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STEROID AND TRITERPENOID COMPOUNDS ISOLATED FROM *AMANITA PANTHERINA* AND THEIR ANTI-INFLAMMATORY ACTIVITY

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Summary

Steroid and Triterpenoid Compounds Isolated from *Amanita pantherina* and their Anti-Inflammatory Activity

Phytochemical research of the ethanolic extract of *Amanita pantherina* sensu Gonnermann & Rabenhorst led to the isolation of six steroids (**1**–**6**) and a triterpenoid (**7**), using various chromatographic separations. Their structures were elucidated to be *β*-sitosterol (**1**), stigmasterol (**2**), cycloeucalenol (**3**), spinasterol (**4**), daucosterol (**5**), stigmasterol 3-*O*-*β*-D-glucoside (**6**), and betulin (**7**) by detailed analysis of NMR spectroscopic records and comparison with those reported. Compound **7** showed the most inhibitory activity (IC_{50} = 33.4 μM) against the LPS-induced NO production in macrophage RAW 264.7 cells, followed by compound **3** with an IC_{50} value of 48.2 μM. Compounds **1**, **2**, and **4** exhibited weak inhibitory activity with IC_{50} values of 92.8, 64.9, and 62.4 μM, respectively, while compounds **5** and **6** were inactive (IC_{50} > 100 μM). This is the first time compounds **3** and **7** have been evaluated for their inhibitory effects on NO production. In addition, compounds **2**, **3**, and **7** showed weak inhibitory activity against tumor necrosis factor-alpha (TNF-*α*) with IC_{50} values of 83.9, 72.8, and 58.1 μM, respectively.

Keywords: *Amanita pantherina* sensu Gonnermann & Rabenhorst, *Sterol, Inflammatory activity, NO production, Cytotoxic, RAW264.7 cells.*

1. Introduction

Amanita pantherina sensu Gonnermann & Rabenhorst has fascinated both traditional cultures and modern researchers due to its striking appearance and complex biochemical properties. Historically, it has been used in rituals and conventional medicine. Today, its bioactivity, particularly muscimol and ibotenic acid, has drawn attention to potential therapeutic applications despite its toxicity [1],[2]. In contemporary research, the bioactivity of *A. pantherina* has garnered attention for its potential therapeutic applications. Studies have revealed that this mushroom contains compounds such as alkaloids (muscimol and ibotenic acid) and sterols [3],[4]. Research has highlighted various health benefits of *A. pantherina*, including its potential neuroactive properties [3]. While primarily recognized for its psychoactive properties, the anti-inflammatory effects of *A. pantherina*, specifically through nitric oxide inhibition, remain underexplored. Given the risks associated with its toxicity, this study focuses on *in vitro* research to evaluate the safety and efficacy of its compounds. Nitric oxide is produced from L-arginine by nitric oxide synthases (NOS), with the inducible form (iNOS) being upregulated by inflammatory stimuli. Elevated NO levels contribute to tissue damage and inflammatory diseases, making iNOS inhibitors of significant interest [5],[6]. Therefore, compounds inhibiting iNOS and reducing NO production greatly interest anti-inflammatory research. This

study elucidates how *A. pantherina* may contribute to anti-inflammatory strategies, linking traditional knowledge with modern scientific research.

2. Materials and Methods

2.1. General experimental procedures

The NMR spectra (^{1}H- and ^{13}C-NMR) were recorded by the Bruker Avance 500 and 600 MHz spectrometers (Bruker Daltonics, Ettlingen, Germany). *Silica gel* (Si 60 F_{254}, 40-63 mesh, Merck, St. Louis, MO, USA), YMCGEL (ODS-A, 12 nm S-150 μm, YMC Co., Ltd., Kyoto, Japan), and Sephadex LH-20 (Sigma-Aldrich, MO, USA) were utilized for column chromatography (CC). Before use, all solvents were redistilled. TLC plates pre-coated, including *Silica gel* 60 F_{254} and RP-C18 F_{254S} (Merck, Darmstadt, Germany), were employed for analytical purposes. Compounds were detected by UV radiation (254 nm and 365 nm) after eluting with the solvent system or spraying with 10% H_2SO_4, followed by heating using a heat gun.

2.2. Plant materials

The fruiting body of *A. pantherina* was collected in February 2023 from Dak Lak province, Vietnam. Botanical identification was performed by Assoc. Prof. Dr. Nguyen Phuong Dai Nguyen at Tay Nguyen University and a voucher specimen (AP-FB-04022023) has been submitted to the Department of Biology, Faculty of Natural Sciences and Technology, Tay Nguyen University.

2.3. Extraction and isolation

The fruiting bodies of *A. pantherina* (0.5 kg) were removed impurities, cut into small pieces, washed, dried, and ground into powder. The dried powder was then extracted using methanol (MeOH, three times, 3 × 2.0 L) by refluxing. After evaporating the solvent, 50 g of MeOH extract was suspended in hot water and then successfully partitioned to obtain dichloromethane- (CH_2Cl_2, 10.5 g), ethyl acetate- (EtOAc, 12.3 g), and water-soluble (27.2 g) fractions. The CH_2Cl_2 fraction (10.5 g) was subjected to *silica gel* column chromatography (CC) with *n*-hexane - acetone (50:1 - 0:1) to obtain eight fractions (APD1-APD8). Fraction APD2 (1.1 g) was purified via *silica gel* CC and eluted with *n*-hexane - EtOAc (30:1 - 10:1), producing four sub-fractions (APD2.1 to APD2.4). The sub-fraction APD2.3 (250 mg) was processed on an ODS column and then eluted with acetone - water (2:1 - 5:1), yielding compounds **1** (10.2 mg) and **2** (8.9 mg). The APD3 fraction (450 mg) was also subjected to *silica gel* CC with *n*-hexane - EtOAc (15:1 to 7:1), resulting in three sub-fractions (APD3.1-APD3.3). Sub-fraction APD3.3 (80 mg) was further purified using an ODS column and eluted with acetone - water (3:1 - 5:1), leading to the isolation of compounds **3** (5.2 mg) and **4** (6.5 mg). The APD5 fraction (1.6 g) was also subjected to *silica gel* CC with *n*-hexane:

EtOAc (5:1 - 1:1) to obtain five sub-fractions (APD5.1-APD5.5). Sub-fraction APD5.4 (120 mg) was further purified using an ODS column and then eluted with MeOH - water (2:1 to 4:1), resulting in the isolation of compounds **5** (5.5 mg) and **6** (6.2 mg). The APD7 fraction (4.5 g) was subjected to *silica gel* CC with CH_2Cl_2-EtOAc (10:1 to 1:1), resulting in fourteen sub-fractions (APD7.1-APD7.14). Sub-fraction APD7.7 (65 mg) was further purified using an ODS column and eluted with methanol:water (1:1 - 4:1), leading to the isolation of compound **7** (3.8 mg).

Compound **1**: White powder; ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H (ppm): 3.51 (1H, m, H-3), 5.35 (1H, t, J = 4.5 Hz, H-6), 0.90 (3H, d, J = 6.4 Hz, CH_3-21), 0.81 (3H, t, J = 7.2 Hz, CH_3-29), 0.83 (3H, d, J = 6.4 Hz, CH_3-26), 0.78 (3H, d, J = 6.4 Hz, CH_3-27), 0.68 (3H, s, CH_3-18), 0.98 (3H, s, CH_3-19); ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C (ppm): 37.3 (C-1), 40.1 (C-2), 72.2 (C-3), 42.5 (C-4), 141.1 (C-5), 121.8 (C-6), 32.1 (C-7), 31.8 (C-8), 50.5 (C-9), 36.9 (C-10), 21.2 (C-11), 40.2 (C-12), 42.5 (C-13), 57.1 (C-14), 26.5 (C-15), 28.6 (C-16), 56.5 (C-17), 36.6 (C-18), 19.5 (C-19), 34.4 (C-20), 24.4 (C-21), 46.2 (C-22), 23.1 (C-23), 12.3 (C-24), 29.3 (C-25), 20.2 (C-26), 19.5 (C-27), 19.1 (C-28), 11.8 (C-29).

Compound **2**: White powder; ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H (ppm): 3.53 (1H, m, H-3), 5.37 (1H, br d, J = 4.5 Hz, H-6), 5.15 (1H, dd, J = 3.5, 15.5 Hz, H-22), 5.02 (1H, dd, J = 3.5, 15.5 Hz, H-23), 0.92 (3H, d, J = 6.5 Hz, CH_3-21), 0.83 (3H, d, J = 6.5 Hz, CH_3-26), 0.81 (3H, d, J = 6.5 Hz, CH_3-27), 0.84 (3H, t, J = 6.5 Hz, H-29), 0.70 (3H, s, CH_3-18), 1.01 (3H, s, CH_3-19); ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C (ppm): 37.3 (C-1), 31.7 (C-2), 71.8 (C-3), 42.4 (C-4), 140.8 (C-5), 121.6 (C-6), 31.9 (C-7), 32.1 (C-8), 50.2 (C-9), 36.6 (C-10), 21.1 (C-11), 39.7 (C-12), 42.4 (C-13), 56.9 (C-14),

24.4 (C-15), 29.0 (C-16), 56.1 (C-17), 12.1 (C-18), 19.4 (C-19), 40.6 (C-20), 21.1 (C-21), 138.4 (C-22), 129.3 (C-23), 51.3 (C-24), 31.9 (C-25), 21.3 (C-26), 19.0 (C-27), 25.4 (C-28), 12.3 (C-29).

Compound **3**: White powder; ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H (ppm): 4.72 (1H, br s, H-30a), 4.67 (1H, br s, H-30b), 3.22 (1H, m, H-3), 2.24 (1H, m, H-17), 1.04 (3H, d, J = 6.5 Hz, CH_3-27), 1.03 (3H, J = 6.5 Hz, CH_3-26), 0.99 (3H, d, J = 6.5 Hz, CH_3-28), 0.98 (3H, s, CH_3-18), 0.91 (3H, d, J = 5.5 Hz, CH_3-21), 0.90 (3H, s, CH_3-29), 0.39 (1H, d, J = 4.5 Hz, H-19a), 0.15 (1H, d, J = 4.5 Hz, H-19b); ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C (ppm): 31.0 (C-1), 35.0 (C-2), 76.8 (C-3), 44.8 (C-4), 43.5 (C-5), 24.9 (C-6), 25.4 (C-7), 47.1 (C-8), 23.8 (C-9), 29.7 (C-10), 28.3 (C-11), 33.1 (C-12), 45.6 (C-13), 49.1 (C-14), 35.6 (C-15), 27.2 (C-16), 52.4 (C-17), 18.0 (C-18), 27.5 (C-19), 36.4 (C-20), 18.6 (C-21), 35.2 (C-22), 31.5 (C-23), 157.1 (C-24), 34.0 (C-25), 22.2 (C-26), 22.1 (C-27), 14.6 (C-28), 19.3 (C-29), 106.1 (C-30).

Compound **4**: White powder; ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H (ppm): 3.59 (1H, m, H-3), 5.15 (1H, overlapped, H-7), 5.15 (1H, overlapped, H-22), 5.03 (1H, dd, J = 5.0, 12.5 Hz, H-23), 1.03 (3H, d, J = 5.5 Hz, CH_3-21), 0.81 (3H, d, J = 5.5 Hz, CH_3-26), 0.80 (3H, d, J = 5.5 Hz, CH_3-27), 0.83 (3H, t, J = 6.5 Hz, CH_3-29); 0.54 (3H, s, CH_3-18), 0.82 (3H, s, CH_3-19); ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C (ppm): 37.2 (C-1), 31.6 (C-2), 71.1 (C-3), 38.1 (C-4), 40.5 (C-5), 29.7 (C-6), 117.5 (C-7), 139.6 (C-8), 49.6 (C-9), 34.3 (C-10), 21.6 (C-11), 39.6 (C-12), 43.2 (C-13), 55.2 (C-14), 23.1 (C-15), 28.6 (C-16), 56.0 (C-17), 12.1 (C-18), 13.0 (C-19), 40.8 (C-20), 21.5 (C-21), 138.3 (C-22), 129.4 (C-23), 51.3 (C-24), 31.9 (C-25), 21.2 (C-26), 19.0 (C-27), 25.4 (C-28), 12.3 (C-29).

Compound **5**: White amorphous powder; ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H (ppm): 3.43 (1H, m, H-3), 5.32 (1H, s, H-6), 0.93 (3H, d, J = 6.5 Hz, CH_3-21), 0.83 (3H, t, J = 7.0 Hz, CH_3-29); 0.84 (3H, t, J = 6.5 Hz, CH_3-26), 0.81 (3H, d, J = 6.5 Hz, CH_3-27), 0.68 (3H, s, CH_3-18), 1.00 (3H, s, CH_3-19), 4.22 (1H, d, J = 7.5 Hz, H-1′), 2.90-3.12 (4H, m, H-2′/H-3′/H-4′/H-5′), 3.40 (1H, m, H-6′a), 3.64 (1H, dd, J = 6.0, 10.0 Hz, H-6′b); ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C (ppm): 36.8 (C-1), 29.3 (C-2), 76.9 (C-3), 38.3 (C-4), 140.4 (C-5), 121.1 (C-6), 31.4 (C-7), 31.3 (C-8), 49.6 (C-9), 36.2 (C-10), 20.6 (C-11), 39.0 (C-12), 41.8 (C-13), 56.2 (C-14), 23.8 (C-15), 27.7 (C-16), 55.4 (C-17), 11.6 (C-18), 18.9 (C-19), 35.4 (C-20), 18.6 (C-21), 33.3 (C-22), 25.5 (C-23), 45.1 (C-24), 28.7 (C-25), 19.7 (C-26), 19.1 (C-27), 22.6 (C-28), 11.7 (C-29), 100.8 (C-1′), 73.4 (C-2′), 76.8 (C-3′), 70.1 (C-4′), 76.7 (C-5′), 61.1 (C-6′).

Compound **6**: White amorphous powder; ^{1}H-NMR (600 MHz, Pyridine-d_5) δ_H (ppm): 3.95 (1H, m, H-3), 5.34 (1H, br d, J = 4.8 Hz, H-6), 5.22 (1H, dd, J = 9.0, 15.0 Hz, H-22), 5.06 (1H, dd, J = 9.0, 15.0 Hz, H-23), 0.97 (3H, d, J = 6.6 Hz, CH_3-21), 0.91 (3H, d, J = 6.6 Hz, CH_3-27), 0.86 (3H, d, J = 6.6 Hz, CH_3-27), 0.89 (3H, t, J = 6.6 Hz, H-29), 0.67 (3H, s, CH_3-18), 0.94 (3H, s, CH_3-19), 5.04 (1H, d, J = 7.5 Hz, H-1′), 4.55 (1H, d, J = 2.4, 11.4 Hz, H-6′a), 4.55 (1H, d, J = 5.4, 11.4 Hz, H-6′b), 3.94-4.27 (4H, m, H-2′/H-3′/H-4′/H-5′); ^{13}C-NMR (150 MHz, Pyridine-d_5) δ_C (ppm): 37.5 (C-1), 30.3 (C-2), 71.8 (C-3), 42.4 (C-4), 141.0 (C-5), 121.9 (C-6), 32.2 (C-7), 32.1 (C-8), 50.4 (C-9), 37.0 (C-10), 21.1 (C-11), 39.4 (C-12), 40.8 (C-13), 57.0 (C-14), 24.6 (C-15), 29.3 (C-16), 56.1 (C-17), 12.2 (C-18), 19.4 (C-19), 39.9 (C-20), 21.3 (C-21), 138.8 (C-22), 129.5 (C-23), 51.4 (C-24), 32.2 (C-25), 21.5 (C-26), 19.2 (C-27), 25.7 (C-28), 12.5 (C-29), 102.6 (C-1′), 78.6 (C-5′), 78.5 (C-3′), 78.1 (C-2′), 75.4 (C-4′), 62.9 (C-6′).

Compound **7**: White powder; ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H(ppm): 4.69 (1H, br s, H-29b), 4.58 (1H, br s, H-29a), 3.81 (1H, d, J = 11.5 Hz, H-28a), 3.36 (1H, d, J = 11.5 Hz, H-28b), 3.21 (1H, dd, J = 11.5, 5.5 Hz, H-3), 1.67 (3H, s, CH_3-30), 1.02 (3H, s, CH_3-26), 0.96 (3H, s, CH_3-27), 0.94 (3H, s, CH_3-23), 0.84 (3H, s, CH_3-25), 0.76 (3H, s, CH_3-24), 0.67 (1H, br d, J = 9.5 Hz, H-5); ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C (ppm): 38.9 (C-1), 27.5 (C-2), 79.0 (C-3), 38.8 (C-4), 55.6 (C-5), 18.6 (C-6), 34.6 (C-7), 39.3 (C-8), 50.7 (C-9), 37.4 (C-10), 21.3 (C-11), 25.6 (C-12), 37.2 (C-13), 41.2 (C-14), 27.1 (C-15), 29.7 (C-16), 47.0 (C-17), 48.7 (C-18), 48.1 (C-19), 150.8 (C-20), 29.9 (C-21), 34.3 (C-22), 28.3 (C-23), 15.6 (C-24), 16.5 (C-25), 16.3 (C-26), 14.9 (C-27), 60.7 (C-28), 109.8 (C-29), 19.4 (C-30).

2.4. Cell culture

The RAW 264.7 cells (from American Type Culture Collection, ATCC, USA) were cultured in Dulbecco's Modified Essential Medium (DMEM) supplemented with streptomycin (100 μg/mL), penicillin (100 units/mL), and 10% heat-inactivated fetal bovine serum (FBS, Cambrex, Charles City, IA, USA). The RAW 264.7 cells were maintained in a humidified 5% CO_2 atmosphere at a temperature of 37 °C.

2.5. Cell viability assay

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was employed to determine the viability of the cells [7]. Briefly, the RAW264.7 cells in DMEM (supplemented with 10% FBS and 1% penicillin-streptomycin) were plated in a 96-well plate. After incubation for 24 hours, the medium was replaced, the compounds (diluted with DMSO at varying concentrations) were added, and continued incubation for 24 hours. 20 µL of MTS was then added to each well and the absorbance was measured at 490 nm by microplate reader.

2.6. NO and TNF-α productions inhibitory activities

Measurement of nitrite in cell culture supernatants to determine the level of NO production. After being seeded in 24-well plates and incubated for 12 h, the RAW 264.7 cells (at a density of 1×10^5 cells/well) were treated with compounds (at concentrations of 1, 3, 10, and 30 µM) in DMEM without FBS. After 1 h treatment, the RAW 264.7 cells were stimulated with or without 1 µg/mL of LPS for 24 hours. The Griess reagent was employed to determine the nitrite levels [7]. Briefly, the mixture, including 100 µL of cell culture medium and 100 µL of Griess reagent, was incubated at room temperature for 10 min, and the absorbance was measured at 540 nm by a microplate reader (Biotek, Winooski, VT, USA). A blank sample (fresh culture medium) was employed in each experiment. A sodium nitrite ($NaNO_2$) standard curve was employed to determine the quantity of nitrite. The amount of TNF-α in the culture supernatant was measured using the ELISA kit (R&D Systems, Minneapolis, MN, USA). N(G)-monomethyl L-arginine (L-NMMA, 1-30 µM) was employed as a positive control in this experiment.

3. Result and Discussion

3.1. Determination of chemical structure

Screening tests of the CH_2Cl_2 soluble fraction from *A. pantherina* extracts led to the isolation of seven secondary metabolites (**1**–**7**) through column chromatography.

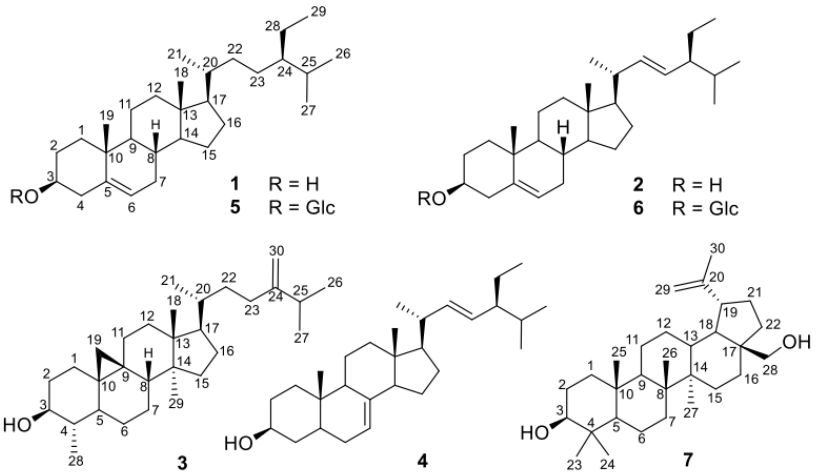

Fig. 1. Structure of the isolated compounds (**1-7**) from *A. pantherina.*

Compound **3** was isolated as a white powder. The ^{1}H-NMR spectrum of compound **3** showed signals for protons on a carbon-carbon double bond [δ_H 4.72 (1H, br s, H-30a), 4.67 (1H, br s, H-30b)], an oxymethine proton [δ_H 3.22 (1H, m, H-3)], and methylene protons [δ_H 0.39 (1H, d, J = 4.5 Hz, H-19a), 0.15 (1H, d, J = 4.5 Hz, H-19b)]. Signals at δ_H 1.04 (3H, d, J = 6.5 Hz, CH_3-27), 1.03 (3H, J = 6.5 Hz, CH_3-26), 0.99 (3H, d, J = 6.5 Hz, CH_3-28), 0.98 (3H, s, CH_3-18), 0.91 (3H, d, J = 5.5 Hz, CH_3-21), and 0.90 (3H, s, CH_3-29) in the ^{1}H-NMR spectrum of **3** indicate six methyl groups (Fig. 1). The ^{13}C-NMR spectrum displayed 30 carbon signals, including an oxymethine carbon [δ_C 76.8 (C-3)], six methyl carbons [C-18/C-21/C-26/C-27/C-28/C-29], and two carbon at δ_C 157.1 (C-24) and 106.1 (C-30), suggesting the presence of an olefinic group at C-24 (Fig. 1). It was confirmed by heteronuclear multiple bond correlation (HMBC) correlations from H-30a, H-

30b, C$\underline{H}_3$-26, and C$\underline{H}_3$-27 to C-24. The HMBC correlation from C$\underline{H}_3$-28 to C-3 confirmed the position of the hydroxyl group at C-3. Furthermore, the HMBC correlations from C$\underline{H}_3$-18 to C-13, C-14, and C-17, C$\underline{H}_3$-21 to C-17 and 20, and C$\underline{H}_3$-29 to C-13 and C-14 were also observed. The further detailed analysis of both 1D- and 2D-NMR spectra provides strong evidence for the structure of compound **3**. Thus, compound **3** was identified as cycloeucalenol [8].

Compound **7** was isolated as a white powder. The ^{1}H-NMR spectrum showed angular methyl protons at δ_H 1.67 (3H, s, CH_3-30), 1.02 (3H, s, CH_3-26), 0.96 (3H, s, CH_3-27), 0.94 (3H, s, CH_3-23), 0.84 (3H, s, CH_3-25), and 0.76 (3H, s, CH_3-24), indicating six methyl groups in the compound. The ^{1}H-NMR showed the proton of H-3 appeared as a doublet of a doublet at δ_H 3.21 (1H, dd, J = 11.5, 5.5 Hz, H-3) and a hydroxymethyl group [δ_H 3.81 (1H, d, J = 11.5 Hz, H-28a), 3.36 (1H, d, J = 11.5 Hz, H-28b)]. It also showed two olefinic protons at δ_H 4.69 (1H, br s, H-29b) and 4.58 (1H, br s, H-29a) representing the exocyclic double bond (Fig. 1). The ^{13}C-NMR spectrum of the compound indicated 30 carbon signals for the terpenoid of lupane skeleton, which includes a carbon bonded to the OH group at C-3 position appearing at δ_C 79.0 ppm, six methyl carbons at δ_C 28.3 (C-23), 15.6 (C-24), 16.5 (C-25), 16.3 (C-26), 14.9 (C-27), and 19.4 (C-30), and a hydroxymethyl carbon at δ_C 60.7 (C-28). The olefinic carbons of the exocyclic double bond appeared at δ_C 150.8 ppm and 109.8 ppm, which are assigned as C-20 and C-29 double bonds of the lupane-type triterpenoid compound (Fig. 1). Thus, the isolated compound was identified as betulin (**7**), which was consistent with the reported literature values [9].

The remaining compounds were identified as *β*-sitosterol (**1**) [10], stigmasterol (**2**) [10], spinasterol (**4**) [11], daucosterol (**5**) [12], and stigmasterol 3-*O*-*β*-D-glucoside (**6**) [13] by detailed analysis of NMR spectroscopic records and comparison with those reported.

3.2. NO and TNF-α productions inhibition activities

In the initial experiment, the cytotoxic effects of the seven isolates (**1**–**7**) were evaluated using the MTS assay [7]. The results indicated that the isolated compounds exhibited no toxicity to the RAW 264.7 cells at concentrations below 100 μM, with cell viability consistently maintained at over 90% (data not shown). Based on these findings, concentrations of 1, 3, 10, and 30 μM were chosen for further experiments to evaluate the effects of the compounds on NO production.

In the second research phase, the anti-inflammatory properties of the seven isolated compounds (**1**–**7**) were thoroughly evaluated using the Griess reaction assay on the RAW 264.7 cell line [7]. The results showed that compound **7** exhibited the most potent inhibitory activity, with an IC_{50} value of 33.4 μM, followed by compound **3** with an IC_{50} value of 48.2 μM, indicating their effectiveness in suppressing inflammatory processes within this cell line. Compounds **1**, **2**, and **4** exhibited weak inhibitory activity with IC_{50} values of 92.8, 64.9, and 62.4 μM, respectively. In contrast, compounds **5** and **6** demonstrated no significant anti-inflammatory activity (IC_{50} > 100 μM) (Table 1). In addition, these compounds were further investigated on the LPS-induced TNF-*α* release. Except for compounds **1** and **4**–**6**, pretreatment of cells with compounds **2**, **3**, and **7** in several concentrations (1–30 μM) decreased the TNF-*α* production (Table 1).

Table 1. *In vitro* inhibitory activity of the isolates (**1**–**7**) on NO and TNF-*α* productions

Compound	NO (IC_{50}, μM)[a]	TNF-*α* (IC_{50}, μM)[a]
1	92.8 ± 3.2	> 100
2	64.9 ± 4.1	83.9 ± 5.2
3	48.2 ± 2.4	72.8 ± 6.0
4	62.4 ± 4.2	> 100
5	> 100	> 100
6	> 100	> 100
7	33.4 ± 1.6	58.1 ± 4.4
L-NMMA[b]	21.5 ± 0.2	14.2 ± 0.8

[a] Results are represented as IC_{50} value (μM); [b] Positive control;
The data are expressed as the mean ± SD of three replicates (n = 3).

In the control group without LPS or compounds, NO accumulation increased approximately 9-fold after incubation for 24 hours. When compounds **3** and **7** were tested at concentrations of 1, 3, 10, and 30 μM, they reduced the nitrite accumulation in a dose-dependent manner following LPS-stimulated RAW 264.7 cells (Fig. 2). This finding further highlights the anti-inflammatory effect of compounds **3** and **7**, as they effectively inhibited NO production even in the presence of LPS-induced inflammatory stimulation.

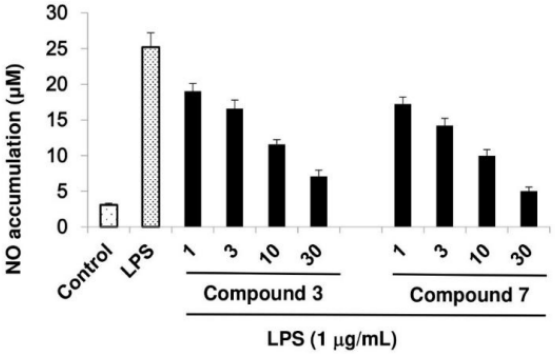

Fig. 2. Inhibition of LPS-induced NO production in RAW 264.7 cells by compounds **3** and **7**. Control values were recorded in the absence of both the compounds and LPS. The results are presented as the mean ± SD of triplicates (n = 3).

4. Conclusion

Seven compounds (**1**–**7**) were isolated from the fruiting body of *A. pantherina*. Their chemical structures were determined by NMR data and compared with the literature. The anti-inflammatory activity of the isolates (**1**–**7**) was evaluated by inhibiting LPS-induced NO production in macrophage RAW 264.7 cells. Compound **7** showed the most potent inhibitory activity on NO production with an IC_{50} value of 33.4 μM, followed by compound **3** with an IC_{50} value of 48.2 μM. Compounds **1**, **2**, and **4** showed IC_{50} values of 94.8, 64.9, and 62.4 μM, respectively, while **5** and **6** were inactive (IC_{50} > 100 μM). This is the first time compounds **3** and **7** have been evaluated for their inhibitory effects on NO production. In addition, compounds **2**, **3**, and **7** showed weak inhibitory activity against TNF-α with IC_{50} values of 83.9, 72.8, and 58.1 μM, respectively. The results suggested that *A. pantherina* and compounds **3** and **7** are promising candidates for further research and potential development as therapeutic agents targeting inflammatory processes.

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