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Four Azadirone-Type Limonoids from *Chisocheton Pentandrus* Stem Bark and Their Cytotoxic Acitivity Against Mcf-7 Breast Cancer Cell Lines

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ABSTRACT. Limonoid group belongs to triterpenoid that has undergone further oxidation accompanied by the loss of four carbon atoms to form a furan ring in the chain. This limonoid compound is often found in the *Chisocheton* genus. Limonoid compounds have been known as compounds with high structural variations and this makes limonoids have diverse and interesting activities, including cytotoxic and anticancer. In the course of our continuing study for limonoid constituents that have cytotoxic activity against cancer cell lines, methanol extract of stem bark from the *Chisocheton pentandrus* plant provides significant activity. The methanol extract was separated using various chromatographic techniques in the normal and reverse stationary phase to produce four azadirone-type limonoid compounds (**1-4**). The elucidation structure of **1-4** was determined using spectroscopic methods including, UV-Visible, IR, and 1D-NMR as well as optical rotation. All four known compounds were established as trichilenone acetate (**1**), toonaciliaton C (**2**), 11α-acetoxiazadiron (**3**), and 16β-hydroxydisobinine (**4**). The cytotoxicity of compounds **1-4** was assessed by examination using the resazurin method, which showed that compound **4** was the promising constituent against the MCF-7 cells with an IC_{50} value of 43.1 μM and was stronger than its positive control.

Keywords: Azadirone-type limonoid, *Chisocheton pentandrus,* cytotoxic activity, Meliaceae.

INTRODUCTION

Limonoid is a derivative of tetracyclic triterpene with a furan ring side in the chain through the degradation of four carbons, as a tetra *nor*-triterperterpenoid (Chong *et al.,* 2012). Limonoids are also popularly known as a highly oxidized tetranortriterpenoid compound and the first type of limonoid was discovered from citrus fruit extract which in 1960 was amed limonin (Naini *et al.*, 2024). Limonoids are a group of compounds with high diversity and are interesting based on the modification of the four rings (Supriatno *et al.*, 2018). Limonoid compounds are found in many plants of the order Rutales (families Meliaceae, Rutaceae, and Simarubaceae), especially in the Meliaceae family (Roy & Saraf, 2006).

The *Chisocheton* genus has 53 species discovered subtropical and in tropical areas, especially in the southern part of the Asian continent such as India, China, Thailand, Papua New Guinea, Malaysia, the Philippines, and Indonesia (Yang *et al.,* 2009). Limonoid compounds are the main compounds of the genus *Chisocheton* along with protolimonoids. The parts of plants that are often investigated for phytochemical constituents are leaves, twigs, bark, wood, root wood, seeds and fruit, while the parts where limonoids are mostly found are the bark and seeds, and few are found in the fruit (Shilpi *et al.*, 2016). Secondary metabolite compounds in the genus *Chisocheton* have been widely known to have interesting activities including antimalarial, insecticide, anti-food, antibacterial (Maneerat *et al.,* 2008; Mohamad *et al.,* 2009; Phongmaykin *et al.,* 2008), anti-inflammatory (Yang *et al.*, 2009), and cytotoxic activity (Wong *et al.*, 2011; Awang *et al.*, 2007; Nurlelasari *et al.*, 2017).

Chisocheton pentandrus is one species of the genus *Chisocheton* that has not been explored yet and can be found in Indonesia, specifically in West Kalimantan (Shilpi *et al.*, 2016). Recent phytochemical investigations of this species carried out by our group

yielded new compounds with interesting cytotoxicity including triterpenoids and limonoids (Supriatno *et al.*, 2018; Supratman *et al.*, 2020; Salam *et al.*, 2021; Harneti *et al.*, 2023; Runadi *et al.*, 2023). These previous findings inspired us to further explore the structure of limonoids from the stem bark of *C. pentandrus* as well as their potential activity in cytotoxic against MCF-7 breast cancer cell lines. We herein describe the isolation and determination structure of four azadirone-type limonoids alongside their cytotoxic activity against MCF-7 cells. The isolation process was performed using various chromatography techniques and characterized using extensive spectroscopic data (optical rotation, UV, IR, MS, and NMR-1D). The elucidation structure in more depth explanation of these four known azadiron-types (**1-4**) of limonoid compound was first presented in this work. In addition, the cytotoxic assay was carried out by using resazurin assay.

EXPERIMENTAL SECTION

General Experimental Procedures

An Everest ATR Thermo scientific FTIR spectrometer was used to measure the IR spectra. JEOL JNM-ECZ 500R/S1 (Tokyo, Japan) and TMS as an internal standard were used to record the NMR spectra at 500 MHz for ^{1}H and 125 MHz for ^{13}C, while mass spectra were obtained by Waters QTOF-HRTOFMS-XEVotm mass spectrometer (Waters, Milford, MA, USA). The UV spectra were performed with a PerkinElmer Lambda 35 UV/VIS double-beam spectrophotometer. The isolation process was carried out by using column chromatography (CC) on silica gel 60 (70-230 mesh and 230-400 mesh, Merck, Darmstadt, Germany) and octadecyl silane (Fuji Sylisia Chemical LTD., Chromatorex® C18 DM1020 M, 200–400 mesh, Tokyo, Japan). The spot detection utilized a thin-layer chromatography (TLC) on precoated silica gel 60 F_{254} (Merck, Darmstadt, Germany) and RP-18 F_{254s} plates (Merck, Darmstadt, Germany) and was observed under UV light at 254 and 265 nm before spraying with 10% H_2SO_4 in ethanol, then heating.

Plant Collection

In June 2016, the Bogor Botanical Garden in Bogor, West Java Province, Indonesia, provided the stem bark of *C. pentandrus*. Mr. Ismail identified a voucher specimen (B0–104), which was then placed in the Bogor Botanical Garden herbarium (Runadi *et al.*, 2023).

Extraction and Isolation

The dried stem bark of *C. pentandrus* (1.8 kg) was powdered and extracted with EtOH (4 x 4 L, 4 days each). The EtOH extract was evaporated under reduced pressure, yielded a 340.01 g crude extract. A partition process was then carried out by adding water 1 : 1, followed by *n*-hexane, EtOAc, and *n*-BuOH, successively. Limonoids were mainly found in the *n*-hexane and EtOAc. Afterwards, these two extracts was subjected to vacuum liquid chromatography (VLC) on silica gel and *n*-hexane: EtOAc: MeOH (10 % stepwise) was used as eluent to obtain eight fractions (A-H).

Fraction E (819.8 mg) was subjected to colum chromatography (CC) on silica gel (70-230 mesh), with CH_2Cl_2: EtOAc (5%, stepwise) to yield five subfractions (E1-E5). Subfraction E4 (215.1 mg) was then subjected to silica gel CC (230-400 mesh) with CH_2Cl_2: EtOAc (2.5%, stepwise) to yield four subfractions (E4a-E4d). Furthermore, subfraction E4c (51.2 mg) was purified on silica gel CC (230-400 mesh) with CH_2Cl_2: EtOAc: MeOH (5: 4: 1) as an eluent to yield compound **1** (5.3 mg). Fraction G (780 mg) was separated on silica gel CC (230-400 mesh) with eluent system CH_2Cl_2: EtOAc (5%, stepwise) to produce seven subfractions G1-G7. Subfraction G3 (310 mg) was chromatographed on silica gel (230-400 mesh) and eluted using CH_2Cl_2: EtOAc: MeOH (8.5: 0.5: 1.5) to obtain four subfractions (G3a-G3d). Subfraction G3d (44 mg) was then purified using CC normal phase (CH_2Cl_2: EtOAc: MeOH, 5: 4.5: 0.5) to produce compound **2** (5.1 mg). Meanwhile, compound **3** (4.5 mg) was afforded by separating subfraction G4 (244 mg) through CC normal phase (CH_2Cl_2: EtOAc: MeOH, 8.5: 0.5: 1).

EtOAc extract (28.1 g) was separated using VLC with eluent system *n*-hexane: EtOAc: MeOH (10% stepwise) to yield six fractions 1-6. Fraction 3 (17.6 g) was separated using CC on silica gel (230-400 mesh) (CH_2Cl_2: EtOAc, stepwise 5%) to obtain five subfractions 3a-3e. Subfraction 3d (1.1 g) was subjected on silica gel (230-400 mesh) using CH_2Cl_2: EtOAc (7.5: 2.5) to produce five subfractions 3d1-3d5. Compound **4** (11.5 mg) was obtained by isolating subfraction 3d5 (59.3 mg) using CC normal phase (CH_2Cl_2: EtOAc: MeOH, 6: 2: 2).

Compound **1** was isolated as a white amorphous. $[\alpha]^{25}_{D}$ +38.5 (*c* 0.15, MeOH). $C_{28}H_{36}O_5$. IR (KBR) ν_{max} 3540, 3450, 2862, 1720, 1690, 1457, 1387, 1247 cm^{-1}. HR-TOFMS at *m/z* 453.2563 $[M+H]^+$ (calculated for $C_{28}H_{37}O_5$ *m/z* 453.2560). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ_H 7.13 (1H, d, *J*=10.0 Hz, H-1), 5.85 (1H, d, *J* =10.0 Hz, H-2), 2.50 (1H, d, *J* =13.0 Hz, H-5), 1.79 (2H, m, H-6), 5.02 (1H, d, *J* = 3.0 Hz, H-7), 1.89 (1H, m, H-9), 4.87 (2H, m, H-11), 1.57 (1H, m, H-12a), 1.79 (1H, m, H-12b), 3.28 (1H, dd, *J* =1.8, 3.6 Hz, H-15), 2.58 (1H, m, H-16a), 2.12 (1H, d, *J*=5.9, H-16b), 2.62 (1H, m, H-17), 0.93 (3H, s, CH_3-18), 1.14 (3H, s, CH_3-19), 7.08 (1H, s, H-21), 6.13 (1H, s, H-22), 7.34 (1H, s, H-23), 1.15 (3H, s, CH_3-28), 1.21 (3H, s, CH_3-29), 1.23 (3H, s, CH_3-30), 2.06 (3H, s, CH_3-1'). ^{13}C-NMR data (Table 1).

Compound **2** was isolated as a white solid. $[\alpha]^{25}_{D}$ +10.2 (*c* 0.11, MeOH). $C_{30}H_{38}O_8$. HR-TOFMS at *m/z* 527.2567 $[M+H]^+$ (calculated for $C_{30}H_{39}O_8$, *m/z* 527.2580). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ_H 7.13 (1H, d, *J*=10.0 Hz, H-1), 5.90 (1H, d, *J*=10.0 Hz, H-2), 2.50 (1H, d, *J*=13.0 Hz, H-5), 5.29 (2H, m, H-6), 5.00 (1H, d, *J*=3.0 Hz, H-7), 2.90 (1H, m, H-9), 4.49

(2H, m, H-11), 2.39 (2H, m, H-12), 3.40 (1H, dd, *J*=1.8, 3.6 Hz, H-15), 2.09 (1H, dd, *J*=10.0; 5.4 Hz, H-16a), 1.60 (1H, dd, *J*=10.0; *J*=5.4 Hz, H-16b), 2.61 (1H, dd, *J*=1.8; 3.6, H-17), 1.03 (3H, s, CH_3-18), 1.24 (3H, s, CH_3-19), 7.16 (1H, s, H-21), 6.19 (1H, s, H-22), 7.33 (1H, s, H-23), 1.28 (3H, s, CH_3-28), 1.22 (3H, s, CH_3-29), 1.32 (3H, s, CH_3-30), 2.02 (3H, s, CH_3-1'), 2.02 (3H, s, CH_3-1''). ^{13}-NMR data (**Table 1**).

Compound **3** was isolated as a white solid. $[\alpha]^{25}_D$ -18.5 (*c* 0.2, MeOH). $C_{30}H_{38}O_6$. HR-TOFMS at *m/z* 495.2667 $[M+H]^+$ (calculated $C_{30}H_{39}O_6$ *m/z* 495.2668). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ_H 7.23 (1H, d, *J*=10.6 Hz, H-1), 5.85 (1H, d, *J*=10.6 Hz, H-2), 2.50 (1H, d, *J*=15.0 Hz, H-5), 1.72 (2H, m, H-6), 5.02 (1H, d, *J*=3.0 Hz, H-7), 1.26 (1H, m, H-9), 4.35 (2H, m, H-11), 1.57 (1H, m, H-12a), 1.79 (1H, m, H-12b), 5.28 (1H, dd, *J*=1.8, 3.6 Hz, H-15), 2.68 (1H, m, H-16a), 2.12 (1H, d, *J*=5.4 Hz, H-16b), 2.62 (1H, m, H-17), 1.34 (3H, s, H-18), 1.23 (3H, s, CH_3-19), 7.08 (1H, s, H-21), 6.13 (1H, s, H-22), 7.34 (1H, s, CH_3-23), 1.15 (3H, s, CH_3-28), 1.21 (3H, s, CH_3-29), 1.23 (3H, s, CH_3-30), 1.94 (3H, s, CH_3-1'), 1.91 (3H, s, CH_3-1''). ^{13}C-NMR data (Table 2).

Compound **4** was isolated as a colorless crystal. $[\alpha]^{25}_D$ +131.5 (*c* 0.2, MeOH). $C_{30}H_{38}O_7$. HR-TOFMS at *m/z* 511.2634 $[M+H]^+$ (calculated for $C_{30}H_{38}O_7$, *m/z* 511.2696). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ_H 8.32 (1H, d, *J*=10.5 Hz, H-1), 5.88 (1H, d, *J*=10.3 Hz, H-2), 2.60 (1H, d, *J*=12.5, H-5), 5.46 (1H, m, H-6), 5.41 (1H, d, *J*=2.7 Hz, H-7), 2.12 (1H, dd, *J*=6.5; 14.5 Hz, H-9), 1.86 (1H, d, *J*=14.5 Hz, H-11a), 2.51 (1H, m, H-11b), 2.37 (2H, m, H-12), 5.48 (1H, d, *J*=7.7 Hz, H-15), 4.49 (1H, t, *J*=7.7 Hz, H-16), 2.82 (1H, dd, *J*=7.7, 11.0 Hz, H-17), 1.33 (3H, s, CH_3-18), 1.28 (3H, s, CH_3-19), 7.28 (1H, s, H-21), 6.30 (1H, d, *J*=1.45, H-22), 7.40 (1H, d, *J*=1.45 Hz, H-23), 0.95 (3H, s, CH_3-28), 1.20 (3H, s, CH_3-29), 1.33 (3H, s, CH_3-30), 2.04 (3H, s, CH_3-1'), 2.07 (3H, s, CH_3-1''). ^{13}C-NMR data (**Table 2**).

Determination of Cytotoxic Assay

The compounds **1-4** were tested for their cytotoxic activity against breast cancer MCF-7 cells using PrestoBlue® reagent depending on monitoring the viability of cells in the presence of a resazurin base. The cells were grown in RPMI-1640 (+10% fetal bovine serum and antibiotics) with 5% CO_2 at 37 °C. Furthermore, both were seeded into 96-well plates with an initial cell density of roughly 3 $\times 10^4$ cells cm^{-3} and left for a 24-hour incubation period. 500, 250, 125, 62.5, 31.25, 15.63, 7.81, and 3.91 µg/mL were the necessary quantities of the compounds that were dissolved in 2% aqueous DMSO. Cisplatin was utilized as a positive control, and the test substance was applied to the cells in triplicate for 48 hours. Following a 48-hour incubation period, add 10 µL of PretoBlue™ cell viability reagent and continue to incubate for an additional one to two hours, or until the color changes. Ultimately, a microplate reader was employed to apply the measured absorbance at 570 nm.

RESULTS AND DISCUSSION

The methanol extract of *C. pentandurs* stem bark was dissolved in water and partitioned successively with *n*-hexane, ethyl acetate, and *n*-butanol. The *n*-hexane extract afforded from the partition process was then separated using chromatographic techniques on a silica gel stationary phase and limonoid compounds **1-4** were produced (**Figure 1**).

Compound **1** was isolated as a white amorphous (MeOH), with the molecular formula $C_{28}H_{36}O_5$, based on the HR-TOFMS spectrum at *m/z* 453.2563 $[M+H]^+$ (calculated $C_{28}H_{37}O_5$ *m/z* 453.2560) and 11 degrees of unsaturation were required. The UV spectrum showed a maximum peak λ_{max} (log ε) at 230 (2.15) nm, which indicated the characteristic *R* band with an $n \rightarrow \pi^*$ transition, suggesting the presence of an *α,β*-unsaturated carbonyl group in the A ring (Tan & Luo, 2011). This assignment was supported by the infrared spectrum with the absorption of conjugated carbonyl groups (ν_{max} 1690 cm^{-1}) and isolated carbonyl (ν_{max} 1720 cm^{-1}) (Tan & Luo, 2011), as well as absorption bands from hydroxyl (ν_{max} 3540 and 3450 cm^{-1}), *gem*-dimethyl (ν_{max} 1457 and 1387 cm^{-1}), and ether (ν_{max} 1247 cm^{-1}) groups. The ^{1}H-NMR spectrum of compound **1** showed the presence of five tertiary methyls at δ_H 1.23 (3H, s, CH_3-30), 1.21 (3H, s, CH_3-29), 1.15 (3H, s, CH_3-28), 1.14 (3H, s, CH_3-19), and 0.93 (3H, s, CH_3-18), three sp^2 methines derived from furan group at δ_H 7.10 (1H, s, H-21), 7.35 (1H, s,

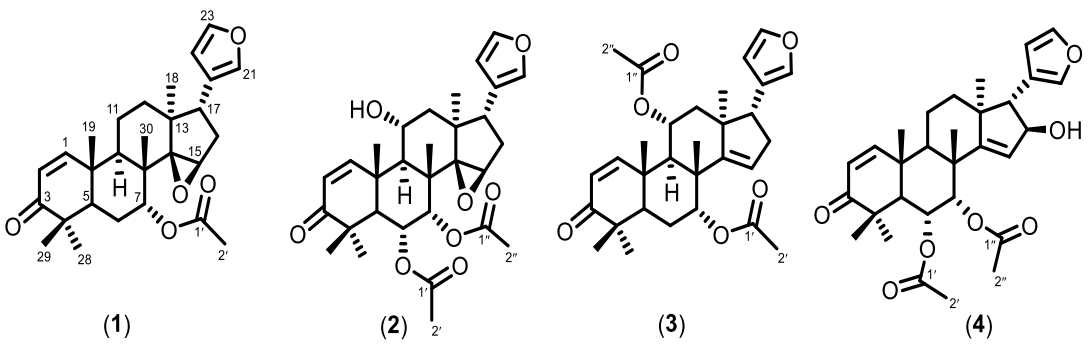

Figure 1. Structures of compound **1-4** (Mulholland *et al.*, 1994; Jiang *et al.*, 2012; Halsall & Troke, 1975; Nurlelasari *et al.*, 2021).

H-22), and 6.16 (1H, s, H-23), together with two additional *sp²* methines at δ_H 5.85 (1H, d, *J*=10.0 Hz, H-2) and 7.13 (1H, d, *J* =10.0 Hz, H-1) confirming a pair of double bond as part of an *α,β*-unsaturated carbonyl moeity. Based on the ^{1}H-NMR data (Table 1), compound **1** showed the existence of an azadirone skeleton (Tan & Luo, 2011). Furthermore, the ^{13}C-NMR with the aid of DEPT 135º showed the presence of 28 carbons, including one carbonyl at δ_C 204.3 (C-3), one carbonyl esther at δ_C 170.4 (C-1'), five tertiary methyls at δ_C 21.9 (C-18), 18.6 (C-19), 18,9 (C-28), 20.3 (C-29), and 21.7 (C-30), five *sp²* methines at δ_C 157.7 (C-1), 126.8 (C-2), 139.6 (C-21), 111.0 (C-22), and 143.1 (C-23), and one quaternary *sp²* carbon at δ_C 123.7 (C-20). These data was accounted for five degrees of unsaturation, while the six remaining of degrees was fulfilled by tetracyclic limonoid (one furan ring) and one additional ring system. The above assignment confirmed that **1** shares an azadirone-type limonoid with the presence of one ring outside of the limonoid intact system. The formation of epoxy at C-14/C-15 as an additional ring was convinced by the signals of one oxygenated quaternary carbon at δ_C 72.9 (C-14) and one oxygenated methine at δ_C 58.9 (C-15)/δ_H 3.39 (1H, s, H-15), together with mass spectrum information. Moreover, the attachment of an *O*-acetyl was pointed by the presence of one oxyenated methine at δ_C 73.7 (C-7)/δ_H 5.02 (1H, d, 13.0 Hz, H-7) and acetyl moeity at [δ_C 170.4 (C-1'), δ_C 21.2 (C-2')/ δ_H 2.06 (3H, s, CH_3-1')]. The 1D-NMR data of **1** was then compared to those of trichilenone acetate (Mulholland *et al.,* 1994), an azadirone-type limonoid with an additional *O*-acetyl at C-7 and epoxy ring at C-14/C-15. The comparison of these two analogs showed high similarity (**Table 1**) and confirmed the structure of **1**. Therefore, compound **1** was identified as trichilenone acetate, isolated from the stem bark of *C. pentandrus.*

Compound **2** was isolated as a white solid (MeOH), with molecular formula of $C_{30}H_{38}O_8$, based on HR-TOFMS at *m/z* 527.2567 $[M+H]^+$ (calculated for $C_{30}H_{39}O_8$, *m/z* 527.2580), referrencing 12 degrees of unsaturation. The ^{1}H-NMR of **2** showed five methyls at δ_H 1.32 (3H, s, CH_3-30), 1.22 (3H, s, CH_3-29), 1.28 (3H, s, CH_3-28), 1.24 (3H, s, CH_3-19), and 1.03 (3H, s, CH_3-18), three *sp²* methines at δ_H 7.16 (1H, s), 7.33 (1H, s), and 6.19 (1H, s) assinged as a furan ring, two additional of a pair vicinal *sp²* methines at δ_H 7.13 (1H, d, *J*= 10.0 Hz) and 5.90 (1H, d, *J*= 10.0 Hz). This data implied that **2** had the same skeleton as **1**, an azadirone-type limonoid (Tan & Luo, 2011). The ^{13}C-DEPT NMR of **2** revelaed the presence of 30 carbons, including three carbonyls (one ketonic and two esters) at δ_C 204.8 (C-3), 170.2 (C-1'), 170.2 (C-1''), seven tertiary methyls at δ_C 21.1 (C-18), 19.6 (C-19), 21.0 (C-28), 31.3 (C-29), 21.7 (C-30), 21.2 (C-2'), and 21.2 (C-2''), five *sp²* methines at δ_C 159.7 (C-1), 125.6 (C-2), 124.6 (C-21), 110.7 (C-22), and 142.8 (C-23), one *sp²* quaternary carbon at δ_C 138.7 (C-20), seven *sp³* methines (four oxygenated carbons) at δ_C 46.9 (C-5), 71.9 (C-6),73.7 (C-7), 39.1 (C-9), 69.1 (C-11), 58.2 (C-15), and 38.4 (C-17), as well as five quaternary of *sp³* carbons (one oxygenated carbon) at δ_C 40.7 (C-4), 45.3 (C-8), 41.9 (C-10), 43.5 (C-13), and 73.8 (C-14). The above data matched the presence of an *α,β*-unsaturated carbonyl ketone of azadirone in compound **2**. Further inspection confirmed that structure of **2** was very close to those of **1**, involving the fromation of C-14/15 epoxide and 7-*O*-acetyl. The differences between these two compounds were an additional of two oxygenated methines at C-6 and C-11, as well as another *O*-acetyl moeity. Toghter with information from mass spectrum, the structure of **2** was then rationalized by an attachment of 6-*O*-acetyl and 11-hydroxyl. The NMR-1D data of **2** and its optical rotation (OR) value of $[\alpha]^{25}_D$ +10.2° were further compared with the related azadirone compound, toonaciliatone C ($[\alpha]^{25}_D$ +5.0°) (Jiang *et al.,* 2012). Consequently, compound **2** was identified as toonaciliatone C due to their chemical shifts and the OR values were nearly identical (**Table 1**).

Compound **3** was characterized as a white solid (MeOH), with a molecular formula of $C_{30}H_{38}O_6$, based on the HR-TOFMS spctrum at *m/z* 495.2667 $[M+H]^+$ (calculated $C_{30}H_{39}O_6$ *m/z* 495.2668), resulting in 12 degrees of unsaturation. The ^{1}H-NMR spectrum of compound **3** the presence of five tertiary methyls at δ_H 1.23 (3H, s, CH_3-30), 1.21 (3H, s, CH_3-29), 1.15 (3H, s, CH_3-28), 1.23 (3H, s, CH_3-19), and 1.34 (3H, s, CH_3-18). Moreover, compound **3** also had similarity to those of previous analogs **1** and **2**, including the presence of a fuan ring at [δ_H 7.08 (1H, s), 7.34 (1H, s), and 6.13 (1H, s)], additional a pair of *sp²* methine at [δ_H 5.85 (1H, d, *J*= 10.6 Hz) and 7.23 (1H, d, *J*= 10.6 Hz)], leading to the existence of an intact limonoid with an *α, β*-unsaturated ketonic group. This conclusion was proved by the ^{13}C-DEPT NMR which revealed the appearance of a furan ring at [δ_C 124.7 (C-20), 139.6 (C-21), 111.0 (C-22), 143.1 (C-23)], a conjugated carbonyl ketone at [δ_C 157.7 (C-1), 126.3 (C-2), 204.8 (C-3)], four *sp³* quaternary carbons non-oxygenated at [δ_C 45.1 (C-4), 43.3 (C-8), 46.9 (C-10), 41.9 (C-13)], three *sp³* methylenes at [δ_C 23.9 (C-6), 34.7 (C-12), 32.2 (C-16)], and five tertiary methyls at [δ_C 21.9 (C-18), 19.3 (C-19), 19.9 (C-28), 20.3 (C-29), 21.7 (C-30)], confirming an intact tetracyclic azadirone-type limonoid. Furthermore, a detailed analysis showed that an epoxy ring at C-14/C-15 in **1** and **2** was replaced by $\Delta^{14,15}$ [δ_C 158.8 (C-14), 119.9 (C-15)/ δ_H 5.28 (1H, dd, *J*=1.8, 3.6 Hz, H-15)] in **3**. Together with the remaining of ^{1}H and ^{13}C signals, compound **3** had two *O*-acetyls which was then compared to another analog 11*α*-asetoksiazadiron (Halsall & Troke, 1975) (**Table 2**). The results showed that these two compounds were resembled in their NMR-1D data, it therefore concluded two *O*-acetyls positioned at C-7 and 11 in **3**. The OR value of **3** at -

18.5° also showed similarity to the reported value at -7.0° of 11α-asetoksiazadiron (Halsall & Troke, 1975). Hence, the structure of **3** was completely identified and elucidated as 11α-asetoksiazadiron.

Compound **4** was isolated as a colorless crystal and its molecular formula was established as $C_{30}H_{38}O_7$ based on HR-TOFMS data at *m/z* 511.2634 $[M+H]^+$ (calculated for $C_{30}H_{38}O_7$, *m/z* 511.2696) as well as in accordance with the NMR-1D data (**Table 2**), resulting in 12 degrees of unsaturation. The NMR-1D data confimed the existance of an azadirone-type limonoid as the main structure of **4** the same as its previous analogs **1-3**. The ^{13}C-DEPT NMR supported by its HSQC of **4** revealed the existance of 30 carbons with 26 signals were assigned for an intact tetracyclic of a limonoid compound. Moreover, compound **4** also showed the presence of an α,β-unsaturated carbonyl ketone system at C_1-C_2-C_3 [δ_C 158.1 (C-1), 124.5 (C-2), 204.4 (C-3)] and an additional $\Delta^{14,15}$ [δ_C 160.6 (C-14), 119.3 (C-15)/ δ_H 5.48 (1H, d, *J*=7.7 Hz, H-15)] that was similar to compound **3**. The remaining four carbon signals implied the attachment of two *O*-acetyls and one hydroxyl in **4** which was confirmed by its mass data. Based on the above preliminary analysis of NMR-1D, compound **4** was then identified as 16β-hydroxydisobinin, previously isolated from *C. macrophyllus* (Nurlelasari *et al.*, 2021). This conclusion determined the 6-*O*-acetyl [δ_C 21.3 (C-1′), 170.1 (C-2′), 69.7 9C-6)] and 7-*O*-acetyl [δ_C 22.4 (C-1″), 170.6 (C-2″), 74.0 (C-7)], as well as 16-OH [δ_C 67.6 (C-16)] substitutions in **4** due to high similarity with those of 16β-hydroxydisobinin (**Table 2**). The above data was then completed by a similar tendency of their OR values, +131.5° vs +122.5° (Nurlelasari *et al.*, 2021). Thus, the complete structure of **4** was fully characterized, as shown in **Figure 1**.

Table 1. The ^{13}C-NMR data of compounds **1** and **2** ($CDCl_3$, 125 MHz) and literatures.

Carbon Position	Compounds			
	1*	Trichilinone acetate **	2*	Toonaciliatone C**
	^{13}C-NMR δ_C (mult)	^{13}C-NMR δ_C (mult)	^{13}C-NMR δ_C (mult)	^{13}C-NMR δ_C (mult)
1	157.7 (d)	158.2 (d)	159.7 (d)	160.2 (d)
2	126.8 (d)	125.6 (d)	125.6 (d)	125.2 (d)
3	204.8 (s)	204.7 (s)	204.5 (s)	204.6 (s)
4	44.6 (s)	44.2 (s)	40.7 (s)	40.5 (s)
5	45.9 (d)	46.8 (d)	46.9 (d)	46.9 (d)
6	22.9 (t)	24.9 (t)	71.9 (d)	71.0 (d)
7	73.7 (d)	73.9 (d)	73.9 (d)	73.3 (d)
8	45.3 (s)	42.8 (s)	45.3 (s)	45.5 (s)
9	39.1 (d)	40.6 (d)	39.1 (d)	39.9 (d)
10	41.9 (s)	39.9 (s)	41.9 (s)	41.6 (s)
11	17.1 (t)	16.5 (t)	69.1 (d)	69.0 (d)
12	30.7 (t)	29.7 (t)	44.2 (t)	44.2 (t)
13	42.9 (s)	42.1 (s)	43.5 (s)	43.2 (s)
14	73.8 (s)	73.3 (s)	73.8 (s)	73.1 (s)
15	58.9 (d)	58.5 (d)	58.2 (d)	57.9 (d)
16	32.2 (t)	32.1 (t)	32.2 (t)	32.1 (t)
17	39.4 (d)	39.8 (d)	38.4 (d)	39.0 (d)
18	21.9 (q)	21.1 (q)	21.1 (q)	21.3 (q)
19	18.6 (q)	19.1 (q)	19.6 (q)	20.0 (q)
20	123.7 (s)	123.7 (s)	138.7 (s)	139.6 (s)
21	139.6 (d)	139.6 (d)	124.6 (d)	124.3 (d)
22	111.0 (d)	111.1 (d)	110.7 (d)	110.8 (d)
23	143.1 (d)	143.0 (d)	142.8 (d)	142.9 (d)
28	18.9 (q)	19.3 (q)	21.0 (q)	20.9 (q)
29	20.3 (q)	20.5 (q)	31.3 (q)	31.9 (q)
30	21.7 (q)	21.7 (q)	21.7 (q)	21.7 (q)
1′	21.2 (q)	21.5 (q)	21.2 (q)	21.2 (q)
2′	170.4 (s)	169.8 (s)	170.2 (s)	170.2 (s)
1"			21.2 (q)	21.3 (q)
2"			170.2 (s)	170.2 (s)

* ($CDCl_3$; ^{1}H-NMR 500 MHz; ^{13}C-NMR 125 MHz)

**($CDCl_3$; ^{1}H-NMR 500 MHz; ^{13}C-NMR 125 MHz)

Table 2. The ^{13}C-NMR data of compounds **3** and **4** ($CDCl_3$, 125 MHz) and literatures.

Carbon Position	Compounds			
	3*	11α-acetoxyazadirone **	4*	16*β*-hydroxydisobinin **
	^{13}C-NMR δ_C (mult)	^{13}C-NMR δ_C (mult)	^{13}C-NMR δ_C (mult)	^{13}C-NMR δ_C (mult)
1	157.7 (d)	157.8 (d)	158.1 (d)	158.3 (d)
2	126.3 (d)	126.5 (d)	124.5 (d)	124.4 (d)
3	204.8 (s)	204.8 (s)	204.4 (s)	204.4 (s)
4	45.1 (s)	45.0 (s)	45.0 (s)	45.0 (s)
5	48.5 (d)	48.5 (d)	47.5 (d)	47.5 (d)
6	23.9 (t)	23.8 (t)	69.7 (d)	69.9 (d)
7	73.7 (d)	73.7 (d)	74.0 (d)	74.1 (d)
8	43.3 (s)	43.3 (s)	41.6 (s)	41.6 (s)
9	39.1 (d)	39.3 (d)	39.4 (d)	39.6 (d)
10	41.9 (s)	41.7 (s)	42.7 (s)	42.7 (s)
11	72.1 (t)	72.0 (t)	18.3 (t)	18.4 (t)
12	34.7 (t)	34.7 (t)	36.4 (t)	36.4 (t)
13	46.9 (s)	46.7 (s)	46.8 (s)	46.6 (s)
14	158.8 (s)	158.9 (s)	160.6 (s)	160.6 (s)
15	119.9 (d)	119.9 (d)	119.3 (d)	119.3 (d)
16	32.2 (t)	32.2 (t)	67.2 (d)	67.1 (d)
17	52.4 (d)	52.6 (d)	51.2 (d)	51.2 (d)
18	21.9 (q)	21.5 (q)	28.7 (q)	28.8 (q)
19	19.3 (q)	19.3 (q)	31.6 (q)	31.7 (q)
20	124.7 (s)	124.7 (s)	124.0 (s)	124.2 (s)
21	139.6 (d)	139.6 (d)	139.7 (d)	139.7 (d)
22	111.0 (d)	111.1 (d)	110.9 (d)	110.9 (d)
23	143.1 (d)	143.1 (d)	142.7 (d)	142.9 (d)
28	19.9 (q)	19.9 (q)	20.5 (q)	20.5 (q)
29	20.3 (q)	20.4 (q)	20.6 (q)	20.8 (q)
30	21.7 (q)	21.7 (q)	20.9 (q)	20.9 (q)
1′	21.2 (q)	21.4 (q)	21.3 (q)	21.4 (q)
2′	170.4 (s)	170.4 (s)	170.1 (s)	170.2 (s)
1"	21.3 (q)	21.3 (q)	22.0 (q)	22.0 (q)
2"	170.2 (s)	170.0 (s)	170.3 (s)	170.3 (s)

* ($CDCl_3$; ^{1}H-NMR 500 MHz; ^{13}C-NMR 125 MHz)
**($CDCl_3$; ^{1}H-NMR 500 MHz; ^{13}C-NMR 125 MHz)

Table 3. The IC_{50} values of compounds **1**-**4** against MCF-7 cells (IC_{50} /μM)

Compounds	IC_{50} (μM)
1	480.3
2	554.4
3	702.2
4	43.1
Cisplatin (+)	53.0

Cytotoxic Activity

All isolated limonoids (**1**-**4**) were evaluated for their cytotoxicity against the MCF-7 human breast cancer cells. The resazurin assay was utilized to examine the viability of a cell using PrestoBlue reagent as described previously (Izdihar *et al.,* 2021; Hidayat *et al.,* 2017; Naini *et al.,* 2023a,b). The MCF-7 cell line is an ideal prototype model to discover new anticancer agents since it is resistant to the chemotherapy agents, such as doxorubicin (Lovitt *et al.,* 2018) and cisplatin (Kobayashi *et al.*, 2022). As shown in **Table 3**, all compounds showed no activity (IC_{50} > 100 μM). against the tested cancer cells, except **4** that had stronger inhibition than cisplatin with an IC_{50} value of 43.1 μM. These results were consistent with the previous works, showing that trichilinone acetate (**1**), toonaciliatone C (**2**), and 11*α*-acetoxyazadirone (**3**) were inactive against MCF-7 cells (Salam *et al.*, 2021; Harneti *et al.*, 2023). In addition, according to the structure-activity relationship, the alteration of epoxy

ring at C-14/C-15 to a double bond pair might not affect on their cytotoxicity. This conclusion was obtained by the inactive activity of **1** and **2** with 14,15-epoxide and **3** with $\Delta^{14,15}$. Subsequently, the presence of hydroxyl at C-16 in **4** was responsible for its significant cytotoxicity.

CONCLUSIONS

Four known azadirone-type limonoids afforded from the *n*-hexane extract of the *Chisocheton pentandrus* stem bark were identified as trichilenone acetate (**1**), toonaciliatone C (**2**), 11α-acetoxyazadiron (**3**), and 16β-hydroxydisobinin (**4**). The cytotoxicity against human breast cancer cells revealed that only compound **4** that had inhibition with an IC_{50} value of 43.1 μM, resulting in a stronger activity compared to its positive control. Additionally, the presence of hydroxyl at C-16 in **4** significantly increased its cytotoxicity against MCF-7 cancer cells.

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