Limonoids Isolated from Chisocheton pentandrus (Meliaceae) Stembarks and its Cytotoxicity Towards MCF-7 Breast Cancer Cell Line

Dudi Runadi  
Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Sumedang 45363, Indonesia

Intan Hawina Anjari  
Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Sumedang 45363, Indonesia

Purnama Purnama  
Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Sumedang 45363, Indonesia

Nurlelasari Nurlelasari  
Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Sumedang 45363, Indonesia

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Dudi Runadi¹, Intan Hawina Anjari¹, Purnama¹, Nurlelasari¹, Desi Haneti¹, Tri Mayanti¹, Harizon², Ace Tatang Hidayat¹, Supriatno Salam⁴, Mohamad Nurul Azmi⁵, and Unang Supratman¹,²*

1. Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Sumedang 45363, Indonesia
2. Central Laboratory, Universitas Padjadjaran, Sumedang 45363, Indonesia
3. Faculty of Teacher Training and Education, Universitas Jambi, Jambi 36361, Indonesia
4. Faculty of Pharmacy, Universitas Mulawarman, Samarinda 75123, Indonesia
5. School of Chemical Sciences, Universiti Sains Malaysia, Minden 11800, Malaysia

*E-mail: unang.supratman@unpad.ac.id

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**Abstract**

*Chisocheton* is a large source of limonoids with various pharmacological effects, such as antiviral, antibacterial, antimalarial, antifungal, and cytotoxic activities. This study aimed to isolate, elucidate, and evaluate the cytotoxicity of limonoids from *C. pentandrus* stembarks. Isolation was carried out using various separation methods including extraction and column chromatography. Spectral data were analyzed by FT-IR, UV, DEPT 135°, ¹H, ¹³C-NMR, and HRTOF-MS and compared with those previously reported to determine the chemical structure. The obtained limonoids were lasiocarpine (1), lasiocarpine B (2), pentandrocin (3), and 16β-hydroxydysobinin (4); all of which were successfully isolated from *C. pentandrus* for the first time. The cytotoxic activity of these limonoids were also evaluated against Michigan Cancer Foundation-7 (MCF-7) breast cancer cells using PrestoBlue method. Lasiocarpine A revealed the strongest cytotoxicity with an IC₅₀ of 42.62 µM in moderate level.

**Keywords:** *Chisocheton pentandrus*, cytotoxicity, limonoid, MCF-7 breast cancer, Meliaceae

**Introduction**

Limonoids are triterpenoid derivative compounds that lost their four carbons on the side chain and are known as tetranor-triterpenoids. They have a diverse chemical structure that is commonly found in oxygenated form [1] and exhibit various pharmacological effects, including antifungal [2], antifeedant, insecticidal [3], antimalarial, antibacterial [4], and cytotoxicity against several human cancer cells [5–7]. Limonoids are widely abundant in the Meliaceae family, particularly in the *Chisocheton* genus. Several limonoids have been obtained from various *Chisocheton* species such as *C. macrophyllus*, *C. lasiocarpus*, *C. siamensis*, *C. erythrocarpus*, *C. ceramicus*, *C. cumingianus*, and *C. paniculatus* [1, 4–10].

*Chisocheton* is a large source of limonoids in Meliaceae. This genus has 53 species spread in Asia, particularly in subtropical and tropical regions [11] in South China, India, Nepal, Myanmar, Southeast Asia, Bhutan, Indonesia, and Papua New Guinea [12–13]. Around 48 species of *Chisocheton* plants grow in Malaysia and Indonesia, spreading in the islands of Sulawesi, Sumatera, Java, Maluku, and Borneo [14–15]. This plant is widely used as traditional medicine for malaria, rheumatism, and stomach illness [16]. Studies have investigated its pharmacological effects, such as antiinflammatory [17], antimycobacterial [4], antimalarial [4, 18], antifungal [19], antifeedant [20–21], antiobesity [22–23], and cytotoxic activities [5–7, 24–25]. In the *Chisocheton* genus, 55 limonoid compounds have been found, including azadirone [6], vilasinin [24], phragmalin [26–27], mexicanolide [27–28], nimbolinin [13], and havanensin [5].

*Chisocheton pentandrus* is one of the species of the *Chisocheton* genus that grow in the tropical rainforest of Indonesia. In Indonesia, this species is used as traditional medicine for the treatment of wounds, stomach ulcers, fever and diarrhea [16]. So far, 38 compounds, including triterpenoids, limonoids, and flavonoids, have been found in *C. pentandrus* [7, 29–30]. The triterpenoid and limonoid, namely, melianodiol and pentandrucine K, isolated from *C. pentandrus* stembarks showed cytotoxicity against MCF-7 cells (IC₅₀ of 16.84 and 19.30
Materials and Methods

General. With methanol as solvent, IR and UV spectra were measured on Thermo Scientific Nicolet spectrophotometer using DTGS KBr detector (Madison, USA) and TECAN Infinite M200 Pro (Mannedorf, Switzerland), respectively. MS spectra were obtained on Waters Xevo HRTOFMS QTOF (Waters, Milford, MA, USA) in positive and negative modes. $^1$H and $^{13}$C-NMR spectra were acquired using JEOL JNM-ECZ500R/S1 (Tokyo, Japan) with CDCl$_3$ as a solvent and tetramethyl silane (TMS) as an internal standard. ODS silica gel 60 70–230 mesh (TMS) from Sylisia Chemical Ltd.) and silica gel 60 GF$_254$ (TMS) as an internal standard. ODS silica gel 60 400 mesh (Fuji Chemical) were used for column chromatography to monitor the isolation procedure. The detection of TLC plate was monitored under UV light and sprayed with p-dimethylaminobenzaldehyde and hydrochloric acid (1:1) in EtOH.

Plant material. *C. pentandrus* stembarks (1.6 kg) were repeatedly macerated using MeOH for 3 days, filtered, and evaporated under vacuum condition to obtain a methanol extract (340.0 g). This extract was then consecutively fractionated using n-hexane, EtOAc, and n-BuOH. All organic fractions were concentrated under vacuum condition to obtain extracts of n-hexane, EtOAc, and n-BuOH with masses of 10.90, 25.18, and 228.63 g, respectively. The schematic of the extraction method is shown in Figure 1. Nonpolar extract (n-hexane) was separated by CC SiO$_2$ with n-hexane, ethyl acetate, and methanol (gradient system) to yield A–H fractions. B fraction was column-chromatographed on SiO$_2$ using a CH$_2$Cl$_2$ and EtOAc (8:2) mixture as a solvent to give four fractions (B1–B4). B1 fraction (45.0 mg) was purified by ODS CC using MeOH:H$_2$O (8:2) to yield compound 1 (5.0 mg), and B2 fraction (50.0 mg) was also purified by ODS CC with MeOH:acetonitrile: H$_2$O (4:3:3) solvent to obtain 2 (3.0 mg). D fraction (5.3 g) was treated with CC SiO$_2$ using a gradient eluent system of n-hexane and EtOAc to gain five combined...
fractions (D1–D5). D2 fraction (174.6 mg) was eluted using n-hexane:DCM:EtOAc (2:7:5:0.5) on SiO₂ to obtain 4 (5.2 mg). E fraction (542.8 mg) was then separated by CC SiO₂ using 5:1:3:5 of n-hexane:CH₂Cl₂:EtOAc. E4 fraction (61.2 mg) was purified with SiO₂ CC and eluted with 6:5:3:5 of CH₂Cl₂:EtOAc to obtain compound 3 (8.4 mg).

**Lasiocarpine A (1):** colorless solid, degraded under its m.p.; [α]$_{D}^{28}$ +12.0 (c 0.50, CHCl₃); UV (MeOH) $λ_{max}$ 230 nm (log ε 2.62); IR (KBr) $ν_{max}$ (cm$^{-1}$) 2920 (C–H sp$^{3}$), 1729 (C=O), 1680 (conjugated C=O), 1450 (C–C sp$^{2}$), and 1082 (C–O). $^1$H-NMR (500 MHz in CDCl₃) $δ_{H}$ 2.50 (1H, d, $J = 12.3$ Hz, H-5), 5.45 and 5.43 (1H, d, $J = 2.3$ Hz, H-6 and H-7), 2.26–2.29 (1H, m, H-9), 5.89 (1H, d, $J = 9.8$ Hz, H-2), 1.70–1.83 (2H, m, H-11), 7.12 (1H, d, $J = 9.8$ Hz, H-1), 1.86–1.93 (2H, m, H-12), 5.37 (1H, d, $J = 3.5$ Hz, H-15), 2.05–2.07 (1H, m, H-16), 2.79 (1H, t, $J = 7.6$ Hz, H-17), 0.87 (3H, s, H-18), 1.26 (3H, s, H-19), 7.20 (1H, d, $J = 2.2$ Hz, H-22), 4.84 (2H, d, $J = 2.2$ Hz, H-23), 1.19 and 1.25 (3H, s, CH$_3$-28 and CH$_3$-29), 2.01 and 2.05 (3H, s, CH-1” and CH-1’”), 1.35 (3H, s, CH$_3$-30); $^{13}$C (125 MHz in CDCl₃) in Table 1; HR-TOFMS (positive ion mode) $m/z$ 511.2691 [M+H]$^+$ (calcd. for C$_{30}$H$_{39}$O$_{8}$ $m/z$ 511.2696).

**Lasiocarpine B (2):** white solid, degraded under its m.p.; [α]$_{D}^{28}$ +17.7 (c 0.22, CHCl₃); UV (MeOH) $λ_{max}$ 230 nm (log ε 2.61); IR (KBr) $ν_{max}$ (cm$^{-1}$) 3320, 1599, 1562, 1417, 1208, 1074, 950, 866, 764, 717 (OH), 3119, 2920, 2858, 1743 (C=O stretch), 1669 (C=C stretch), 1589 (C=C stretch), 1499 and 1370 (C=C dimethyl), 1250 (C–O stretch). $^1$H-NMR (CDCl₃, 500 MHz) $δ_{H}$ 8.31 (3H, d, $J = 10.3$ Hz, H-1), 5.89 (1H, d, $J = 10.3$ Hz, H-2), 2.59 (1H, d, $J = 12.4$ Hz, H-5), 5.47 (1H, m, H-6), 5.40 (1H, d, $J = 2.7$, H-7), 2.13 (1H, dd, $J = 6.3$, 14.4 Hz, H-9), 1.87 and 2.49 (2H, d, $J = 14.4$ Hz, H-11), 2.36 and 1.68 (2H, m, H-12), 5.49 (1H, d, $J = 7.7$ Hz, H-15), 4.49 (1H, d, $J = 7.7$ Hz, H-16), 2.81 (1H, dd, $J = 7.7$, 11.0 Hz, H-17), 1.32 (3H, s, H-18), 1.27 (3H, s, H-19), 7.30 (1H, d, $J = 1.3$ Hz, H-22), 7.39 (1H, d, $J = 1.3$ Hz, H-23), 0.94 and 1.19 (3H, s, CH$_2$-28 and CH$_2$-29), 1.32 (3H, s, CH$_3$-30), 2.03 and 2.06 (3H, s, CH$_3$-1’ and CH$_3$-1”); $^{13}$C (125 MHz in CDCl₃), in Table 1; HR-TOFMS (positive ion mode) $m/z$ 511.2673 [M+H]$^+$ (calcd. for C$_{30}$H$_{39}$O$_{8}$ $m/z$ 511.2696).

**Cytotoxic activity test by prestoblue assay.** PrestoBlue™ method was used to determine the cytotoxic activities of 1–4 against MCF-7 cells [Authentication of the cell lines: MCF-7 (ATCC HTB-22)]. MCF-7 cells were acquired from American Type Culture Collection (ATCC® HTB 22), passage number P7–3, official distributor in Indonesia. The morphology of the MCF-7 cells was observed under an inverted microscope for the authentication. The cancer cells were seeded into 96-well plates on Rosewell Park Memorial Institute medium and incubated for 48 hours (temperature 37 °C and 5% CO₂ gas) until they reached 10,000 cells/well. The cells were then treated with the sample (DMSO 0.5%–0.2% as solvent and PBS as cosolvent) at a concentration range of 7.5 ppm to 1000 ppm and a positive control (cisplatin 200 ppm). The treated cells were incubated for 2 days. Resazurin reagents were added, and the incubation was carried out for 2 hours until the color changed from blue to purple.

**Data analysis.** The IC$_{50}$ of all compounds was determined by calculating the percent cell viability according to the absorbance results. Absorbances were recorded by a multimode reader (wavelength 570 and 600 nm). In addition, the Z factor of PrestoBlue assay or resazurin assay was calculated from the data following 120 min incubation with 50 µM resazurin according to Equation (1):

$$Z = 1 - (3(σ_p + σ_o)/(µ_p + µ_o)),$$

where $σ_p$ and $σ_o$ indicate the standard deviation of the positive and negative controls, respectively, and $µ_p$ and $µ_o$ indicate the mean of the positive and negative controls,

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respectively. Z-factor calculation is shown in detail in Supplementary Data (S1). Moreover, the Z-factor was calculated based on the normalized data, that is, 100% viability for positive control and 0% viability for negative control.

Results and Discussion

Four known limonoids (1–4) were isolated from C. pentandrus stembarks for the first time. These compounds were lasiocarpine (1), lasiocarpine B (2), pentandricine (3), and 16β-hydroxydysobinin (4). The chemical structures of 1–4 are shown in Figure 2.

Compound 1 (C_{30}H_{38}O_{3}) with a mass spectrum m/z 511.2691 [M+H]^+ (calcd. C_{30}H_{38}O_{3}; m/z 511.2696; mass error 0.9779 ppm) has 12 unsaturated degrees. UV absorption (λ_{max} 230 nm, log ε 2.62) indicate the presence of a furan ring in compound 2 at δC 127.4 (C-20), 138.8 (C-21), 109.9 (C-22), and 143.0 (C-23) on the side chain. In addition, the different chemical shift of olefinic group at δC 116.1 (C-12) and 151.4 (C-13) indicates the different position of the olefinic group. According to literature comparison, this olefinic group is positioned at C-12 and C-13 on the C-ring of azadirone-type limonoid [6]. The presence of one additional oxygenated methine at δH 3.21 (3H, s, CH_{2}-1') and 2.05 (3H, s, CH_{3}-2') and two ester carbonyls at 170.2 (C-2') and 170.4 (C-2''). These functional groups are bonded to C-6 and C-7, which are also supported by the presence of two oxygenated methines at 69.8 (C-6) and 74.7 (C-7). Moreover, the deshielded methines sp^{2} 7.20 (H-22) and 7.12 (H-1) indicate the existence of two α,β-unsaturated carbonyls, supported by the presence of two remaining carbonyls observed as ketone 204.6 (C-3) and ester 174.3 (C-21) carbonyls. The position of α,β-unsaturated ketone is on A-ring, and α,β-unsaturated ester is on a lactone ring in the side chain. In addition, the two remaining olefinic carbons indicate the presence of one double bond positioned at D-ring as revealed by literature comparison [6]. The structure determination including the absolute configuration of 1 was carried out using the approach for azadirone-type limonoids in the Chisocheton genus and its biosynthetic pathway [33].

Compound 2 was purified as a white solid. Its molecular formula is C_{30}H_{38}O_{3}; according to the mass spectrum m/z 511.2685 [M+H]^+ (calcd. 511.2696; mass error 2.1515 ppm), indicating 12 degrees of unsaturation. ^1H and ^13C-NMR detailed with DEPT 135° of 2 show the similarities of its chemical shift to that of compound 1. The main difference of these two compounds is the presence of a furan ring in compound 2 at δC 127.4 (C-20), 138.8 (C-21), 109.9 (C-22), and 143.0 (C-23) on the side chain. In addition, the different chemical shift of olefinic group at δC 116.1 (C-12) and 151.4 (C-13) indicates the different position of the olefinic group. According to literature comparison, this olefinic group is positioned at C-12 and C-13 on the C-ring of azadirone-type limonoid [6]. The presence of one additional oxygenated methine at δH 3.21 (3H, s, CH_{2}-1') and 2.05 (3H, s, CH_{3}-2') and two ester carbonyls at 170.2 (C-2') and 170.4 (C-2''). These functional groups are bonded to C-6 and C-7, which are also supported by the presence of two oxygenated methines at 69.8 (C-6) and 74.7 (C-7). Moreover, the deshielded methines sp^{2} 7.20 (H-22) and 7.12 (H-1) indicate the existence of two α,β-unsaturated carbonyls, supported by the presence of two remaining carbonyls observed as ketone 204.6 (C-3) and ester 174.3 (C-21) carbonyls. The position of α,β-unsaturated ketone is on A-ring, and α,β-unsaturated ester is on a lactone ring in the side chain. In addition, the two remaining olefinic carbons indicate the presence of one double bond positioned at D-ring as revealed by literature comparison [6]. The structure determination including the absolute configuration of 1 was carried out using the approach for azadirone-type limonoids in the Chisocheton genus and its biosynthetic pathway [33].

Compound 3 was identified as a colorless solid. Mass spectrum m/z 440.2130 [M+H]^+ (calcd. C_{20}H_{20}O_{6}; m/z 440.2121; mass error 2.0444 ppm) shows that the molecular formula of 3 is C_{20}H_{20}O_{6} and indicates 11 unsaturated degrees. Compound 3 was elucidated as pentandricine. Several similar functional groups and characteristics have been discovered, including three double bonds, two carbonyls, and six rings. A limonoid generally has five methyls, and the existence of four methyls indicates the absence of one methyl. This finding is supported by the presence of one oxygenated methylene, the product of ether formation, and one oxygenated methine 4.46 (1H, dd, H-6) with J= 12.3, 3.6 Hz. The chemical shift and J coupling value of this ether group indicate the existence of additional cyclic in ether

![Chemical Structure of Compounds 1–4](image-url)
form. The spectroscopic data of 3 reveal two α,β-
unsaturated carbonyls with a similar characteristic to the
α,β-unsaturated carbonyls of 1. The main differences are
the chemical shifts of α,β-unsaturated ketone at 203.6 (C-
1), 129.1 (C-2), and 153.3 (C-3), indicating the different
position of the ketone group. In addition, the different
chemical shifts of α,β-unsaturated ester at 171.7 (C-21),
132.8 (C-20), 118.7 (C-22) show the influence of the
hydroxyl group at lactone ring (C-23). The existence of
OH is supported by one anomic oxygenated methine at
99.5 (C-23). Apart from the oxymethylene at C-6 and C-23,
one remaining oxymethylene is positioned at C-7 as
determined from the biosynthesis and literature approach.
The absolute configurations of each asymmetric carbons
were also determined using the same technique [7, 33].

Compound 4 (C_{20}H_{33}O_{3}) was purified as a colorless
crystal. Mass spectrum m/z 511.2673 [M+H]^+ (calcd.
511.2696; mass error 4.4986 ppm) reveals 12 unsaturated
degrees. All NMR data indicate seven unsaturated degrees,
including four double bonds and three carbonyls. The
five remaining unsaturated degrees correspond to
pentacyclic skeleton. Moreover, the spectroscopic data of
compound 4 show similarity to those of compound 1.
The main difference is the presence of a furan ring, which is
supported by the existence of furan moiety at 7.29 (1H, s,
H-21), 7.39, and 6.29 (1H, d, J = 1.3 Hz, H-22 and H-23),
and a hydroxyl group, which is supported by the
existence of additional oxygenated methine at 67.1 (C-16)
on compound 4. The configuration of the furan ring was
suggested as α-orientation according to the biosynthetic
approach, and the OH group exhibited β-orientation
according to literature comparison [5, 33]. Therefore,
compound 4 was obtained as a havanensis-type limonoid,
namely, 16β-hydroxydysobinin. This compound has
been previously isolated from C. macrophyllus seeds.

Table 1. Comparison of 13C-NMR Data for Compounds 1–4 (CDCl₃, 125 MHz) with Published Data [5–7]

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*a* in CDCl₃, 125 MHz
The cytotoxicity of 1–4 were tested against MCF-7 cells using resazurin assay as previously described [34,35]. Table 2 shows the results. Lasiocarpine A (1) has the highest cytotoxicity in moderate level with an IC\textsubscript{50} of 42.62 µM, followed by 16β-hydroxydysobinin (4), lasiocarpine B (2), and pentandricine (3) with IC\textsubscript{50} values of 46.08, 170.34, 368.95 µM, respectively. Lasiocarpine B has a weak cytotoxicity, and pentandricine is inactive. A comparison of chemical structure between 1 and 4 revealed that the cytotoxicity of limonoids is influenced by the side chain form and the presence of hydroxyl group on C-16. Compound 1 is more active than 4, indicating that these functional groups (furan ring and hydroxyl group on C-16) increase the activity, albeit insignificantly. Meanwhile, the different positions of the unconjugated olefinic group and hydroxyl group reveal the significant effect as determined from the comparison between 2 and 4. Compound 2 is less active than 4, indicating that the position of unconjugated olefinic on C-14/C-15 and the hydroxyl group on C-16 (compound 4) contribute to the increased cytotoxicity compared with the unconjugated olefinic on C-12/C-13 with the hydroxyl group (C-15) on 2. The complicated modifications on 3, such as the different position of α,β-unsaturated ketone on A-ring, additional ether formation on C-28/C-6, and presence of hydroxyl group on C-7 and C-23, significantly decrease its cytotoxic activity. Nevertheless, all these compounds are less active than cisplatin (positive control).

All these limonoids are known compounds, and their cytotoxic activity against MCF-7 breast cancer cell line has been reported previously. Unfortunately, the previous studies did not explain the bioassay results in detail. To verify the exact IC\textsubscript{50} value of all compounds, we also reported the Z-factor in Table 2 to confirm the quality of the bioassay methods we used. An assay with a Z-factor over 0.5 is excellent, and that with a Z factor between 0.4 and 0.5 is sufficiently reliable for semiquantitative studies [35]. According to Table 2, the Z-factor values of the assay are higher than 0.5 (in the range of 0.93–0.98) for all the compounds including the positive control. Considering these results, we concluded that the performance of resazurin assay utilized in this investigation is excellent for the cytotoxicity determination of secondary metabolites against MCF-7 breast cancer cells.

### Conclusion

Four known limonoids including two azadirone types and two havanensin types have been successively isolated from C. pentandrus stem bark for the first time using several extraction and column chromatography methods. These limonoids were identified as lasiocarpine (1), lasiocarpine B (2), pentandricine (3), and 16β-hydroxydysobinin (4) based on spectroscopic analysis. Furthermore, the cytotoxicity of 1–4 against MCF-7 cells was determined using PrestoBlue assay. Among these compounds, lasiocarpine A showed the highest cytotoxicity with an IC\textsubscript{50} of 42.62 µM. The absence of hydroxyl group on C-16, the position of unconjugated olefinic at C-14/C-15, and the presence of lactone ring with α,β-unsaturated ester on the side chain significantly contribute to this cytotoxicity.

### Acknowledgements

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### References


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