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# Cytotoxic Evaluation of Steroids Isolated from *Dysoxylum alliaceum* (Blume) Blume ex A.Juss.

**Sandra Amalia Riyadi[1,2], Al Arofatus Naini[2], Tri Mayanti[1], Ronny Lesmana[2,3], Mohamad Nurul Azmi[4], Unang Supratman[1,2*]**

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor 45363, Sumedang, Indonesia.
[2]Central Laboratory, Universitas Padjadjaran, Jatinangor 45363, Sumedang, Indonesia.
[3]Physiology Division, Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran, Jatinangor 45363, Indonesia
[4]School of Chemical Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia

[*]Corresponding author email: unang.supratman@unpad.ac.id

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**ABSTRACT.** *Dysoxylum alliaceum* belongs to the *Dysoxylum* genus (Meliaceae) and there are few reports concerning the phytochemical components of this plant. To examine the chemical constituents of *Dysoxylum alliaceum* stem bark, a phytochemical study has been conducted and identified five known steroids, 3*β*,16*β*-dihydroxy-24(*S*)-methyl cholestenol (**1**), ergosta-5,22-dien-3*β*-ol (**2**), ergosta-7,24(28)-dien-3*β*-ol (**3**), 22(*E*)-ergosta-6,22-dien-3*β*,5*α*,8*α*-triol (**4**), and 20*α*-dihydroprogesterone (**5**) from its ethanolic extract. Spectroscopic data such as FT-IR, HR-ESI-MS, 1D, and 2D NMR as well as comparison with previously published spectral data were used to identify the chemical structures of compounds **1**–**5**. Furthermore, these steroids **1**-**5** were assessed *in vitro* regarding their cytotoxic effect against A549 lung cancer cell lines and revealed weak to inactive with $IC_{50}$ values ranging from 68.52 to >150 $\mu$M.

**Keywords:** A549 cell lines, cytotoxic evaluation, *Dysoxylum alliaceum* (blume) blume ex a.juss, meliaceae, steroid

## INTRODUCTION

The genus *Dysoxylum* comprises more than 200 species distributed in India, South China, Australia, Malaysia, and Indonesia (Lakshmi *et al.,* 2009). The genus *Dysoxylum* is plentiful in triterpenoids (Naini *et al.,* 2022a; 2022b), sesquiterpenoids (Naini *et al.,* 2023; Riyadi *et al.,* 2023), limonoids (Nagakura *et al.,* 2010), steroids (Kurimoto *et al.,* 2011), and macrolides (Riyadi *et al.,* 2024a). Many of these plant species, like *D. binectariferum*, have been utilized as traditional medicines by the indigenous people of Asia to cure skin conditions and nasty ulcers (Hu *et al.,* 2014), *D. gaudichaudianum* leaves for the majority of aches, pains, and lung issues (Chen *et al.,* 2007) and *D. richii* for stiff limbs and skin irritations (Lakshmi *et al.,* 2009).

*Dysoxylum alliaceum* (Blume) Blume ex. A.Juss. var. Lanceolatum Koord. & Valeton (Figure 1) is a large tree species growing up to 38 m in height and known as the onion scented tree, that belongs to the family Meliaceae of class Magnoliopsida and order Sapindales its widely distributed in Indonesia and has rarely been systematically investigated (Backer & Bakkuizen, 1963). Previous studies of *D. alliaceum* have reported sesquiterpene phenol (Nishizawa *et al.,* 1983), unsymmetrical dimeric sesquiterpenoids (Nishizawa *et al.,* 1985), tirucallane-type triterpenoids (Riyadi *et al.,* 2024b), and five new highly oxidized mexicanolide-type limonoids (Riyadi *et al.,* 2024c). Although several steroids were obtained from *Dysoxylum* plants with numerous cytotoxic activities, most of the biological activity investigations were limited to the distinction of cancer cell lines. Considering the lack of phytochemical and biological study of *D. alliaceum*, a comprehensive examination of the chemical constituents of *D. alliaceum* is required. Furthermore, all the isolated compounds were examined for cytotoxic potency against A549 lung cancer cell lines. A brief explanation of the structure-activity relationship between compounds **1**–**5** and these cell lines was also given. Based on these results, the isolation and structural identification of four ergostanes and one pregnane-type steroid revealed new information regarding the phytochemical constituent of *D. alliaceum* and their cytotoxic activity.

## EXPERIMENTAL SECTION

### Material and Methods

Using an ESI+ mode and microchannel plates MCPs detector, a Waters Xevo Q-TOF direct probe/MS system (Milford, MA, USA) was utilized to produce

high-resolution mass spectra (HR-ESI-MS). Moreover, NMR spectra were obtained at 500 MHz for $^{1}H$ and 125 MHz for $^{13}C$ using JEOL ECZ and Bruker Topspin spectrometers, with TMS as an internal standard. With the $CDCl_3$ solvent as a reference, chemical shifts were determined in δ (ppm) using signals $\delta_H$ 7.26 and $\delta_C$ 77.16. Silica gel 60 (Merck, 70-230 and 230-400 mesh) and Octa decyl silane (Fuji sylisia chemical LTD., Chromatorex® C18 DM1020 M, 100-200 mesh) were used in column chromatography (CC). Precoated silica gel 60 $F_{254}$ (Merck) and RP-18 $F_{254}$ (Merck) plates were used for thin layer chromatography (TLC), and 254 nm UV light was used for detection. In addition, the IR spectroscopy was recorded using an Everest ATR Thermo scientific FT-IR spectrophotometer.

## Plant Materials

After being collected in September 2021 from the National Forest Pangandaran in West Java Province, Indonesia (longitude 108.65845, latitude -7.70344, and elevation 125 m), the stem bark of *Dysoxylum alliaceum* (Blume) Blume ex A.Juss. (Meliaceae) was placed at the herbarium Universitas Padjadjaran (Number of collection 14/HB/07/2021) by Mr. Joko Kusmoro

## Extraction and Isolation

The 7.50 kg of air-dried stem barks were ground into a powder, and after being extracted for 24 hours at room temperature 5 times (in 12 L, each), the solvent was eliminated using an evaporator under decompression. Following a suspension in $H_2O$, the evaporated MeOH extract (1.51 kg) was divided into three parts: *n*-hexane, EtOAc, and *n*-butanol. To get extracts of *n*-hexane (35.1 g), EtOAc (254.3 g), and *n*-butanol (236.1 g), respectively, the organic layer was evaporated under reduced pressure. Using vacuum liquid chromatography over silica gel, the *n*-hexane fraction (35.1 g) was fractionated with a gradient of *n*-hexane-EtOAc (10:0–1:9, 10% v/v) to produce nine fractions (A–I). Six subfractions (B1-B6) were obtained by separating fraction B (3.1 g) using a gradient solvent of *n*-hexane-EtOAc (10:0-9:1, 2.5% v/v). B2 and B6 fractions were fractionated by an open ODS CC (MeOH-$H_2O$, 8:2) to produce **1** and **2** (5.6 mg and 6.7 mg). Fraction C (2.1 g) was purified to get seven subfractions (C1-C7) with a gradient elution of *n*-hexane-EtOAc (10:0–7:3, 5% v/v). C3 (200 mg) was separated by the silica gel of column chromatography (CC) (230-400 mesh) and eluted with *n*-hexane-dichloromethane-EtOAc (9:0.5:0.5) to produce seven subfractions (Ca-Cg). Furthermore, Cb (90 mg) and Cc (80.4 mg) were subjected to the ODS column (MeOH–$H_2O$, 8:2) to produce compounds **3** (4.5 mg), **4** (5 mg), and **5** (7.5 mg).

**3*β*,16*β*-dihydroxy-24(*S*)-methylcholestenol** (**1**) was isolated as white powder and gave molecular ion peak HR-ESI-QTOFMS *m/z* 417.3717 $[M+H]^+$ (calcd. $C_{28}H_{48}O_2$, *m/z* 417.3733); IR $\nu_{max}$ 3430, 2929, 1465, 1021 $cm^{-1}$; $^{1}H$-NMR ($CDCl_3$, 500 MHz), $\delta_H$ (ppm): 1.84 (1H, m, H-1a), 1.79 (1H, m, H-1b), 1.99 (1H, m, H-2a), 1.83 (1H, m, H-2b), 3.51 (1H, m, H-3), 2.27 (2H, m, H-4), 5.32 (1H, m, H-6), 1.94 (1H, m, H-7a), 1.79 (1H, m, H-7b), 1.49 (1H, m, H-8), 0.92 (1H, m, H-9), 0.99 (2H, m, H-11), 2.01 (1H, m, H-12a), 1.97 (1H, m, H-12b), 0.83 (1H, m, H-14), 2.15 (1H, m, H-15a), 1.15 (1H, m, H-15b), 4.33 (1H, m, H-16), 0.97 (1H, m, H-17), 0.87 (3H, s, $CH_3$-18), 1.03 (3H, s, $CH_3$-19), 1.52 (1H, m, H-20), 0.77 (3H, d, *J*=6.4 Hz, $CH_3$-21), 2.34 (2H, m, H-22), 1.30 (2H, m, H-23), 1.19 (1H, m, H-24), 1.23 (1H, m, H-25), 0.96 (3H, d, *J*=7.0 Hz, $CH_3$-26), 0.83 (3H, d, *J*=7.0 Hz, $CH_3$-27), 0.78 (3H, d, *J*=7.0 Hz, $CH_3$-28). $^{13}C$-NMR ($CDCl_3$, 125 MHz) $\delta_C$ (ppm): 37.2 (C-1), 31.6 (C-2), 71.8 (C-3), 42.3 (C-4), 140.9 (C-5), 121.5 (C-6), 31.8 (C-7), 32.4 (C-8), 50.1 (C-9), 36.5 (C-10), 20.7 (C-11), 40.0 (C-12), 42.3 (C-13), 54.6 (C-14), 36.9 (C-15), 72.6 (C-16), 61.3 (C-17), 13.1 (C-18), 19.4 (C-19), 31.5 (C-20), 18.2 (C-21), 33.8 (C-22), 30.8 (C-23), 38.9 (C-24), 30.1 (C-25), 18.3 (C-26), 20.3 (C-27), 15.4 (C-28).


A B C

**Figure 1.** Whole plant of *D. alliaceum* (**A**) with bark (**B**) and stem barks (**C**) (Private Photograph courtesy).

**Ergosta-5,22-dien-3*β*-ol** (**2**) was gained colorless crystalline and gave molecular ion peak HR-ESI-QTOFMS *m/z* 399.3474 $[M+H]^+$, (calcd. $C_{28}H_{46}O$, *m/z* 399.3638); IR $\nu_{max}$ 3326; 2955; 1434; 1048 $cm^{-1}$; $^1$H-NMR ($CDCl_3$, 500 MHz), $\delta_H$ (ppm): 1.38 (1H, m, H-1a), 1.13 (1H, m, H-1b), 1.56 (1H, m, H-2a), 1.31 (1H, m, H-2b), 3.52 (1H, m, H-3), 2.23 (2H, m, H-4), 5.35 (1H, m, H-6), 2.04 (1H, m, H-7a), 1.79 (1H, m, H-7b), 1.40 (1H, m, H-8), 1.44 (1H, m, H-9), 1.52 (2H, m, H-11), 1.56 (1H, m, H-12a), 1.31 (1H, m, H-12b), 1.45 (1H, m, H-14), 1.75 (1H, m, H-15a), 1.50 (1H, m, H-15b), 1.60 (2H, m, H-16), 1.51(1H, m, H-17), 0.87 (3H, s, $CH_3$-18), 1.01 (3H, s, $CH_3$-19), 2.33 (1H, m, H-20), 0.77 (3H, d, *J*=6.4 Hz, $CH_3$-21), 5.14 (1H, m, H-22), 5.02 (1H, m, H-23), 1.86 (1H, m, H-25), 1.02 (3H, d, *J*=7.0 Hz, $CH_3$-26), 0.92 (3H, d, *J*=7.0 Hz, $CH_3$-27), 0.79 (3H, d, *J*=7.0 Hz, $CH_3$-28). $^{13}$C-NMR ($CDCl_3$, 125 MHz) $\delta_C$ (ppm): 37.4 (C-1), 29.9 (C-2), 72.0 (C-3), 42.4 (C-4), 140.9 (C-5), 121.9 (C-6), 31.8 (C-7), 32.1 (C-8), 50.3 (C-9), 36.7 (C-10), 20.9 (C-11), 40.1 (C-12), 42.5 (C-13), 57.0 (C-14), 23.9 (C-15), 29.1 (C-16), 55.9 (C-17), 11.9 (C-18), 19.6 (C-19), 40.3 (C-20), 21.1 (C-21), 136.0 (C-22), 131.9 (C-23), 43.0 (C-24), 33.3 (C-25), 20.1 (C-26), 19.5 (C-27), 17.8 (C-28).

**Ergosta-7,24(28)-dien-3*β*-ol** (**3**) was obtained as an amorphous white powder. The mass spectrum displays parent molecular weight HR-ESI-QTOFMS *m/z* 399.3617 $[M+H]^+$, (calcd. $C_{28}H_{46}O$, *m/z* 399.3627); IR $\nu_{max}$ 3325, 2959, 1454, 1049 $cm^{-1}$; $^1$H-NMR ($CDCl_3$, 500 MHz), $\delta_H$ (ppm): 1.56 (1H, m, H-1a), 1.31 (1H, m, H-1b), 1.72 (1H, m, H-2a), 1.47 (1H, m, H-2b), 3.57 (1H, m, H-3), 1.68 (2H, m, H-4), 1.46 (1H, m, H-5), 2.04 (1H, m, H-6a), 1.79 (1H, m, H-6b), 5.18 (1H, m, H-7), 1.93 (1H, m, H-9), 1.42 (2H, m, H-11), 1.34 (1H, m, H-12a), 1.09 (1H, m, H-12b), 2.17 (1H, m, H-14), 1.64 (1H, m, H-15a), 1.39 (1H, m, H-15b), 1.60 (1H, m, H-16a), 1.35 (1H, m, H-16b), 1.47 (1H, m, H-17), 0.54 (3H, s, $CH_3$-18), 0.83 (3H, s, $CH_3$-19), 1.64 (1H, m, H-20), 0.91 (3H, d, *J*=6.4 Hz, $CH_3$-21), 1.54 (2H, m, H-22), 1.96 (2H, m, H-23), 2.56 (1H, m, H-25), 0.97 (3H, d, *J*=6.8 Hz, $CH_3$-26), 1.02 (3H, d, *J*=6.8 Hz, $CH_3$-27), 4.71 (1H, s, H-28a), 4.66 (1H, s, H-28a). $^{13}$C-NMR ($CDCl_3$, 125 MHz) $\delta_C$ (ppm): 36.4 (C-1), 37.4 (C-2), 71.1 (C-3), 39.0 (C-4), 43.3 (C-5), 38.7 (C-6), 117.5 (C-7), 139.1 (C-8), 49.6 (C-9), 34.8 (C-10), 19.0 (C-11), 30.9 (C-12), 40.2 (C-13), 39.7 (C-14), 21.7 (C-15), 22.9 (C-16), 29.7 (C-17), 12.0 (C-18), 13.2 (C-19), 56.0 (C-20), 19.3 (C-21), 34.8 (C-22), 39.8 (C-23), 157.1 (C-24), 55.0 (C-25), 22.1 (C-26), 21.8 (C-27), 106.3 (C-28).

**22(*E*)-ergosta-6,22-dien-3β,5*α*,8*α*-triol** (**4**) was isolated as white powder and gave molecular ion peak HR-ESI-QTOFMS *m/z* 453.3358 $[M+Na]^+$, (calcd. $C_{28}H_{46}O_3$, *m/z* 453.3345); IR $\nu_{max}$ 3421, 2956, 1456, 1044 $cm^{-1}$; $^1$H-NMR ($CDCl_3$, 500 MHz), $\delta_H$ (ppm): 1.96 (1H, m, H-1a), 1.69 (1H, m, H-1b), 1.99 (1H, m, H-2a), 1.24 (1H, m, H-2b), 3.95 (1H, m, H-3), 1.92 (2H, m, H-4), 6.60 (1H, m, H-6), 6.33 (1H, m, H-7), 1.59 (1H, m, H-9), 1.62 (2H, m, H-11), 1.52 (2H, m, H-12), 1.19 (1H, m, H-14), 1.22 (2H, m, H-15), 1.23 (2H, m, H-16), 1.51 (1H, m, H-17), 0.87 (3H, s, $CH_3$-18), 0.89 (3H, s, $CH_3$-19), 1.82 (1H, m, H-20), 0.93 (3H, d, *J*=6.4 Hz, $CH_3$-21), 5.18 (1H, m, H22), 5.22 (1H, m, H23), 2.02 (1H, m, H24), 1.85 (1H, m, H25), 0.84 (3H, d, *J*=6.5 Hz, $CH_3$-26), 0.81 (3H, d, *J*=6.5 Hz, $CH_3$-27), 1.01 (3H, d, *J*=6.5 Hz, $CH_3$-28). $^{13}$C-NMR ($CDCl_3$, 125 MHz) $\delta_C$ (ppm): 34.7 (C-1), 39.3 (C-2), 66.5 (C-3), 36.9 (C-4), 82.2 (C-5), 135.4 (C-6), 130.7 (C-7), 79.4 (C-8), 51.7 (C-9), 37.0 (C-10), 20.6 (C-11), 30.1 (C-12), 44.6 (C-13), 55.9 (C-14), 22.9 (C-15), 28.6 (C-16), 33.1 (C-17), 12.9 (C-18), 18.2 (C-19), 42.8 (C-20), 17.6 (C-21), 132.3 (C-22), 135.2 (C-23), 39.7 (C-24), 51.5 (C-25), 19.1 (C-26), 19.6 (C-27), 20.9 (C-28).

**20*α*-dihydroprogesterone** (**5**) was gained colorless crystalline and gave molecular ion peak HR-ESI-QTOFMS *m/z* 317.2412 $[M+H]^+$, (calcd. $C_{21}H_{32}O_2$, *m/z* 317.2481). IR $\nu_{max}$ 3420; 2968; 1708 $cm^{-1}$; $^1$H-NMR ($CDCl_3$, 500 MHz), $\delta_H$ (ppm): 1.49 (1H, m, H-1a), 1.24 (1H, m, H-1b), 2.42 (1H, m, H-2a), 2.31 (1H, m, H-2b), 5.71 (1H, s, H-4), 2.01 (1H, m, H-6a), 1.91 (1H, m, H-6b), 1.42 (1H, m, H-7a), 1.17 (1H, m, H-7b), 1.41 (1H, m, H-8), 1.40 (1H, m, H-9), 1.52 (2H, m, H-11), 1.56 (2H, m, H-12), 1.44 (1H, m, H-14), 1.60 (2H, m, H-15), 1.35 (2H, m, H-16), 1.56 (1H, m, H-17), 0.78 (3H, s, $CH_3$-18), 1.17 (3H, s, $CH_3$-19), 3.71 (1H, m, H-20), 1.13 (3H, d, *J*=6.5 Hz, $CH_3$-21). $^{13}$C-NMR ($CDCl_3$, 125 MHz) $\delta_C$ (ppm): 35.7 (C-1), 34.0 (C-2), 199.7 (C-3), 123.8 (C-4), 171.6 (C-5), 32.7 (C-6), 32.1 (C-7), 35.5 (C-8), 53.8 (C-9), 38.5 (C-10), 21.6 (C-11), 39.7 (C-12), 42.4 (C-13), 55.4 (C-14), 24.5 (C-15), 25.6 (C-16), 58.4 (C-17), 12.5 (C-18), 17.4 (C-19), 70.5 (C-20), 23.2 (C-21).

### Cytotoxic Activity Assay

Using the Resazurin (PrestoBlue) technique, the cytotoxicity of compounds **1**–**5** and *n*-hexane extract was assessed against human cancer cell lines A549 lung cancer cell. The cells were grown in RPMI-1640 with 5% $CO_2$ at 37 °C, antibiotics, and 10% fetal bovine serum added. Furthermore, both were first seeded onto 96-well plates with a cell density of roughly $3x10^4$ cells $cm^{-3}$ and left for a full day of incubation. The compounds were dissolved at the necessary concentrations of 500, 250, 125, 62.5, 31.25, 15.63, 7.81, and 3.91 µM in 2% aqueous DMSO. For 48 hours, the test chemical was applied to the cells in triplicate, with doxorubicin serving as a positive control. Following a 48-hour incubation period, 10 microliters of PrestoBlueTM. The addition of the Cell Viability Reagent was followed by a further one to two hours of incubation, or until the color changed. In a microplate reader, the measured absorbance was then applied at 570 nm. By differentiating the plotted graph of the percentage of living cells and the control group (which acquired just

PBS and DMSO) with the assessed drug concentration (μM), the $IC_{50}$ values were determined.

## RESULT AND DISCUSSION

This study intends to extract, separate, and purify steroid components from *D. alliaceum* stem bark and identify their chemical structure using various spectroscopic techniques. Subsequently, the *n*-hexane extract of *D. alliaceum* stem bark was subjected to repeated column chromatography to yield compounds **1-5** (**Figure 2**).

Compound **1** was isolated as a white powdered, with the result of HR-ESI-QTOFMS, obtained from its molecular ion $[M+H]^+$ *m/z* 417.3717, (calcd. *m/z* 417.3733), with a molecular formula of $C_{28}H_{49}O_2$, which required five degrees of unsaturation containing one double bond and tetracyclic rings. The FT-IR of **1** spectrum presented the hydroxyl group (3430 $cm^{-1}$), CH $sp^3$ (2929 $cm^{-1}$, and 1465 $cm^{-1}$), and C-O (1021 $cm^{-1}$) (Suzuki et al., 2019). The $^1$H-NMR data revealed the signals of six methyl groups containing tertiary methyls at $\delta_H$ 0.87 (s, H-18), 1.03 (s, H-19), and secondary methyls 0.77 (d, *J*=6.4 Hz, $CH_3$-21), 0.96 (d, *J*=7.0 Hz, $CH_3$-26), 0.83 (d, *J*=7.0 Hz, $CH_3$-27), 0.78 (d, *J*=7.0 Hz, $CH_3$-28), two oxymethines at $\delta_H$ 3.51 (m, H-3) and 4.33 (m, H-16), one $sp^2$ proton at $\delta_H$ 5.32 (m, H-6). The $^{13}$C and DEPT 135° NMR data showed the presence of 28 signals, which comprised six methyl signals at $\delta_C$ (13.1, 15.4, 18.2, 18.3, 19.4, 20.3), nine methylene groups at $\delta_C$ (20.7, 30.8, 31.6, 31.8, 33.8, 36.9, 37.2, 40.0, 42.3), ten methines including two oxygenated as well as one olefinic at $\delta_C$ (32.4, 50.1, 54.6, 36.9, 61.3, 38.9, 30.1, 71.8, 72.6, 121.5), and three quaternary carbons at $\delta_C$ (36.5, 42.3, 140.9) (**Table 1**). The presence of 1D-NMR data in association with the HMQC spectrum of **1** suggested that **1** should be a $C_{28}$ steroid (Wang *et al.,* 2020). Furthermore, in addition to the one olefinic, the remaining four indicated **1** to be tetracyclic, which suggested an ergostane skeleton for **1**. The planar structure of **1** was extrapolated from the observed HMBC and $^1$H-$^1$H COSY correlations (**Figure 3**).

The selected HMBC correlations from the olefinic proton H-6 to C-10, C-7, C-4 and a combination with observed $^1$H-$^1$H COSY cross-peak of H-6/H-7 confirmed the presence of double bond across C-5/C-6. The attachment of OH- at C-3 was established by vicinal proton cross-peaks H-2/H-3/H-4, along with HMBC correlations of H-6 to C-4. Furthermore, one remaining hydroxyl group was assigned at C-16 based on the HMBC correlations from proton H-16 to C-13 and C-14, along with the vicinal cross-peaks of H-15/H-16/H-17 from the $^1$H-$^1$H COSY spectrum. The NOESY spectrum showed cross-peaks from H-9/α to H-1 and H-14, H-1/α to H-3, and H-14/H-17α to H-16 were observed, which suggested that H-3 and H-16 should be an α oriented. Conversely, the two hydroxyls at C-3 and C-16 were placed on the opposite side as β orientation. In addition, the observed NOESY correlation between $CH_3$-21 and $CH_3$-28 was not enough to confirm the orientation of these two protons owing to the natural free rotation of the single bond in the side chain (**Figure 4**).

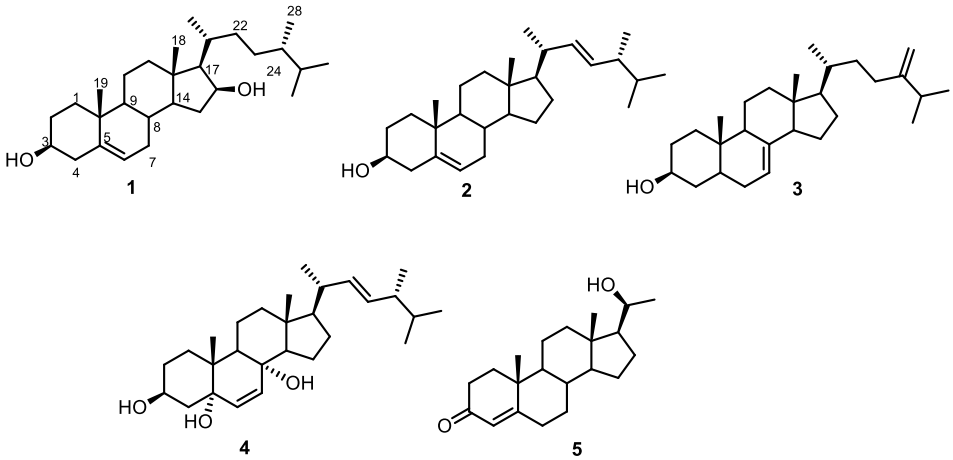



**Figure 2.** Structure of steroids isolated from *D. alliaceum* stem bark **1-5**.

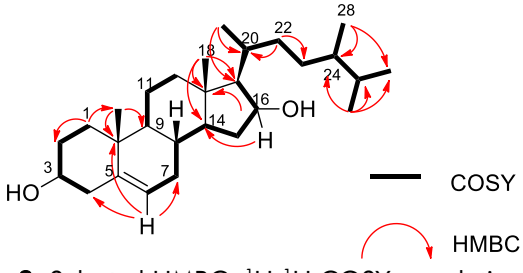
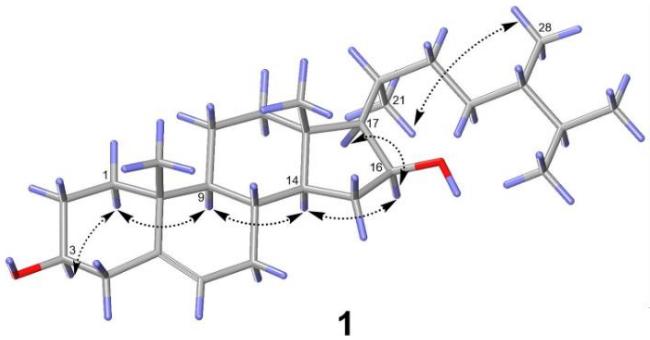



**Figure 3**. Selected HMBC, $^1$H-$^1$H COSY correlations of **1**




**1**

**Figure 4**. Selected NOESY correlation of **1**

**Table 1**. $^{1}H$ ($CDCl_3$, 500 MHz) and $^{13}C$-NMR ($CDCl_3$, 125 MHz) data of compound **1**

| Position Carbon | **1** | |
|---|---|---|
| | $\delta_C$ (type) | $\delta_H$ ($J$ in Hz) |
| 1 | 37.2 ($CH_2$) | 1.84 (1H, m, H-1a) |
| | | 1.79 (1H, m, H-1b) |
| 2 | 31.6 ($CH_2$) | 1.99 (1H, m, H-2a) |
| | | 1.83 (1H, m, H-2b) |
| 3 | 71.8 (CH) | 3.51 (1H, m, H-3) |
| 4 | 42.3 ($CH_2$) | 2.27 (2H, m, H-4) |
| 5 | 140.9 (C) | - |
| 6 | 121.5 (CH) | 5.32 (1H, m, H-6) |
| 7 | 31.8 ($CH_2$) | 1.94 (1H, m, H-7a) |
| | | 1.79 (1H, m, H-7b) |
| 8 | 32.4 (CH) | 1.49 (1H, m, H-8) |
| 9 | 50.1 (CH) | 0.92 (1H, m, H-9) |
| 10 | 36.5 (C) | - |
| 11 | 20.7 ($CH_2$) | 0.99 (2H, m, H-11) |
| 12 | 40.0 ($CH_2$) | 2.01 (1H, m, H-12a) |
| | | 1.97 (1H, m, H-12b) |
| 13 | 42.3 (C) | - |
| 14 | 54.6 (CH) | 0.83 (1H, m, H-14) |
| 15 | 36.9 ($CH_2$) | 2.15 (1H, m, H-15a) |
| | | 1.15 (1H, m, H-15b) |
| 16 | 72.6 (CH) | 4.33 (1H, m, H-16) |
| 17 | 61.3 (CH) | 0.97 (1H, m, H-17) |
| 18 | 13.1 ($CH_3$) | 0.87 (3H, s, $CH_3$-18) |
| 19 | 19.4 ($CH_3$) | 1.03 (3H, s, $CH_3$-19) |
| 20 | 31.5 (CH) | 1.52 (1H, m, H-20) |
| 21 | 18.2 ($CH_3$) | 0.77 (3H, d, 6.4, $CH_3$-21) |
| 22 | 33.8 ($CH_2$) | 2.34 (2H, m, H-22) |
| 23 | 30.8 ($CH_2$) | 1.30 (2H, m, H-23) |
| 24 | 38.9 (CH) | 1.19 (1H, m, H-24) |
| 25 | 30.1 (CH) | 1.23 (1H, m, H-25) |
| 26 | 18.3 ($CH_3$) | 0.96 (3H, d, 7.0, $CH_3$-26) |
| 27 | 20.3 ($CH_3$) | 0.83 (3H, d, 7.0, $CH_3$-27) |
| 28 | 15.4 ($CH_3$) | 0.78 (3H, d, 7.0, $CH_3$-28) |

The $\alpha$ orientations of $CH_3$-21 ($\delta_C$ 18.2) and $CH_3$-28 ($\delta_C$ 15.4) were then elucidated as the same as its analogs of those reported ergostane [$CH_3$-21$\alpha$ ($\delta_C$ 18.3) and $CH_3$-28$\alpha$ ($\delta_C$ 15.8)] by an unambiguous ORTEP representation of X-ray experiment (Mei *et al.,* 2019). Compound **1** was identified by data comparison with a previously reported compound from soft coral *Cladiella krempfi* and identified as 3$\beta$,16$\beta$-dihydroxy-24(*S*)-methylcholestenol (Sarma *et al.,* 1995), however, the 1D NMR data was reported for the first time in this study.

Compound **2** was obtained as a colorless crystalline, with the result of HR-ESI-QTOFMS spectra showed $[M+H]^+$ *m/z* 399.3474, (calcd. *m/z* 399.3638), with molecular formula $C_{28}H_{47}O$, which required six degrees of unsaturation including two double bond and tetracyclic rings. The $^1H$, $^{13}C$, and DEPT 135° NMR spectra of **2** (**Table 2**) were almost identical to those of **1**, except for the presence of one more olefinic group $\delta_H$ 5.14 (m, H-22) and 5.02 (m, H-23) and the absence of hydroxyl group at C-16. The $^1H$-NMR spectrum of **2** showed the presence of five methyl groups at $\delta_H$ 0.87 (s, $CH_3$-18), 1.01 (s, $CH_3$-19), including secondary methyl groups at $\delta_H$ 0.77 (d, *J*=6.4, $CH_3$-21), 1.02 (d, *J*=7.0, $CH_3$-26), and 0.92 (d, *J*=7.0, $CH_3$-27), one oxygenated methine at $\delta_H$ 3.52 (m, H-3), and three olefinic methines at $\delta_H$ 5.35 (m, H-6) also 5.14 (m, H-22) and 5.02 (m, H-23). The $^{13}C$-NMR spectrum of **2** presented 28 signals of carbons, attributed to six methyl at $\delta$c (12.2, 17.8, 19.6, 19.8, 21.1, 20.1), eight methylenes at $\delta$c (21.2, 24.4, 28.7, 29.9, 31.8, 37.4, 39.8), eleven methines together with one oxygenated and three olefinic methines at $\delta$c (32.1, 33.3, 40.3, 43.0, 50.3, 57.0, 56.2, 72.0, 121.9, 136.0, 131.9), four quaternary carbons at $\delta$c (36.7, 42.5, 140.9). Thus, compound **2** was assigned as ergosta-5,22-dien-3$\beta$-ol according to data from the literature which is identical to ergostane-type steroid isolated from the fungus *Gyromitra esculenta* (Suleimen *et al.,* 2021).

Compound **3** was gained as a white amorph its molecular formula was identified as $C_{28}H_{46}O$ according to the HR-ESI-QTOFMS spectra *m/z* 399.3617 $[M+H]^+$, (calcd. $C_{28}H_{46}O$, *m/z* 399.3627), which represents six unsaturated degrees. The $^1H$-NMR spectra of **3** showed the existence of two tertiary methyls resonance at $\delta_H$ 0.54 (s, H-18), 0.83 (s, H-19) and three secondary methyls at $\delta_H$ 0.97 (3H, d, *J*=7.0 Hz, $CH_3$-26), 1.02 (3H, d, *J*=7.0 Hz, $CH_3$-27), and 0.91 (3H, d, *J*=6.4 Hz, $CH_3$-21). The $^{13}C$-NMR spectra detailed with DEPT 135º of **3** demonstrated 28 carbons, including five methyls at $\delta_C$ (12.0, 19.3, 13.2, 22.1, 22.0), eleven methylenes including one methylene olefinic at $\delta_C$ (19.0, 21.7, 22.9, 30.9, 36.4, 34.8, 37.2, 39.0, 38.7, 39.8, 106.1), eight methines consisting one oxygenated methine and one $sp^2$ methine at $\delta_C$ (29.7, 39.7, 43.4, 49.6, 56.0, 55.0, 71.2, 117.5), four quaternary carbons including two olefinic carbons at $\delta_C$ (34.8, 40.2, 139.1, 157.1). The above data showed the characteristics of ergostane-type steroid, specifically the presence of one pair of olefinic bond at $\delta_C$ 117.5 (C-7) and 139.1 (C-8). Furthermore, one remaining degree of unsaturation was fulfilled by another terminal olefinic positioned at $\delta_C$ 157.1 (C-24) and 106.1 (C-28). After careful analysis of 1D-NMR data showed that compound **3** was identical with ergosta-7,24(28)-dien-3$\beta$-ol isolated from *Neurospora crassa* (Morris *et al.,* 1974) which was isolated for the first time in the *Dysoxylum* genus, particularly in *D. alliaceum*.

Compound **4** was purified as a white powder with a molecular formula $C_{28}H_{46}O_3$, determined by its HR-ESI-QTOFMS *m/z* 453.3358 $[M+Na]^+$ (calcd. *m/z* 453.3345) which indicated six unsaturated degrees. Compound **4** has the same skeleton as **2** and showed the existence of olefinic at position C-22 (132.3) and C-23 (135.2) referring to ergostane-type steroid. The difference of **4** was the existence of two quaternary oxygenated carbons, along with two pair methines olefinic at $\delta_C$ 135.4 (C-6) and 130.7 (C-7), leading to the polyhydroxy ergostane-type at $\delta_C$ 82.2 (C-5) and 79.4 (C-8). Afterward, additional examination and a review of the literature revealed that the 1D-NMR data of 4 (**Table 3**) had strong similarities to 22(*E*)-ergosta-6,22-dien-3$\beta$,5$\alpha$,8$\alpha$-triol from the ethanolic extract of *Letinus edodes* (Shiitake) (Rivera *et al.,* 2009). To the best of our knowledge, 22(*E*)-ergosta-6,22-dien-3$\beta$,5$\alpha$,8$\alpha$-triol (**4**), as a polyhydroxylated ergostane-type steroid was first ever reported from *D. alliaceum*.

Compound **5** was gained as a colorless crystal with a molecular formula of $C_{21}H_{32}O_2$ which was identified by HR-ESI-MS *m/z* 317.2412 $[M+H]^+$ (calcd. *m/z* 317.2481), indicating six unsaturated degrees. The FT-IR spectra of **5** displayed the absorption bands of OH- (3420 $cm^{-1}$), CH $sp^3$ (2968 $cm^{-1}$), and C=O (1708 $cm^{-1}$) groups. Furthermore, the $^1H$-NMR spectra of **5** demonstrated the signals of two tertiary methyls at $\delta_H$ 0.78 (s, H-18) and 1.17 (s, H-19), one secondary methyl at $\delta_H$ 1.13 (3H, d, *J* =6.5 Hz, $CH_3$-21), one olefinic methine at $\delta_H$ 5.71 (s, H-4) and one oxygenated methine at $\delta_H$ 3.71 (1H, m, H-20). In addition, the $^{13}C$-NMR and DEPT 135º spectra of **5** exhibited the presence of 21 carbons, containing three methyl carbons at $\delta_C$ (12.5, 17.4, 23.8), eight methylene carbons at $\delta_C$ (20.9, 24.5, 25.6, 35.7, 32.1, 32.9, 34.0, 39.7), six methine carbons counting for one oxygenated and one olefinic methine at $\delta_C$ (35.5, 53.8, 55.4, 58.4, 70.51, 123.8), four quaternary carbons including olefinic and carbonyl ketone at $\delta_C$ (35.5, 42.4, 171.6, 190.7). The $^1H$, $^{13}C$, and DEPT 135º NMR data above suggested that **5** is a pregnane derivative. The presence of one double bond and one carbonyl was calculated for two unsaturated degrees, therefore the four unassigned hydrogen deficiency indexes corresponded to the tetracyclic ring systems. The location of carbonyl ketone moiety was determined at C-3 based on the biogenesis pathway of the pregnane type-steroid

**Table 2**. $^{13}$C-NMR ($CDCl_3$, 125 MHz) data comparison of compounds **2** and **3** with literature

| Position Carbon | **2** $\delta_C$ (type) | ergosta-5,22-dien-3$\beta$-ol $\delta_C$ (type) | **3** $\delta_C$ (type) | ergosta-7,24(28)-dien-3$\beta$-ol $\delta_C$ (type) |
|---|---|---|---|---|
| 1 | 37.4 ($CH_2$) | 37.4 ($CH_2$) | 36.4 ($CH_2$) | 36.3 ($CH_2$) |
| 2 | 29.9 ($CH_2$) | 30.1 ($CH_2$) | 37.4 ($CH_2$) | 37.3 ($CH_2$) |
| 3 | 72.0 (CH) | 72.0 (CH) | 71.1 (CH) | 71.2 (CH) |
| 4 | 42.4 ($CH_2$) | 42.4 ($CH_2$) | 39.0 ($CH_2$) | 38.0 ($CH_2$) |
| 5 | 140.9 (C) | 140.9 (C) | 43.4 (CH) | 40.4 (CH) |
| 6 | 121.9 (CH) | 121.9 (CH) | 38.7 ($CH_2$) | 38.0 ($CH_2$) |
| 7 | 31.8 ($CH_2$) | 32.0 ($CH_2$) | 117.5 (CH) | 117.6 (CH) |
| 8 | 32.1 (CH) | 32.0 (CH) | 139.1 (C) | 139.7 (C) |
| 9 | 50.3 (CH) | 50.3 (CH) | 49.6 (CH) | 49.6 (CH) |
| 10 | 36.7 (C) | 36.7 (C) | 34.8 (C) | 34.8 (C) |
| 11 | 20.9 ($CH_2$) | 21.2 ($CH_2$) | 19.0 ($CH_2$) | 19.0 ($CH_2$) |
| 12 | 40.1 ($CH_2$) | 39.8 ($CH_2$) | 30.9 ($CH_2$) | 31.2 ($CH_2$) |
| 13 | 42.5 (C) | 42.4 (C) | 40.2 (C) | 40.4 (C) |
| 14 | 57.0 (CH) | 57.0 (CH) | 39.7 (CH) | 39.7 (CH) |
| 15 | 23.9 ($CH_2$) | 24.4 ($CH_2$) | 21.7 ($CH_2$) | 21.7 ($CH_2$) |
| 16 | 29.1 ($CH_2$) | 28.7 ($CH_2$) | 22.9 ($CH_2$) | 23.1 ($CH_2$) |
| 17 | 55.9 (CH) | 56.2 (CH) | 29.7 (CH) | 29.8 (CH) |
| 18 | 11.9 ($CH_3$) | 12.1 ($CH_3$) | 12.0 ($CH_3$) | 12.0 ($CH_3$) |
| 19 | 19.6 ($CH_3$) | 19.6 ($CH_3$) | 13.2 ($CH_3$) | 13.2 ($CH_3$) |
| 20 | 40.3 (CH) | 40.3 (CH) | 56.0 (CH) | 56.2 (CH) |
| 21 | 21.1 ($CH_3$) | 21.1 ($CH_3$) | 19.3 ($CH_3$) | 19.0 ($CH_3$) |
| 22 | 136.0 (CH) | 136.0 (CH) | 34.8 ($CH_2$) | 34.8 ($CH_2$) |
| 23 | 131.9 (CH) | 131.9 (CH) | 39.8 ($CH_2$) | 39.7 ($CH_2$) |
| 24 | 43.0 (CH) | 43.0 (CH) | 157.1 (C) | 157.0 (C) |
| 25 | 33.3 (CH) | 33.3 (CH) | 55.0 (CH) | 55.2 (CH) |
| 26 | 20.1 ($CH_3$) | 20.1 ($CH_3$) | 22.1 ($CH_3$) | 22.0 ($CH_3$) |
| 27 | 19.5 ($CH_3$) | 19.8 ($CH_3$) | 21.8 ($CH_3$) | 22.1 ($CH_3$) |
| 28 | 17.8 ($CH_3$) | 17.8 ($CH_3$) | 106.3 ($CH_2$) | 106.2 ($CH_2$) |

(Kurimoto *et al.,* 2011). After careful analysis of 1D-NMR data showed that compound **5** was identical with 20$\alpha$-dihydroprogesterone (Nakano *et al.,* 1988) which was reported as the first pregnane-type steroid isolated from *D. alliaceum*.

## Cytotoxic Activity

Using the A549 lung cancer cell lines, the cytotoxic activity of all isolated compounds **1**–**5** was assessed with previously reported methodology (Kautsari *et al.,* 2024; Hidayat *et al.*, 2017) and doxorubicin of 2.10 $\mu$M was used as the positive control. The A549 cell line has a poor prognosis due to its resistance to current therapies (Huang & Zou, 2011). As shown in **Table 4**, compound **5** had the most potential cytotoxic activity against A549 lung cancer cell lines with an $IC_{50}$ value of 68.52 $\mu$M, while compounds **1**-**4** were inactive. These results were in line with previous data, that the ergostane-type steroids were deemed inactive due to the presence of extra C-28 and $\Delta^{5,6}$ displayed a completed decrease of the activity in compounds **1**-**4** against A549 cell lines (Yan *et al.,* 2014).

The structure-activity relationship studies showed that the existence of a hydroxyl group at the C-16 position in ergostane-type steroid distinctly of compound **1** might increase its cytotoxic activity. Meanwhile, two pairs of double bond appearance at C-7, C-8, and C-24, C-28 in compound **3,** as well as two pairs of double bond resonance at C-6, C-7, and C-22, C-23 in compounds **2** and **4** extremely decreased their cytotoxic activity. Among all the characterized steroids, pregnane-type showed the lowest cytotoxic with an $IC_{50}$ value of 68.52 $\mu$M.

**Table 3**. $^{13}$C-NMR ($CDCl_3$, 125 MHz) data comparison of compounds **4** and **5** with literature

| Position Carbon | **4** | 22(E)-ergosta-6,22-dien-3β,5α,8α-triol | **5** | 20α-dihydroprogesterone |
|---|---|---|---|---|
| | $\delta_C$ (type) | $\delta_C$ (type) | $\delta_C$ (type) | $\delta_C$ (type) |
| 1 | 34.7 ($CH_2$) | 34.7 ($CH_2$) | 35.7 ($CH_2$) | 35.7 ($CH_2$) |
| 2 | 39.3 ($CH_2$) | 39.3 ($CH_2$) | 34.0 ($CH_2$) | 34.0 ($CH_2$) |
| 3 | 66.5 (CH) | 66.5 (CH) | 199.7 (C) | 198.9 (C) |
| 4 | 36.9 ($CH_2$) | 36.9 ($CH_2$) | 123.8 (CH) | 123.8 (CH) |
| 5 | 82.2 (C) | 82.2 (C) | 171.6 (C) | 170.6 (C) |
| 6 | 135.4 (CH) | 135.4 (CH) | 32.7 ($CH_2$) | 32.7 ($CH_2$) |
| 7 | 130.7 (CH) | 130.7 (CH) | 32.1 ($CH_2$) | 31.9 ($CH_2$) |
| 8 | 79.4 (C) | 79.4 (C) | 35.5 (CH) | 35.5 (CH) |
| 9 | 51.7 (CH) | 51.7 (CH) | 53.8 (CH) | 53.6 (CH) |
| 10 | 37.0 (C) | 37.0 (C) | 38.5 (C) | 38.5 (C) |
| 11 | 20.6 ($CH_2$) | 20.6 ($CH_2$) | 21.6 ($CH_2$) | 21.0 ($CH_2$) |
| 12 | 30.1 ($CH_2$) | 30.1 ($CH_2$) | 39.7 ($CH_2$) | 38.6 ($CH_2$) |
| 13 | 44.6 (C) | 44.6 (C) | 42.4 (C) | 43.8 (C) |
| 14 | 55.9 (CH) | 56.2 (CH) | 55.4 (CH) | 55.9 (CH) |
| 15 | 22.9 ($CH_2$) | 23.4 ($CH_2$) | 24.5 ($CH_2$) | 24.8 ($CH_2$) |
| 16 | 28.6 ($CH_2$) | 28.6 ($CH_2$) | 25.6 ($CH_2$) | 25.2 ($CH_2$) |
| 17 | 33.1 (CH) | 33.1 (CH) | 58.4 (CH) | 57.9 (CH) |
| 18 | 12.9 ($CH_3$) | 12.9 ($CH_3$) | 12.5 ($CH_3$) | 12.2 ($CH_3$) |
| 19 | 18.2 ($CH_3$) | 18.2 ($CH_3$) | 17.4 ($CH_3$) | 17.3 ($CH_3$) |
| 20 | 42.8 (CH) | 42.8 (CH) | 70.5 (CH) | 70.0 (CH) |
| 21 | 17.6 ($CH_3$) | 17.6 ($CH_3$) | 23.2 ($CH_3$) | 22.8 ($CH_3$) |
| 22 | 132.3 (CH) | 132.3 (CH) | | |
| 23 | 135.2 (CH) | 135.2 (CH) | | |
| 24 | 39.7 (CH) | 39.7 (CH) | | |
| 25 | 51.5 (CH) | 51.1 (CH) | | |
| 26 | 19.9 ($CH_3$) | 19.9 ($CH_3$) | | |
| 27 | 19.6 ($CH_3$) | 19.6 ($CH_3$) | | |
| 28 | 20.9 ($CH_3$) | 20.9 ($CH_3$) | | |

**Table 4**. Cytotoxic evaluation of compounds **1**-**5** against A549 lung cancer cell lines.

| Compounds | $IC_{50}$ (μM) |
|---|---|
| 3β,16β-dihydroxy-24(*S*)-methylcholestenol (**1**) | 72.24 |
| Ergosta-5,22-dien-3β-ol (**2**) | >150 |
| Ergosta-7,24(28)-dien-3β-ol (**3**) | >150 |
| 22(*E*)-ergosta-6,22-dien-3β,5α,8α-triol (**4**) | 130.40 |
| 20α-dihydroprogesterone (**5**) | 68.52 |
| Doxorubicin* | 2.10 |

*Positive control

## CONCLUSIONS

In these phytochemical studies, we report the isolation and characterization of five known steroids from the *Dysoxylum alliaceum* stem bark including 3β,16β-dihydroxy-24(*S*)-methylcholestenol (**1**), ergosta-5,22-dien-3β-ol (**2**), ergosta-7,24(28)-dien-3β-ol (**3**), 22(*E*)-ergosta-6,22-dien-3β,5α,8α-triol (**4**) and 20α-dihydroprogesterone (**5**). Ergostane-type steroids (**1**-**4**) and progesterone-type steroid (**5**) were gained for the first time from this plant species. Compound **5** showed the potential cytotoxic activity with $IC_{50}$ 68.52 μM, while the remaining isolated compounds showed no inhibition with $IC_{50}$ values ranging from 72.24 to > 150 μM against the A549 lung cancer cell line. The structure-activity relationship showed that the existence of addition C-28 and 5,6 double bonds in ergostane-type steroids can significantly decrease the cytotoxic activity.

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## CONFLICT OF INTEREST

The authors declare that they hold no competing interests.

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