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Comparative Antileukemic Activity of a Tetranorditerpene Isolated from *Polyalthia longifolia* Leaves and the Derivative against Human Leukemia HL-60 Cells

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Abstract: The discovery of potent cytotoxic isolates from botanicals provides an opportunity to explore this viable tool for cancer chemoprevention. The antileukemic potential of clerodane diterpene from *Polyalthia longifolia* leaves has already been established. However, in this present study, utilizing chromatographic techniques we report for the first time, the isolation of a rare tetranorditerpene (compound 1) from *P. longifolia*. The structure of compound 1 was elucidated and confirmed by spectrophotometric data. UPLC-MS analysis was conducted on the methanolic extract, ethyl acetate fraction, and isolated tetranorditerpene showed that the tetranorditerpene is one of the major constituents of the plant with a retention time of 30.78 min. In addition, a methyl ester derivative (compound 2) of the isolated tetranorditerpene was synthesized. Using the CCK-8 assay, we compared the cytotoxic potential of isolated tetranorditerpene (1) and methyl ester derivative (2) with the previously isolated clerodane diterpenes. Our results showed that the methyl ester derivative (2) displayed the highest inhibitory activity against human leukemia HL-60 cells. The isolated tetranorditerpene (1) did not exhibit significant inhibitory effect against HL-60 cells. Morphological examination indicated chromatin condensation and nuclear fragmentation suggesting induction of apoptosis in compound 2 treated HL-60 cells. The methyl esterification of the isolated tetranorditerpene (1) conferred on it a significant level of antileukemic activity suggesting the possibility of a synergistic relationship between pure compound isolation and synthetic reaction in the discovery of new chemopreventive agents.

Key words: *Polyalthia longifolia*, tetranorditerpene, antileukemic activity, human leukemic HL-60 cells

1 INTRODUCTION

Leukemia constitutes approximately 33 percent of estimated cancer cases in children and adolescents younger than 15 years of age. Although leukemia is the most common cause of cancer related deaths in children, it occurs in older adults, too[1]. Despite significant advances in the chemotherapeutic management of leukemia, most children and adults still die of this disease. Thus, there is an unmet need for agents with higher efficacy and reduced side effects to serve either as chemopreventive therapies or cytotoxic agents.

Phytochemicals play an important role as a rich source of bioactive agents. Exploration of natural active compounds from plants to treat cancer has attracted considerable attention worldwide[2]. Among cancer chemotherapeutic agents, the proportion of natural product-derived drugs is high[3]. Several conventional chemotherapeutic agents have their origin from medicinal plants. Some examples include vincristine, vinblastine and vinorelbine which are vinca alkaloids derived from a periwinkle plant *Catharanthus roseus*[4]; paclitaxel and docetaxel are diterpenes originally identified from plants of genus *Taxus*[5], topotecan and irinotecan are cytotoxic quinoline alkaloid obtained from the stem of *Camptotheca acuminate*[6]. Thus isolated

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phytochemicals have shown great efficacy in cancer chemotherapy.

Polyalthia longifolia also referred to as mast tree or masquerade tree is native to several continents including Africa and Asia. *P. longifolia* belongs to the *Annonaceae* family and includes approximately 120 species of shrubs and trees found in the tropic and sub-tropic regions[7]. It is a tall (grows as long as 12 meters), columnar and evergreen ornamental tree. The entire length of the plant is covered by long and wavy leaves. As an ornamental tree it is used for beautification and also serves as a wind breaker. Ethnopharmacological findings have shown that various parts of *P. longifolia* have been used for several purposes including fever, skin diseases, diabetes, hypertension and helminthiasis[8, 9]. The extract of *P. longifolia* leaves has been reported to show anti-inflammatory, antimicrobial, and antitumor activities[10−12]. Furthermore, a protective effect of the leaves extracts has been described against glucose-induced cataractogenesis[13]. There are reports on the presence of numerous chemical constituents with interesting biological activities. For example, alkaloids pendulamine A and B isolated from the root extract exhibited significant antibacterial activities[14] while flavonoids and their glycosides isolated from leaves possess free radical scavenging ability[15]. Cytotoxic cycloartane triterpenes and clerodane diterpenes were also isolated from the leaves with marked growth inhibitory activity against four cancer cell lines including leukemic cells *in vitro*[16]. All these aforementioned adduce to the wealth of bioactive constituents present in *P. longifolia*.

This present study sets out to investigate and compare the effects of newly isolated phytochemical from *P. longifolia* leaves and a synthesized derivative against human leukemia HL-60 cells and possibly delineate its mechanism of cytotoxic activity.

2 EXPERIMENTAL PROCEDURES

2.1 Plant Materials

P. longifolia leaves were collected from a residential apartment in Ilorin, Kwara State, Nigeria in August 2015.

2.2 Reagents and Assay kits

RPMI 1640 and dimethyl sulphoxide (DMSO) were obtained from Wako Pure Chemical industries, Ltd. HI bovine serum, phosphate buffer solution (PBS) were obtained from GIBCO (New Zealand). Cell Counting Kit-8 (CCK-8) for cell proliferation and cytotoxicity assays was obtained from Dojindo Molecular Technologies Inc (Kumamoto, Japan). UV-visible absorption was measured using Emax precision microplate reader (490 nm), Molecular Devices. Induction of apoptosis was observed under a fluorescence microscope (Axiovert 40, Carl Zeiss).

2.3 General Instrumentation

^{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 the Waters UPLC system (Aquity UPLC XevoQtof). IR spectra were recorded on JASCO FT/IR- 460 plus spectrometer. Column chromatography (CC) was performed on silica gel 60N (40-50 μm, spherical, neutral, Kanto chemical, Co., INC.) and on Sephadex LH-20 (GE Healthcare, Japan). Thin layer chromatography (TLC) was performed on silica gel 60 F254 for analytical chromatography (200 μm thickness; Merck). Preparative thin-layer chromatography (PTLC) was performed on silica gel 60 F254 (1 mm layer thickness; Merck). All the chemicals used in this study were purchased from Wako Pure Chemical Industries, Ltd and Sigma-Aldrich. To assess the chromatographic profile, the MeOH extract, EtOAc fraction and isolated compound were analyzed by UPLC-ESI-TOFMS. Analyses were conducted using the Waters UPLC system (Aquity UPLC XevoQtof) using a UPLC BEH C18 RP column (1.7 μm, 2.1 × 100 mm) and 260 nm detection.

2.4 Extraction and isolation

Air dried leaves (710 g) were milled to a powdery form and macerated in methanol at room temperature for 7 days. The residue was reconstituted in methanol for 7 more days; the filtrate from the two weeks' maceration was concentrated *in vacuo* and weighed to give a methanolic extract (99.9 g, 14.1% yield). The MeOH extract was subjected to solvent-solvent partitioning using *n*-hexane, ethyl acetate and *n*-butanol successively to give *n*-hexane fraction (63.9 g; 64.0% yield), ethyl acetate fraction (10.9 g; 10.9% yield) and *n*-butanol fraction (18.1 g; 18.0%). The ethyl acetate fraction (10.9 g) was fractionated on silica gel, eluted with chloroform, acetone and methanol mixture of increasing polarity. This separation process yielded 21 vials of the ethyl acetate fraction. Vials having the same R_f values were combined and 8 fractions were obtained (F1, F3, F6, F12, F16, F18, F20 and F21). Fraction F16 was further re-columned to give 2 fractions F161 and F162, F162 was purified using preparative TLC with solvent system (chloroform:acetone = 8:2) to give a pure compound **1** (1-Naphthaleneacetic-7-oxo-1,2,3,4,4a,7,8,8a-octahydro-1,2,4a,5-tetramethyl acid; 34 mg).

2.5 Methyl Esterification of Compound 1

Iodomethane (0.285 mmol) was added to a solution of compound **1** (0.057 mmol) and K_2CO_3 (0.171 mmol) in acetone (4 mL). Under a N_2 atmosphere, the reaction mixture was stirred for 20 h at room temperature. The resultant solution was poured into distilled water, partitioned with EtOAc, and washed with brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified using PTLC ($CHCl_3$/MeOH = 10/1),

to yield compound **2** (Methyl 7-oxo-1,2,3,4,4a,7,8,8a-octahydro-1,2,4a,5-tetramethyl-1-naphthaleneacetate, 0.0323 mmol: 57%).

2.6 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 10% heat inactivated HI bovine serum, 1% antibiotics, penicillin-streptomycin and kept in the incubator for 4 days at 37℃ in 5% CO_2. Cell viability was measured using the Cell Counting Kit- 8 (CCK-8) assay method.

2.7 CCK-8 assay

The HL-60 cells (2.0×10^4 cells/mL) were seeded in 96-well plates. After 24 h, sample solutions were added. Following 48-h incubation, CCK-8 solution (10 μL) was added, and the plates were incubated for an additional 4 h. Visible absorption (490 nm) was measured using a microplate reader.

2.8 Morphological examination

For the morphological examination of cell death, the HL-60 cells (1.0×10^5 cells/mL) were seeded in 48-well plates, cells were treated with compound **2** (50 μM) and incubated for 48 h. Hoechst 33342 solution (Sigma-Aldrich, USA) was added to the culture medium at 5 μg/mL concentration to stain cellular nucleus. After incubation for 30 min, the collected cells were washed with PBS and then observed under a fluorescence microscope (Axiovert 40, Carl Zeiss).

3 RESULTS AND DISCUSSION

3.1 Isolation and Structural Elucidation

The ethyl acetate fraction of *Polyalthia longifolia* leaves was repeatedly subjected to column chromatography (CC) on silica gel or Sephadex LH-20, continued with PTLC. The chemical structure of isolated compound **1** was elucidated using IR, ^{1}H NMR, ^{13}C NMR, 2D NMR, and HRMS. Compound **1** was isolated as a colorless oil, and its molecular formula was established as $C_{16}H_{24}O_3$ from HRESITOFMS for the peak at *m/z* 263.1664 $[M-H]^-$ (calcd. $C_{16}H_{23}O_3$, 263.1647). The IR spectrum displayed intense absorption bands for hydroxyl (3387 cm^{-1}) and α,β-unsaturated carbonyl (1652 cm^{-1}) functionalities. Its ^{13}C NMR and DEPT spectra exhibited 16 carbon signals, including a ketone carboxyl group (δ_C 201.7), a carboxyl group (δ_C 171.6), a double bond (δ_C 174.2 and 125.4), four methyls, four methylenes, two methines, and two quaternary carbons. An olefinic proton at δ_H 5.72 correlated to the ketone carboxyl carbon and a methyl carbon (δ_C 19.2) in HMBC, suggesting the existence of methylated α,β-unsaturated ketone moiety. In addition, the COSY and HMBC from the ketone structure revealed that compound **1** possessed a 7-oxo-5,6-dehydrodecalin core. The occurrence and substitution of $-CH_2COOH$ unit were deduced from the following HMBC correlations: δ_H 5.93 (COO*H*) with δ_C 171.6 and 21.3; δ_H 0.83 (*CH*$_3$) and 1.53-1.50 (C*H*) with δ_C 21.3. These spectroscopic studies resulted in the structural determination of compound **1** as 1-naphthaleneacetic-7-oxo-1,2,3,4,4a,7,8,8a-octahydro1,2,4a,5-tetramethyl acid. This structure was confirmed from previous literature[17], as shown in Fig. 1.

3.2 Derivatization of an Isolated Compound 1

Comparative analyses with molecules possessing different functional groups are necessary to establish the efficacy of these compounds. Therefore, we prepared a methyl ester derivative of compound **1** as shown in Fig. 1. Reaction of compound **1** with 5.0 equiv. of iodomethane (MeI) in the presence of 3.0 equiv. of potassium carbonate (K_2CO_3) was carried out in acetone at room temperature for 20 h. Through purification by PTLC, the derivative was obtained in 57% yield. A 3H singlet observed at δ_H 3.80 showed the HMBC correlation to a carbonyl carbon at δ_C 165.2, indicating that the reaction conditions successfully produced the corresponding methyl ester derivative, methyl 7-oxo-1,2,3,4,4a,7,8,8a-octahydro-1,2,4a,5-tetramethyl-1-naphthaleneacetate (compound **2**).

3.3 Antileukemic Assay

We have previously established the cytotoxic potential of clerodane diterpenes isolated from *P. longifolia* leaves against human leukemia HL-60 cells *via* the induction of apoptosis. Our results showed that 16α-hydroxycleroda-3,13(14)-*Z*-dien-15,16-olide (**3**) had the highest activity when compared with polyalthialdoic acid (**4**) and kolavenic acid (**5**)[18]. It was further noted that the chloroform extract of *P. longifolia* leaves induced apoptosis through mitochondrial dependent pathway in HL-60 cells[19]. In the present study, we evaluated the growth inhibitory activity of compounds **1**, **2**, and **3** against human leukemia HL-60 cells. The results showed that the dose dependent inhibitory effect for the methyl ester derivative (**2**) was significantly higher when compared with the previously isolated compound **3** and also newly isolated tetranorditerpene (**1**). The latter did not exhibit any significant activity against

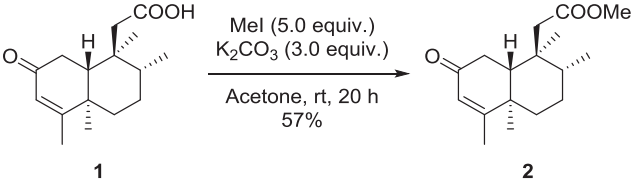

Fig. 1 Chemical structure of isolated compound **1** and methyl esterification.

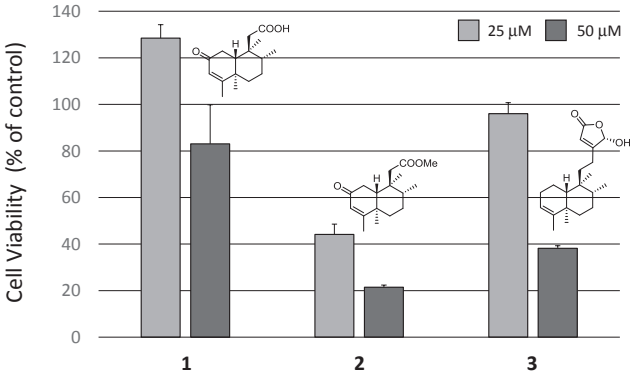

Fig. 2 Antiproliferative effects of tetranorditerpene (1), its methyl ester derivative (2) and a previously isolated clerodane diterpene (16α-hydroxy-cleroda-3,13(14)-*Z*-dien-15,16-olide) (3) against human leukemia HL-60 cells (means ± SEMs, $n = 5$).

HL-60 cells, as summarized in Fig. 2. The IC_{50} values of compounds 1, 2, and 3 were 71.1 μM, 17.7 μM, and 44.8 μM, respectively. This suggests that methyl esterification of the compound 1 conferred on it a potent anti-proliferative activity against HL-60 cells. This activity can be likened to the mechanism seen in Bulsufan, an alkylating anticancer agent which acts *via* a nucleophilic attack on the DNA molecule causing a DNA-DNA intrastrand cross-link preventing DNA replication and subsequently leading to apoptosis[20]. Morphological examination showed alteration of cellular morphology when treated with compound 2 using Hoechst 33342 staining. Cell shrinkage, blebbing and formation of apoptotic bodies were observed under the fluorescence microscope. The cells shrink, and finally the blebs separate forming apoptotic bodies. These are early signs of an apoptotic event[21]. The induction of apoptotic morphology in HL-60 cells upon treatment with compound 2 is shown in Fig. 3.

3.4 UPLC-MS Analysis

To assess the chromatographic profile, the methanolic extract, EtOAc fraction and isolated tetranorditerpene (1) were analyzed by UPLC-ESI-TOFMS. The best conditions were obtained using gradient elution with MeCN/H_2O (solvent A: distilled water and solvent B: MeCN). The liner gradient system employed was: 0-30 min 90% solvent A to 50% solvent A and 10% solvent B to 50% solvent B, hold for 5 minutes, 35-45 min 50% solvent A to 30% solvent A and 50% solvent B to 70% solvent B, and hold for 5 minutes. Figure 4 shows the peak of isolated tetranorditerpene (compound 1) which was detected at the retention time of 30.78 min. This was also detected in the EtOAc fraction and methanolic extract at 30.80 and 30.82 minutes, respectively. From the EtOAc fraction we further detected the retention times for previously isolated 16 α-hydroxy-cleroda-3,13(14)-*Z*-dien-15,16-olide (3, 43.00 min), polyalthialdoic acid (4, 41.69 min), and kolavenic acid (5, 37.68 min). Chemical fingerprints and simple UPLC-MS analytical method have a positive impact on the conservation of this plant.

4 CONCLUSION

We successfully isolated a tetranorditerpene (compound 1) from *P. longifolia* for the first time. We also synthesized a methyl ester derivative (2) of 1 and compared their cytotoxic potentials with the previously isolated clerodane diterpenes. Our studies showed that the methyl derivative (2) had the highest antiproliferative activity against human leukemic HL-60 cells. We suggest therefore the possibility of a synergistic relationship between pure compound isolation and synthetic reaction in the discovery of novel cytotoxic and chemopreventive agents.

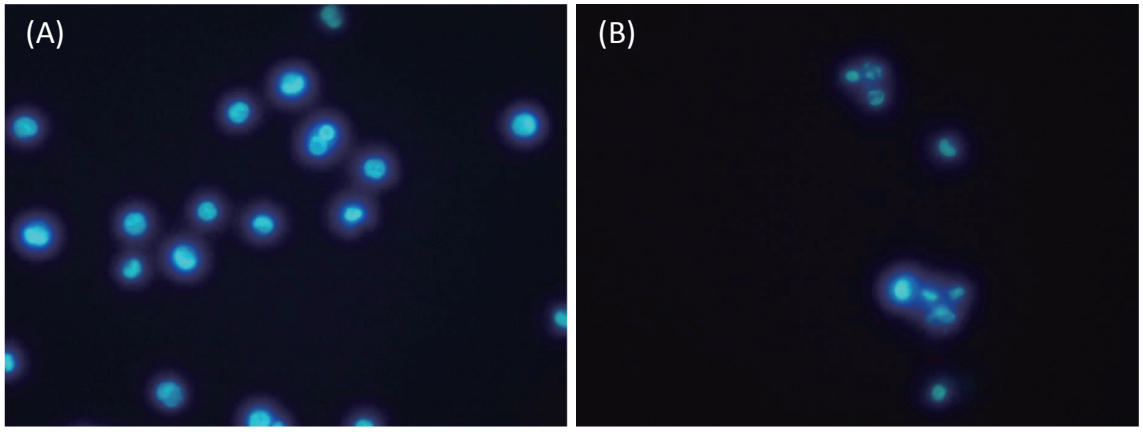

Fig. 3 Morphological changes of HL-60 cells induced by the methyl ester derivative (2) at a final concentration of 50 μM. (A) Blank (DMSO alone); (B) compound 2 in HL-60 cells.

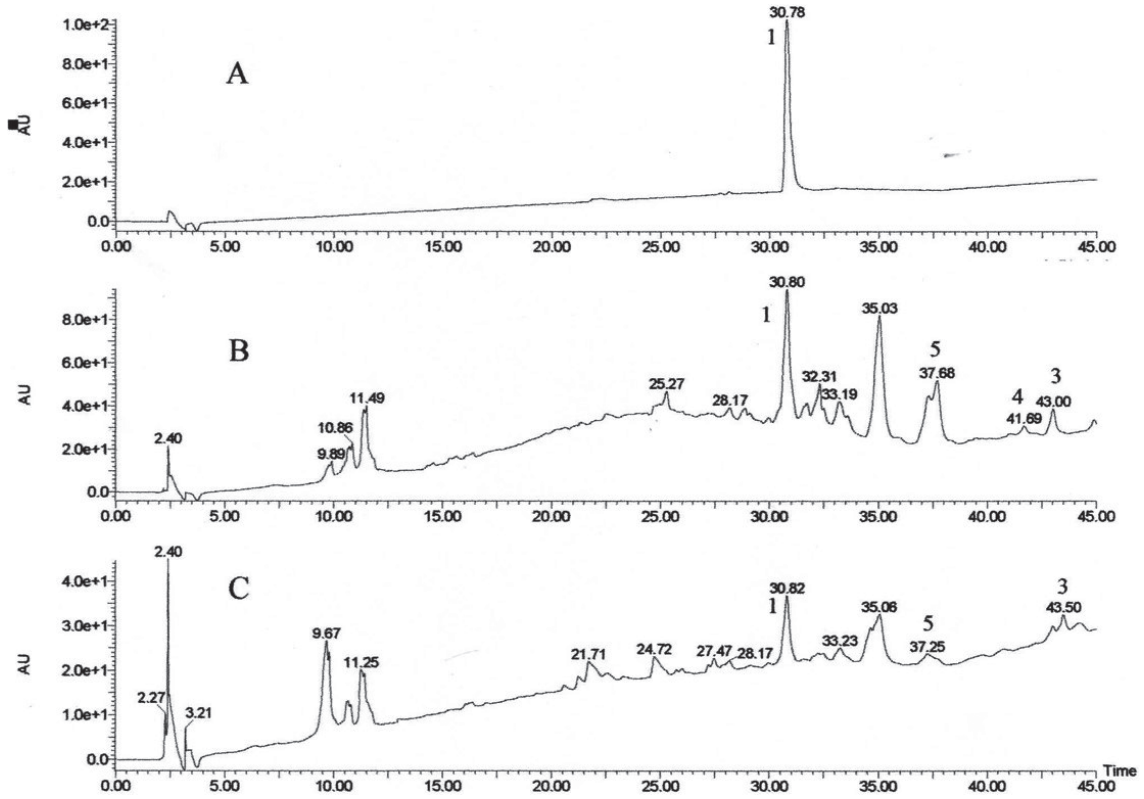

Fig. 4 UPLC analysis of A: tetranorditerpene (**1**); B: EtOAc fraction and C: MeOH extract. Retention times for tetranorditerpene (**1**), 16α-hydroxy-cleroda-3,13(14)-*Z*-dien-15,16-olide (**3**), polyalthialdoic acid (**4**), and kolavenic acid (**5**).

SUPPORTING INFORMATION

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