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NOTE

Clerodane Diterpenes Isolated from *Polyalthia longifolia* Induce Apoptosis in Human Leukemia HL-60 Cells

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Abstract: *Polyalthia* is a versatile genus of shrubs and trees found in tropic and sub-tropic regions. In this study, three clerodane diterpenes, kolavenic acid (1), polyalthialdoic acid (2), and 16α-hydroxy-cleroda-3,13(14)*Z*-dien-15,16-olide (3) isolated from *Polyalthia longifolia* leaves were evaluated for their apoptotic potential against human leukemia HL-60 cells. Compounds 2 and 3 inhibited cell proliferation with IC_{50} values of 21.8 and 13.7 μM, respectively. Morphological changes and DNA fragmentation analysis indicated that these diterpenes induce apoptotic cell death in the HL-60 cells. Our results revealed the importance of *P. longifolia* as a chemopreventive medicinal plant.

Key words: *Polyalthia longifolia*, clerodane diterpene, HL-60 cell, antileukemic activity, apoptosis

1 INTRODUCTION

Polyalthia belongs to the *Annonaceae* family and includes approximately 120 species of shrubs and trees found in tropic and sub-tropic regions[1], of which *Polyalthia longifolia* Thw. is a tall, handsome, columnar, and evergreen ornamental tree. Traditionally, various parts of *P. longifolia* have been used for several medicinal purposes to treat fever, skin diseases, diabetes, hypertension, and helminthiasis[2, 3]. The extract of *P. longifolia* showed antioxidative, antimicrobial, antitumor, anti-inflammatory, and anticataractogenesis properties[4−8]. Previous phytochemical investigations of this plant revealed the presence of numerous chemical constituents with interesting biological activities. For example, new azafluorene alkaloids were isolated from the stem bark[9]. Alkaloids pendulamine A and B isolated from the root extract were found to be the antibacterial constituents[10]. Flavonoids and their glycosides isolated from the leaves possessed antioxidative potential[11]. Cytotoxic cycloartane triterpenes and clerodane diterpenes were isolated from the leaves[12, 13]. Thus, *P. longifolia* is a confirmed natural resource of biologically active compounds for human health.

Within the scope of our ongoing program aimed at the chemical study of Indonesian plants with a medicinally interesting profile, we investigated the chemical constituents of *P. longifolia*. In this research, the isolated clerodane diterpenes were evaluated for their antiproliferative activity against human leukemia HL-60 cells. We strived to better elucidate the biological properties of *P. longifolia*.

2 EXPERIMENTAL PROCEDURES

2.1 General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solvent using JEOL ECA 400 spectrometer with tetramethylsilane (TMS) as an internal standard. MS spectra were recorded on JEOL JMS-700/GI mass spectrometer. IR spectra were recorded on JASCO FT/IR-460 Plus spectrometer. Column chromatography (CC) was performed on silica gel 60 N (40-50 μm, spherical, neutral, Kanto Chemical Co., INC.,) and on Sephadex LH-20 (GE Healthcare, Japan). Thin layer

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chromatography (TLC) was performed on silica gel 60 F_{254} for analytical chromatography (200 μm layer thickness; Merck). Preparative thin-layer chromatography (PTLC) was performed on silica gel 60 F_{254} (1 mm layer thickness; Merck). All the chemicals used in this study were purchased from Wako Pure Chemical Industries, Ltd. and Sigma-Aldrich.

2.2 Plant material

The leaves of *P. longifolia* were collected from the Padang region, West Sumatra, Indonesia, in October 2012.

2.3 Extraction and isolation

Air-dried leaves (1 kg) were grounded and macerated successively at room temperature with *n*-hexane, ethyl acetate, and methanol. Ethyl acetate fraction was filtered and evaporated *in vacuo*. A portion of the ethyl acetate extract (60 g) was fractionated on silica gel, eluted with $CHCl_3$, acetone, and MeOH mixture of increasing polarity. This fractionation process provided 20 vials of the extract. Fractions having the same *Rf* value were combined and 9 fractions (FEA_1–FEA_9) were obtained. Fraction FEA_3 was subjected to CC on silica gel (*n*-Hexane:$CHCl_3$ = 1:1) to afford 12 (F_1–F_{12}) fractions. Fraction F_7 (0.1967 g) was further purified on Sephadex LH-20 using methanol as the eluent, followed by repeated separations on CC (*n*-Hexane: $CHCl_3$ = 1:1). This fractionation process gave 10 fractions ($F_{7.1}$–$F_{7.10}$). Fractions $F_{7.4}$–$F_{7.5}$ were furthermore separated using PTLC, $CHCl_3$: MeOH (30:1), whereby kolavenic acid (1, 40.8 mg) was obtained as pale yellow oil. A portion of fraction F_8 was subjected to CC (*n*-Hexane:$CHCl_3$ = 1:1) to give 8 fractions. The elutes were combined based on TLC analysis ($CHCl_3$: MeOH = 10:1) and further purified with PTLC using $CHCl_3$: MeOH (10:1) to afford compound **2** (Polyalthialdoic acid, 73.8 mg) as pale yellow oil. FEA_4 was further purified with silica gel (*n*-Hexane: $CHCl_3$ = 1:1) to yield 21 fractions. The fractions were combined based on TLC analysis to yield 4 fractions (Fc–Ff). A portion of Fc was next fractionated using PTLC with $CHCl_3$: MeOH (10:1) as the eluent, whereby 16α-hydroxy-cleroda-3,13 (14) *Z*-dien-15,16-olide (**3**, 138.2 mg) was isolated as pale yellow oil.

Kolavenic acid (1): Pale yellow oil; IR (KBr): 3273, 2957, 2926, 2873, 1689, 1638, 1252 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 0.73 (3H, s, H-20), 0.80 (3H, d, *J* = 5.96 Hz, H-17), 0.99 (3H, s, H-19), 1.30–1.33 (1H, m, H-10), 1.42–1.47 (3H, m, H-7 and H-8), 1.58 (3H, s, H-18), 1.57–1.60 (2H, m, H-1), 1.68–1.70 (2H, m, H-12), 1.71–1.73 (2H, m, H-6), 1.99–2.02 (2H, m, H-11), 2.02–2.06 (2H, m, H-2), 2.17 (3H, s, H-16), 5.19 (1H, s, H-3), 5.68 (1H, s, H-14); ^{13}C NMR (100 MHz, $CDCl_3$): δ 16.0 (C-17), 18.0 (C-18), 18.3 (C-1 and C-20), 19.5 (C-16), 19.9 (C-19), 26.9 (C-2), 27.5 (C-7), 35.0 (C-11), 36.3 (C-8 and C-12), 36.8 (C-6), 38.2 (C-5), 38.8 (C-9), 46.5 (C-10), 114.8 (C-14), 120.4 (C-3), 144.5 (C-4), 164.7 (C-13), 172.1 (C-15); HREIMS: *m/z* 304.2408 $[M]^+$ (calcd for $C_{20}H_{32}O_2$, 304.2402).

Polyalthialdoic acid (2): Pale yellow oil; IR (KBr): 3371, 2924, 1698 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 0.68 (3H, s, H-20), 0.84 (3H, d, *J* = 6.88 Hz, H-17), 0.99 (3H, s, H-19), 1.25–1.29 (2H, m, H-11), 1.38–1.46 (5H, m, H-1, H-7, and H-10), 1.59 (3H, s, H-18), 1.68–1.75 (1H, m, H-8), 1.73–1.79 (1H, m, H-6), 2.05–2.14 (2H, m, H-2), 2.53–2.61 (2H, m, H-12), 5.21 (1H, s, H-3), 6.47 (1H, s, H-14), 9.53 (1H, s, H-16); ^{13}C NMR (100 MHz, $CDCl_3$): δ 15.9 (C-17), 18.1 (C-1, C-18, and C-20), 19.2 (C-12), 19.9 (C-19), 26.8 (C-2), 27.6 (C-7), 36.3 (C-8), 36.8 (C-6), 37.1 (C-11), 38.2 (C-5), 39.3 (C-9), 46.6 (C-10), 120.8 (C-3), 134.1 (C-14), 144.3 (C-4), 157.5 (C-13), 170.6 (C-15), 194.3 (C-16); HREIMS: *m/z* 318.2218 $[M]^+$ (calcd for $C_{20}H_{30}O_3$, 318.2195).

16α-Hydroxy-cleroda-3,13 (14) *Z*-dien-15,16-olide (3): Pale yellow oil; IR (KBr): 3350, 2925, 1739, 1126 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 0.76 (3H, s, H-20), 0.80 (3H, br s, H-17), 1.00 (3H, s, H-19), 1.15–1.20 (1H, m, H-6α), 1.35 (1H, br s, H-10), 1.40–1.45 (1H, m, H-8), 1.43–1.47 (2H, m, H-7), 1.45–1.48 (2H, m, H-1), 1.50–1.53 (1H, m, H-11α), 1.58 (3H, s, H-18), 1.69–1.73 (2H, m, H-6β and H-11β), 1.95–2.05 (2H, m, H-2), 2.28–2.35 (2H, m, H-12), 5.18 (1H, s, H-3), 5.82 (1H, s, H-14), 6.03 (1H, s, H-16); ^{13}C NMR (100 MHz, $CDCl_3$): δ 16.0 (C-17), 18.0 (C-18), 18.2 (C-1 and C-20), 19.8 (C-19), 21.3 (C-12), 26.7 (C-2), 27.3 (C-7), 34.7 (C-11), 36.2 (C-8), 36.6 (C-6), 38.1 (C-5), 38.6 (C-9), 46.4 (C-10), 99.4 (C-16), 116.7 (C-14), 120.4 (C-3), 144.2 (C-4), 171.2 (C-13), 172.3 (C-15); HREIMS: *m/z* 318.2198 $[M]^+$ (calcd for $C_{20}H_{30}O_3$, 318.2195).

2.4 General biological methods

RPMI 1640, DMSO, isopropanol and HCl were obtained from Wako Pure Chemical Industries, Ltd. HI Bovine Serum, Phosphate Buffer Saline (PBS) were obtained from GIBCO (New Zealand). Trypan Blue Solution (0.4%) and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were provided by Sigma-Aldrich. UV–visible absorption was measured using a PerkinElmer EnVision microplate reader (570 nm). DNA was extracted using Get *pure*DNA Kit-Cell, Tissue kit from Dojindo Molecular Technologies, INC. Induction of apoptosis was observed under a fluorescence microscope (Axiovert 40, Carl Zeiss).

2.5 Cell culture

Human leukemia cell line (HL-60) was provided by DS Pharma Biomedical Co., Ltd. (Japan) and cultured in RPMI 1640 media (with L-glutamine and phenol red) supplemented with 5% heat-inactivated HI Bovine Serum, and kept in incubator for 4 days at 37℃ in 5% CO_2. Cell viability was measured by the trypan blue dye exclusion method.

2.6 MTT assay

The cell-growth-inhibition effect of compounds was evaluated by colorimetrically using the MTT assay. Cell suspen-

sions (100 μL, 2×10^4 cells/mL) were incubated in 96-well plates. After 24 h, various concentrations of the compounds (2 μL) were added and DMSO was added in the blank and control wells. Following re-incubated for 48 h, MTT solution (10 μL) was added and the plates were again incubated for a period of 5 h. Acidic isopropanol (100 μL) was added to each well and the absorbance (570 nm) was recorded using microplate reader. The IC_{50} values were used to determine concentration of compounds that inhibit 50% of cell proliferation.

2.7 Morphological changes

For the morphological examination of cell death, 20 μL of compounds 2 or 3 were added to cultured HL-60 cells (1 mL, 2×10^5 cells/mL), and the plate was incubated for 24 h. Hoechst 33342 solution (5 μL) was added to the culture medium to stain cellular DNA, which was incubated for 30 min. After centrifugation, the collected cells were washed with PBS and observed under a fluorescence microscope.

2.8 DNA fragmentation assay

The effects of isolated compounds on DNA damage of the cells were analyzed by DNA fragmentation assay. HL-60 cells (1 mL, 1×10^6 cells/mL) were incubated in 24-well plates in RPMI 1640 media for 24 h. Subsequently, these cells were incubated with 20 μL of compounds 2 or 3 (final concentration, 25 μM) and DMSO (as control). After 24 h, cells were washed with PBS and centrifuged at 1,500 rpm for 5 min. We used Get *pure*DNA Kit-Cell, Tissue kit for DNA extraction and followed the manufacturer's instructions. Lysis buffer (250 μL) and Proteinase K solution (10 μL) were added to the collected cells and incubated for 10 min at 65℃. Post inoculation, cells treated with RNase solution (2 μL) at room temperature for 2 min. Protein precipitation solution (50 μL) was added successively, and the solution was centrifuged at 15,000 rpm for 5 min. The supernatant containing DNA was precipitated twice using ethanol. Dried DNA samples were dissolved in Tris/EDTA (TE) buffer. After mixing with loading buffer, DNA fragmentation was observed by electrophoresis for 30 min using 2% agarose gel in Tris-Borate/EDTA (TBE) buffer. Stained cells (in *GelRed*) were then observed under UV light.

3 RESULTS AND DISCUSSION

The ethyl acetate extract of the *P. longifolia* leaves was repeatedly subjected to CC on silica gel or Sephadex LH-20 and continued with PTLC. The chemical structures of three isolated compounds (1–3) were elucidated using IR, ^{1}H NMR, ^{13}C NMR, 2D NMR, and HRMS. Compound 1 was identified as diterpenes bearing 20 carbon atoms, and its molecular formula was established as $C_{20}H_{32}O_2$ from HREIMS for the peak at m/z 304.2408 $[M]^+$ (calcd for $C_{20}H_{32}O_2$, 304.2402). The structure of compound 1 ($C_{20}H_{32}O_2$) was determined from reference data[14], and it was identified as kolavenic acid (16-methyl-cleroda-3,13 (14)*E*-15-carboxylic acid). Comparison of the ^{1}H and ^{13}C NMR data of compound 2 ($C_{20}H_{30}O_3$) with that of compound 1 allowed the identification of a structure with the similar clerodane skeleton. The difference was in C-16, where the methyl group was substituted by an aldehyde group observed at δ_c 194.3; compound 2 was identified as polyalthialdoic acid[15]. Compound 3 ($C_{20}H_{30}O_3$), which had the similar structure with compounds 1 and 2, was assigned as 16α-hydroxy-cleroda-3,13 (14)*Z*-dien-15,16-olide[16] (Fig. 1). Diterpenes 2 and 3 have only been isolated from the genus *Polyalthia*, and are characteristic compounds of *Polyalthia* plants. Compound 3 appears to be a major constituent of *P. longifolia* (e.g., Misra *et al.*, obtained compound 3 (3.56 g) from 16 kg air-dried leaves.)[16]. However, from a chemotaxonomic point of view, the limited distribution of these clerodane diterpenes is quite interesting.

Previously, we have reported that clerodermic acid isolated from an Indonesian plant *Enicosanthum membranifolium* demonstrated unique antileukemic activity. Clerodermic acid-induced growth inhibition observed in HL-60 cells was mainly due to caspase-independent apoptosis[17]. Therefore, we were interested in investigating the effects of the isolated diterpenes against HL-60 cells. Three isolated diterpenes 1–3 were investigated for growth inhibition effect on HL-60 cells by using the MTT cell-viability assay. Cells in the rapid phase of growth were exposed to test compounds for 48 h. The treatment of the HL-60 cells with each compound (1–3) indicated that cell proliferation was inhibited by polyalthialdoic acid (2) and 16α-hydroxy-cleroda-3,13 (14)*Z*-dien-15,16-olide (3) in a dose-dependent manner, with IC_{50} values of 21.8 and 13.7 μM, respectively (Fig. 2), whereas kolavenic acid (1) was inactive at all concentrations. In contrast, the anti-cancer drug, 5-fluorouracil had an IC_{50} of 9.5 μM toward HL-60 cells[18]. Compounds 1 and 2 have the same basic skeleton and carboxyl position (C-15), but a methyl group in 1 (C-16) is replaced by an aldehyde group in 2, proposing that an aldehyde group in

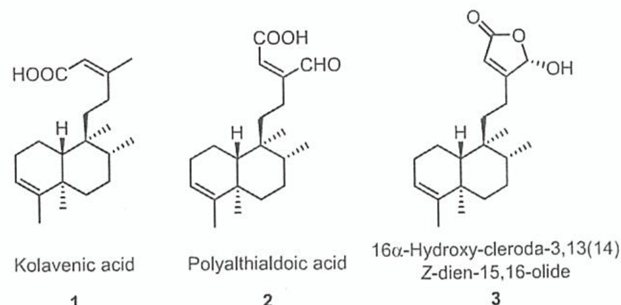

Fig. 1 Clerodane diterpenes isolated from *P. longifolia* leaves.

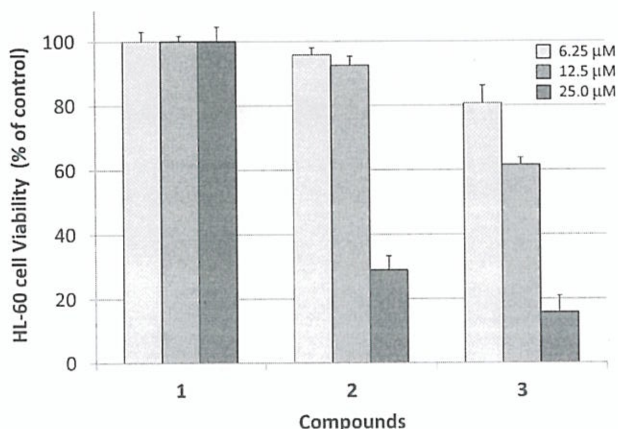

Fig. 2 Cytotoxic effects of the isolated clerodane diterpenes against HL-60 cells (means ± SEMs, n = 9).

the skeleton contributes to the cytotoxic effect. Compound 3, having the same molecular formula as 2, showed more potential for anticancer activity, probably because of the existence of one γ-lactone in the skeleton.

Next, we investigated the mechanism through which these diterpenes inhibited cell proliferation in HL-60 cells. Apoptosis is a form of programmed cell death that occurs through the activation of the suicide machinery within cells[19]. Cells undergoing apoptosis display profound structural changes, including a rapid blebbing of the plasma membrane and nuclear disintegration. In order to investigate the mechanisms of these compounds, we observed morphological changes in the cells stained with Hoechst 33342[17]. At 25 μM concentration of compounds 2 and 3, HL-60 cells showed different shapes compared with the control cells (C) which exhibited general pattern of cell growth and normal morphology. HL-60 cells treated with 2 and 3 (A and B) showed the splitting cells into the smaller form (Fig. 3). The chromatin was condensed, the nucleus was severely fragmented, and cells shrunk, thereby indicating the progression of apoptosis[20]. Apoptosis-induced cells also appear to be initiated by DNA damage (genotoxic stress)[21]. DNA fragmentation assay was performed on HL-60 cells treated with compounds 2 or 3 for 24 h. The DNA fragmentation laddering property was observed in treated apoptotic cells after separation of the extracted DNA on agarose gels (Fig. 4, Lanes 2, 3, 6, and 7).

In recent years, Verma *et al.* claimed the anticancer po-

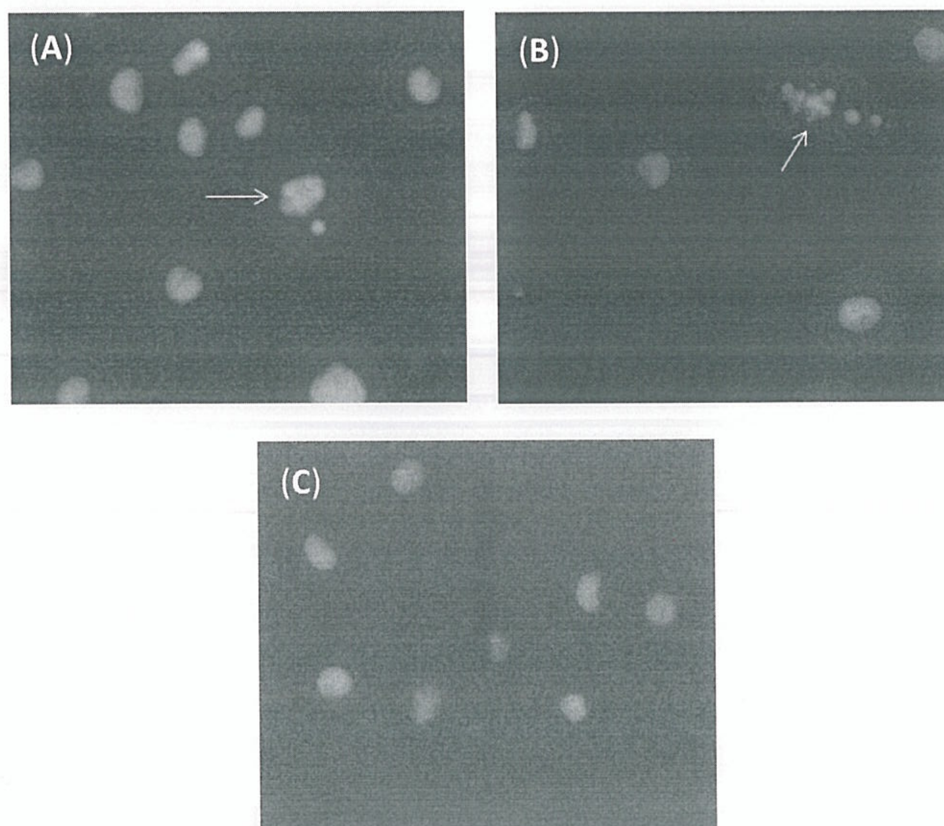

Fig. 3 Apoptosis of HL-60 cells induced by the isolated diterpenes (25 μM) after 24 h. Morphological examination was carried out by using Hoechst 33342 solution. (A) Polyalthialdoic acid (**2**), (B) 16α-hydroxy-cleroda-3,13(14)Z-dien-15,16-olide (**3**), (C) Control.

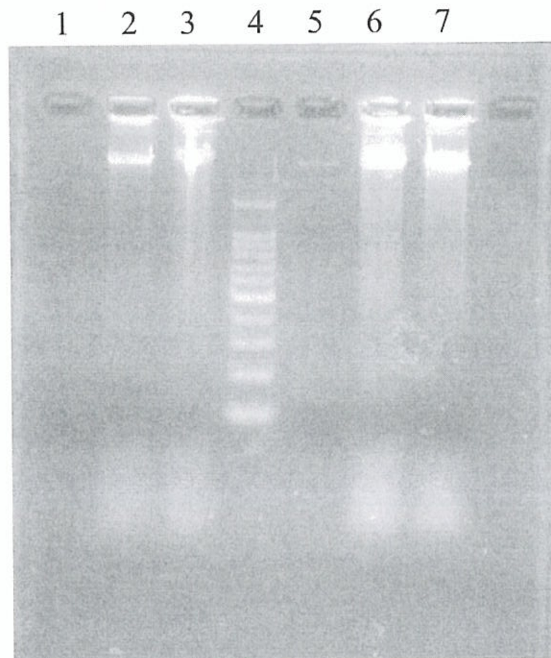

Fig. 4 DNA fragmentation in the isolated diterpenes treated HL-60 cells. Untreated cells (Lanes 1, 5), cells treated with 25 μM of polyalthialdoic acid (**2**) (Lanes 2, 6), cells treated with 25 μM of 16α-hydroxy-cleroda-3,13(14)Z-dien-15,16-olide (**3**) (Lanes 3, 7), and DNA size marker (Line 4).

tential of *P. longifolia* leaves extracts. They described the chloroform extract induced apoptosis through the mitochondrial-dependent pathway in HL-60 cells[22]. Our findings indentified the potential compounds responsible for these effects. Although there are a few reports about cytotoxicity of diterpenes **2** and **3** against cancer cell lines, including human lung carcinoma (A549), human breast carcinoma (MCF-7), human colon adenocarcinoma (HT-29) cells[15, 23], these reports did not indicate induction of apoptosis. In addition, the methanol extract of *P. longifolia* leaves and isolated 16α-hydroxy-cleroda-3,13(14)*Z*-dien-15,16-olide (**3**) have been reported to be non-toxic and safe in *in vivo* models[16, 24]. Thus, *P. longifolia* seems to be a chemopreventive and orally active medicinal plant.

4 CONCLUSION

In the present study, we successfully demonstrated the cytotoxic potential of clerodane diterpenes (**2** and **3**) isolated from *P. longifolia* leaves in HL-60 cells *via* induction of apoptosis. The apoptosis-inducing property of the isolated polyalthialdoic acid (compound **2**) is reported in this article for the first time. Our work highlights the significant potential of these diterpenes as the plausible antileukemic agents. Additionally, we suggest the chemopreventive potency of *P. longifolia* as alternative medicine.

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