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 Penicillium sp of leaves of kunyit putih (Curcuma zedoaria 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 C. zeodoria and compound **2** is new compound.*

Keywords: *endophytic fungi; Penicillium sp; Curcuma zedoaria*

ABSTRAK

*Telah dilakukan isolasi dua senyawa dari kultifat jamur endofitik Penicillium sp dari daun kunyit putih (Curcuma zedoaria. 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 C. zeodoria 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-(2,4-dihydroxy-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-3β,5α,6β-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%, NaOCl, 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 F_{254}, *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 (^{1}H) and 125 MHz (^{13}C) 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 H_2O, 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 vacuum-filtered 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 2nd (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

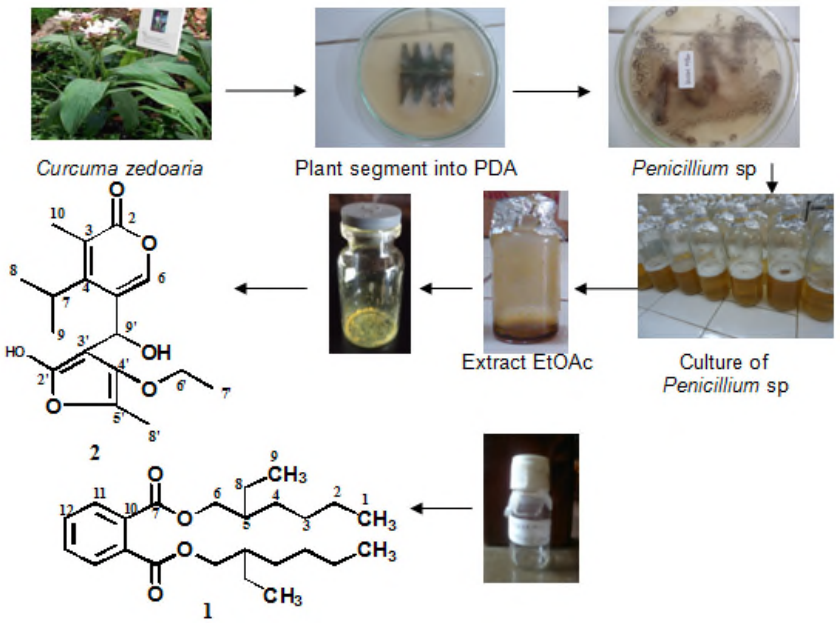

Fig 1. Isolation of the compounds from ethyl acetate extract of *Penicillium* sp from the leaves of *C. zedoaria*

Tablel 1. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz), spectral data of compound **1**. recorded in CD_3OD

Carbon no.	δ_H ppm (ΣH, multiplicity, *J* in Hz)		δ_C (ppm)	
	1	**1***	**1**	**1***
1	0.89 (3H, *t*)	0.84 (3H, *t*, 4.3)	14.1	14.1
2	1.30 (2H, *m*)	1.23 - 1.44 (2H, *m*)	23.1	24.8
3	1.29 (2H, *m*)	1,23 - 1.44 (2H, *m*)	29.0	22.7
4	1.38 (2H, *m*)	1.23 - 1.44 (2H, m)	23.8	29.5
5	1.67 (1H, *m*)	2.60 (1H, *m*)	38.8	40.8
6	4.21 (2H, *m*)	4,15 (2H, *m*)	68.1	65.2
7			167.8	171.1
8	1.34 (2H, *m*)	2.30 (2H,*dq*, 4.3)	30.4	29.7
9	0.91 (3H, *t*)	0.93 (3H, *t*, 4.3)	11.0	20.8
10			132.5	124.8
11	7.69 (1H, *dd*, 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* [19]

of spectroscopic analysis including UV, IR, ^{1}H-NMR, ^{13}C-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 semi-mangrove 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 carbonyl ester (1722 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 ^{1}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

Table 2. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz), spectral data of compound **2** recorded in CDCl3

Carbon no.	δ_C ppm	DEPT	δ_H ppm (ΣH, multiplicity, *J* in Hz)	HMBC	COSY
2	183.4	C			
3	123.1	C			
4	139.1	C			
5	107.4	C			
6	162.8	CH	8.23 (1H, *s*)	81.7, 107.4, 139.1	
7	34.6	CH	2.98 (1H, *q*, *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	C			
4'	171.2	C			
5'	177.2	C			
6'	60.4	CH_2	4.11 (2H, *q*, *J* = 7.15 Hz)	171.2	1.25
7'	14.2	CH_3	1.25 (3H, *t*)	60.4	
8'	21.1	CH_3	2.04 (3H, *s*)	171.2	
9'	81.7	CH	4.77 (1H, *q*, *J* = 7.15 Hz)	139.1, 162.8	1.34

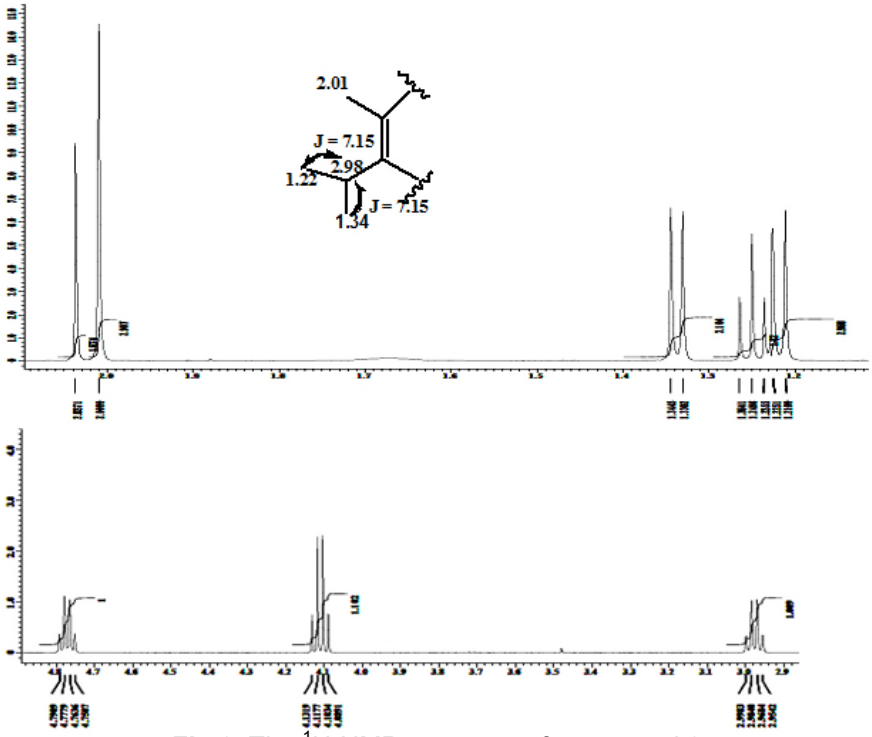

Fig 2. The ^{1}H-NMR spectrum of compound **2**

alcohol group. Furthermore, the presence proton signal at δ_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 ^{13}C-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 ^{1}H and ^{13}C-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.

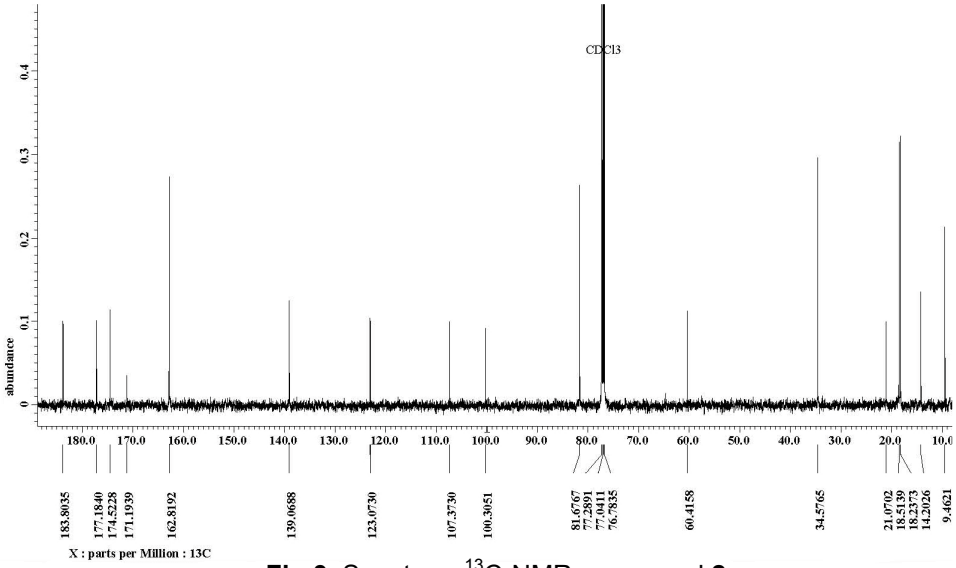

Fig 3. Spectrum ^{13}C-NMR compound **2**

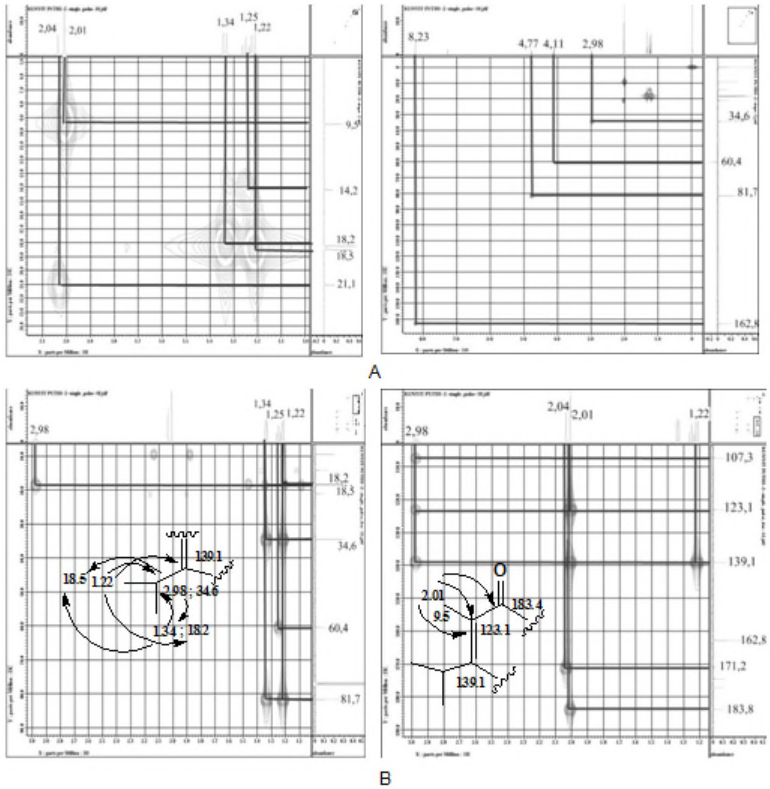

Fig 4. HMQC correlation of proton at δ_H 1.23–8.23 ppm (A) and HMBC correlation of proton at δ_H 1.23–2.98 ppm (B) compound **2**

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 ν_{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). ^{1}H-NMR (DMSO, 500 MHz) δ_H ppm and ^{13}C-NMR (DMSO, 125 MHz) δ_C ppm (see Table 2).

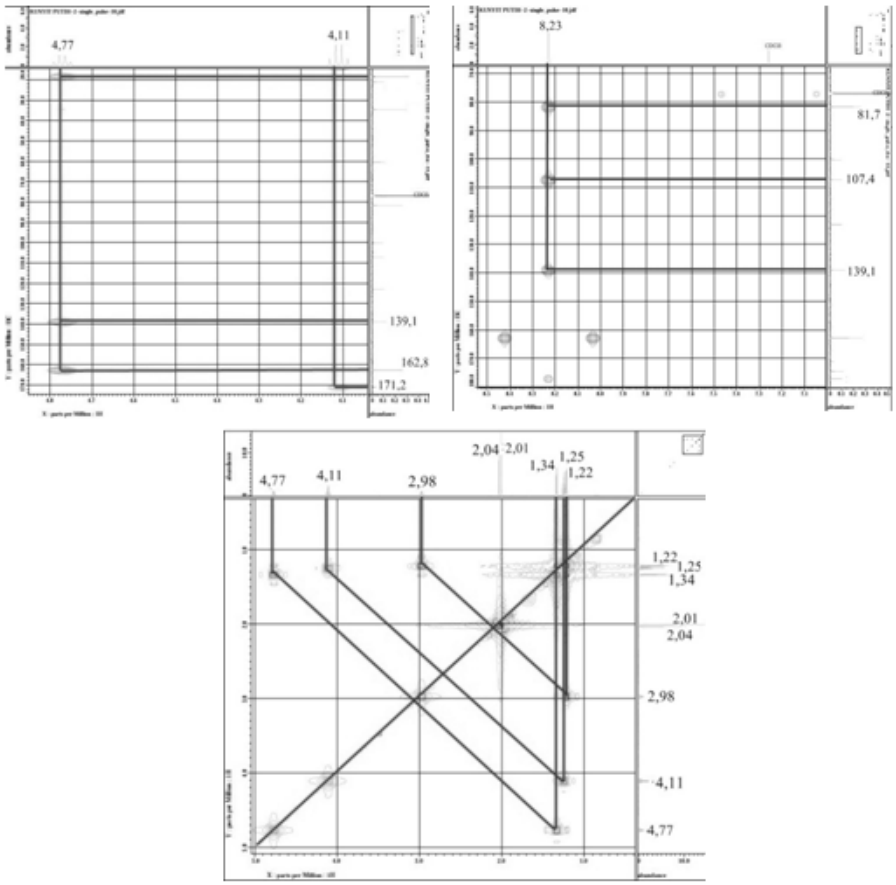

Fig 5. HMBC correlation of proton at 4.01–8.23 ppm and COSY correlation compound **2**

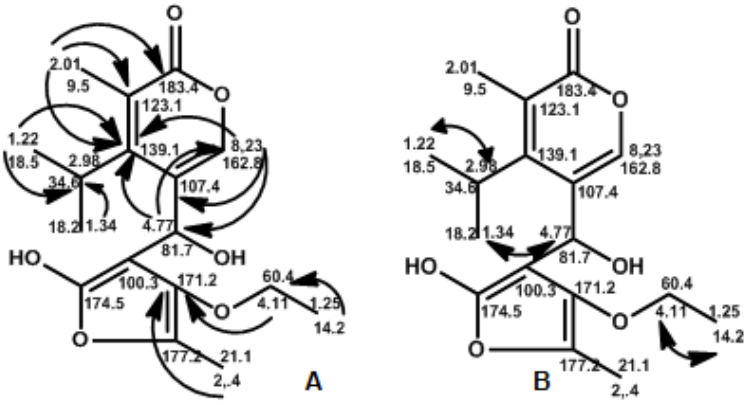

Fig 6. HMBC (**A)**, and COSY (**B)** correlations and δ-assignment of compound **2**

The ^{1}H-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 ^{13}C-NMR (Fig. 3), DEPT 135 spectrum, and HMQC spectrum (Fig. 4) showed 17 signal consist that nine signal as C sp^2 and 8 signal as C sp^3. 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 δ_H 4.11 correlation to carbon at δ_C 171.2, proton at δ_H 4.77 ppm showed correlation to carbon at δ_C 139.1; 162.8 ppm, while proton at δ_H 8.23 ppm to correlation to carbon at δ_C 81.7; 107.4 and 139.1 ppm. Analysis of ^{1}H–^{1}H COSY spectrum (Fig. 5) also to indication of two proton spin system corresponding at δ_H 1.22 with proton at δ_H 2.98 ppm. And proton at δ_H 1.25 to correlation to proton at 4.11 ppm. HMBC and COSY correlation and δ-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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