck (Art.5554) silica gel 60 F₂₅₄, *n*-hexane, ethyl acetate, and methanol. The organic solvents were used from distilled technical grade.

Instrumentation

The apparatus in the research were counter colony, autoclave, incubator, water bath, microscope, magnetic hotplate, UV lamp, column chromatography and generally apparatus in organic and microbiology laboratory, melting point was determined using Fisher John Apparatus. UV spectra were determined with Varian Conc 100 instrument. IR spectra were determined on FTIR-Perkin Elmer-Spectrum One and NMR spectra were recorded at 500 MHz (¹H) and 125 MHz (¹³C) on JEOL JNM ECA-500 spectrometer, UV light at λ 254 nm and 365 nm.

Procedure

Isolation of endophytic fungus

The leaves sample was washed before it was processed and surface sterilized in 70% ethanol for 3 min and 0.5% NaOCl for 1 min and rinsed thoroughly with sterile distilled water. The segment sample placed on petri-plates containing potato dextrose agar medium (PDA) (200 g potato, 20 g dextrose, and 15 g agar in 1 L of H₂O, supplemented with 100 mg/L of chloramphenicol to suppress bacterial growth). The plates were incubated at 25 ± 2 °C until fungus growth appeared. The plant segments were observed once a day for the growth of endophytic fungus. Colony fungus showed difference characteristic furthermore to pure with the plated segments were immediately transferred into new PDA plates and then subcultured until pure cultures were obtained [13].

Identification of the endophyte

The endophytic fungal strain was identified by the morphological method. The morphological examination was performed by scrutinizing the fungal culture, the mechanism of spore production, and the characteristics of the spores. All experiments and observations were repeated at twice [14].

Cultivation of pure fungal strain

The purified fungus (a small park) was transferred under sterile conditions to the PDB medium. For chemical investigations, the fungal strains were static cultivated into 15 flasks (1 L each) containing 400 mL of PDB medium for 3 weeks at room temperature [12-14].

Extraction, isolation, purification, and structure elucidation

Fungus in the 3 weeks cultures were vacuumfiltered and the filtrate fractionated thrice by liquid-liquid partition with ethyl acetate (1:1). Then the solvent phase was evaporated under reduced pressure using rotary vacuum evaporator at 40 °C to produce the ethyl acetate fraction of liquid cultures. The EtOAc fraction (3.0 g) was preabsorbed on silica gel and then purification by column chromatography (silica gel, eluted *n*-hexane : EtOAc = 5:5 - 1:9), EtOAc 100%, EtOAc : MeOH = 9:1 - 1:9 and MeOH 100%). Based on detection by TLC using the eluent system, to give five fractions F1-F5. The 1st fraction to yield compound **1** (20 mg). Furthermore, fraction 2^{nd} (0.2 g) was rechromatographed using the same method (silica gel, eluted with EtOAc : MeOH (8:2 - 1:9) and MeOH (100%) to yield three fractions F2.1 - F2.3. Fraction F2.1 to yield pure compound 2 (10 mg). The molecular structure of compounds were established on the basis



Fig 1. Isolation of the compounds from ethyl acetate extract of Penicillium sp from the leaves of C. zedoaria

(<u>000 Mil 12</u>		-), opeotral data of compo-			
Carbon	<i>δ_H</i> ppm (ΣΗ, mι	δ_H ppm (Σ H, multiplicity, J in Hz)		δ _C (ppm)	
no.	1	1*	1	1*	
1	0.89 (3H, <i>t</i>)	0.84 (3H, <i>t</i> , 4.3)	14.1	14.1	
2	1.30 (2H, <i>m</i>)	1.23 - 1.44 (2H, <i>m</i>)	23.1	24.8	
3	1.29 (2H, <i>m</i>)	1,23 - 1.44 (2H, <i>m</i>)	29.0	22.7	
4	1.38 (2H, <i>m</i>)	1.23 - 1.44 (2H, m)	23.8	29.5	
5	1.67 (1H, <i>m</i>)	2.60 (1H, <i>m</i>)	38.8	40.8	
6	4.21 (2H, <i>m</i>)	4,15 (2H, <i>m</i>)	68.1	65.2	
7			167.8	171.1	
8	1.34 (2H, <i>m</i>)	2.30 (2H,dq, 4.3)	30.4	29.7	
9	0.91 (3H, <i>t</i>)	0.93 (3H, <i>t</i> , 4.3)	11.0	20.8	
10			132.5	124.8	
11	7.69 (1H, <i>dd</i> , 5.9 – 3.3)	6.96 (1H, dd, 6.3 – 2.2)	128.8	119.0	
12	7,51 (1H, dd, 5,9 – 3.3)	7.11(1H, dd, 6.3 – 2.2)	130.9	132.6	
1 [10]					

Tablel 1. ¹H-NMR (500 MHz) and ¹³C-NMR (125 MHz), spectral data of compound **1**. recorded in CD₃OD

1 [19]

of spectroscopic analysis including UV, IR, ¹H-NMR, ¹³C-NMR, DEPT, HMQC, HMBC, and COSY.

RESULT AND DISCUSSION

The fungus strain was identified as *Penicillium sp. Penicillium* species isolated as endophytes were obtained from several plant species such as, *Melia azedarach* [15-16]. Zingiberaceae family [14] meliaceae family, although in marine organisms as the semimangrove plant *Hibiscus tiliaceus* [17]. Fungus *Penicillium sp* after that cultivated on 5 L of PDB medium and then extracted with ethyl acetate to afford 3.0 g of residue. The extract (3.0 g) was separated by column chromatography to yield compound **1** (20 mg) and compound **2** (10 mg). The isolation of the compounds from ethyl acetate extract of *Penicillium* sp from the leaves of *C. zedoaria* described in Fig. 1.

Compound 1 was obtained as colorless oil liquid. The UV spectra of 1 exhibited absorption at λ_{max} nm : 206, 225, and 274. The bathochromic shift in addition of NaOH showed there is no wave length shift, it can concluded that there was no phenolic group. The IR spectrum showed the functional group such as ester (1722 carbonyl cm⁻¹), C=C aromatic (1598-1462 cm⁻¹), C-O (1273 cm⁻¹), C-H aromatic (3070 cm⁻¹), and C-H aliphatic (2927–2860 cm⁻¹). The H-NMR data (Table 1) disclosed the presence of two protons as AB spin system at δ_H 7.69 (1H, dd, 5.9 & 3.3 Hz) and 7.51 (1H, dd, 5.9 & 3.3 Hz) that characteristic for aromatic proton at ortho substituted ring. The proton signal at δ_{H} 4.21 ppm (2H, m) is assigned to a methylene group geminal to the ester

Carbon no.	δ _C ppm	DEPT	δ_H ppm (Σ H, multiplicity, J in Hz)	HMBC	COSY
2	183.4	С			
3	123.1	С			
4	139.1	С			
5	107.4	С			
6	162.8	СН	8.23 (1H, <i>s</i>)	81.7, 107.4, 139.1	
7	34.6	СН	2.98 (1H, g, J = 7.15 Hz)	107.4, 123.1, 139.1	1.22
8	18.5	CH_3	1.22 (3H, $d, J = 7.15$ Hz)	139.1, 34.6, 81.7	
9	18.2	CH_3	1.34 (3H, d, J = 7.15 Hz)	34.6	
10	9.5	CH ₃	2.01 (3H, s)	123.1, 139.1, 183.8	
2'	174.5	C		, ,	
3'	100.3	С			
4'	171.2	С			
5'	177.2	С			
6'	60.4	CH_2	4.11 (2H, <i>q</i> , <i>J</i> = 7.15 Hz)	171.2	1.25
7'	14.2	CH_3	1.25 (3H, <i>t</i>)	60.4	
8'	21.1	CH ₃	2.04 (3H, s)	171.2	
9'	81.7	CH	4.77 (1H, a , $J = 7.15$ Hz)	139.1. 162.8	1.34

Table 2. ¹H-NMR (500 MHz) and ¹³C-NMR (125 MHz), spectral data of compound 2 recorded in CDCI3



alcohol group. Furthermore, the presence proton signal at $\delta_{\rm H}$ 1.67 ppm (1H, *m*) for proton methine, signal at 1.2–1.4 ppm for four methilene group, and signal at 0.89 and 0.91 as pair of multiplate (3H, *m*) for two methyl groups.

The ¹³C-NMR spectrum of compound **1** (Table 1), confirming the symmetry of the molecule, exhibited the expected 12 carbon resonance. DEPT spectrum showed to two quaterner, three methane, five methylene carbons, and two methyl groups. These spectroscopic data, by comparison of ¹H and ¹³C-NMR data to those

published in literature [19] and showed similarity, in conclusion compound **1** was identified as Di-(2-ethylhexyl)phthalate (DEHP). DEHP (compound **1**) is a well known synthetic plasticizes, so already reported to be present in *Calotropis gigantean* [15], *Alchornea cordifolia* [16], and *Aloe vera* [17]. The effective presence of compound **1** in endophytic fungus *Penicillium* sp of leave *C. zedoaria*, not as a contaminant from solvents and endophytic fungus *Penicillium* sp not cultivated in plastic bags, so these could be discounted as a source of DEHP.



Fig 4. HMQC correlation of proton at δ_H 1.23–8.23 ppm (A) and HMBC correlation of proton at δ_H 1.23–2.98 ppm (B) compound 2

Compound **2** was obtained as a yellow crystal, mp. 171-172 °C. The Spectra UV (MeOH) of **2** exhibited absorption at λ_{max} nm: 213, 253, and 321. The bathochromic shift in addition of NaOH exhibited absorption at λ_{max} nm: 213, 253, and 321. Base on Spectroscopic data UV indicate this compound was no

phenolic group. The IR spectra (KBr) showed v_{max} cm⁻¹: 3466.08 (OH), 2980.02 and 2935.66 (C-H aliphatic), 1625.99 (conjugated C=O), 1579.70; 1521.12; 1438.90 (C=C conjugation), and 1180.44 (C-O ether). ¹H-NMR (DMSO, 500 MHz) δ_{H} ppm and ¹³C-NMR (DMSO, 125 MHz) δ_{C} ppm (see Table 2).



Fig 5. HMBC correlation of proton at 4.01–8.23 ppm and COSY correlation compound 2



Fig 6. HMBC (A), and COSY (B) correlations and δ-assignment of compound 2

The ¹H-NMR spectrum, (Fig. 2) showed signal two methyl doublet at δ_{H} 1.22 and 1.34 ppm (3H, *d*, 7.15 Hz) and signal methine quartet at δ_{H} 2.98 ppm (1H, *q*, 7.15 Hz) and one methine singlet at δ_{H} 8.23 (1H, *s*). At spectrum also showed signal for methyl triplet at δ_{H} 1.25 ppm (3H, *t*, 7.15 Hz), and two methyl singlet at δ_{H} 2.01 and 2.04 ppm, (3H, *s*) and one signal methylene quartet at δ_{H} 4.11 ppm (2H, *q*, 7.15 Hz).

The ¹³C-NMR (Fig. 3), DEPT 135 spectrum, and HMQC spectrum (Fig. 4) showed 17 signal consist that nine signal as C sp² and 8 signal as C sp³. Analysis spectrum DEPT 135 showed 8 signal C quarternary at δ_C 100.3; 107.4; 123.1; 139.1; 171.2; 174.5; 177.2 and 183.8 ppm, 5 signal methyls carbon at δ_C 9.5; 14.2; 18.2; 18.5 and 21.1 ppm, 3 signal methines carbon at δ_C 34.6; 81.7 and 162.8 ppm, and one signal

methylene carbon at δ_c 60.4 ppm. Signal carbon at δ_c 183.4 ppm indicated these compound have C=O carbonyl.

NMR 2D analysis for HMQC spectrum (Fig. 4) showed the proton at δ_{H} 1.34 ppm correlation to carbon at δ_H 18.2 ppm and proton at δ_H 1.22 ppm correlation to carbon at δ_c 18.5. HMBC spectrum showed correlation from proton at δ_H 1.22 and 1.34 ppm to carbon at δ_{C} 34.6 and 139.1 ppm. Proton at δ_{H} 1.22 also correlation to carbon at δ_c 18.2 ppm and proton at δ_H 1.34 ppm showed correlation with carbon at δ_{C} 18.5. This data to indicated that proton δ_{H} 1.22 and 1.34 ppm bound to carbon fasten carbon δ_C 34.6 ppm. Proton at 1.25 ppm correlation to carbon at δ_c 60.4 ppm. Further HMBC spectrum showed correlation proton at δ_{H} 2.01 (3H, s) to carbon at δ_c 123.1; 139.1 and 183.8 ppm, and correlation proton at δ_H 2.04 ppm to carbon at δ_{C} 171.2 ppm. Proton at δ_{H} 2.01 and 2.04 (3H, s) at HMQC spectrum showed fastened with carbon at δ_c 9.5 and 21.1 ppm.

Proton at $\overline{\delta}_{H}$ 4.11 correlation to carbon at δ_{C} 171.2, proton at $\overline{\delta}_{H}$ 4.77 ppm showed correlation to carbon at $\overline{\delta}_{C}$ 139.1; 162.8 ppm, while proton at $\overline{\delta}_{H}$ 8.23 ppm to correlation to carbon at $\overline{\delta}_{C}$ 81.7; 107.4 and 139.1 ppm. Analysis of ¹H–¹H COSY spectrum (Fig. 5) also to indication of two proton spin system corresponding at $\overline{\delta}_{H}$ 1.22 with proton at $\overline{\delta}_{H}$ 2.98 ppm. And proton at $\overline{\delta}_{H}$ 1.25 to correlation to proton at 4.11 ppm. HMBC and COSY correlation and $\overline{\delta}$ -assignment of compound showed Fig. 6. These spectroscopic data, therefore suggested that compound **2** is 5-(4'-ethoxy-2'-hydroxy-5'-methyl-2',3'dihydrofuran-3'-il (hydroxy) methyl-4-isopropyl-3-methyl-2-pyran-2-on).

Compound **1** is not new compound, but it is new for endophytic fungus from *C. zedoaria* and base on Dictionary Natural Products data base, 5-(4'-ethoxy-2'hydroxy-5'-methyl-2',3'-dihydrofuran-3'-il (hydroxy) methyl-4-isopropyl-3-methyl-2-pyran-2-on) (**2**) is new compound. Exploration of secondary metabolites research needs to be done in order to get the profile of organic compounds produced by endophytic fungus of *C. zedoaria*.

CONCLUSION

Two compounds have been isolated from the endophytic fungus *Penicillium* sp from the leaves of kunyit putih (*C. zedoaria*). Based on spectroscopic analysis and comparison data to those published in literature compound **1** was identified as Di-(2-ethylhexyl)phthalate and compound **2** as 5-(4'-ethoxy-2'-hydroxy-5'-methyl-2',3'-dihydrofuran-3'-il (hydroxy) methyl-4-isopropyl-3-methyl-2-pyran-2-on).

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