

New tigliane-type diterpenoids from *Euphorbia aellenii* Rech. f. with immunomodulatory activity

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Abstract

The cytotoxic chloroform fraction of *Euphorbia aellenii* Rech. F. (Euphorbiaceae) afforded two new phorbol diterpenoids: 4-deoxy-4 α -phorbol-12-(2,3-dimethyl) butyrate-13-isobutyrate and 17-hydroxy-4-deoxy-4 α -phorbol-12-(2,3-dimethyl) butyrate-13-isobutyrate. Their structures were elucidated by NMR and other spectroscopic methods. The immunomodulating potentials of the isolated compounds were tested using standard proliferation and chemiluminescence assays. Compound 2 showed moderate inhibitory activity against both T-cell proliferation and reactive oxygen species (ROS) production in whole blood with IC₅₀ of 14.0 \pm 0.57 and 44.1 \pm 3.8 μ g/ml, respectively, while compound 1 was relatively inactive with IC₅₀ >50 μ g/mL for T-cell proliferation, and >100 μ g/mL for ROS.

Keywords: *Euphorbia aellenii*; Immunomodulatory activity; Tigliane-type diterpenoid; Phorbol diterpene

INTRODUCTION

Many of the published studies on *Euphorbia* family have highlighted their immunomodulating, anti-tumor, and anti-HIV properties, more probably related to the presence of certain types of polycyclic diterpenes (1). Diterpenes constitute a group of C₂₀ compounds arising from geranyl geranyl pyrophosphate. Cyclization of the diterpenes leads to the formation of polycyclic structures by intramolecular nucleophilic substitution i.e. jatrophane, lathyrane, mirisinane and tigliane diterpenes in *Euphorbia* family (2). Among them the tigliane nucleus is the carbon framework of phorbol whose derivatives occur widely in Euphorbiaceae and are renowned for their tumor promoting and irritant activities (2-3).

Recently, lathyrane diterpenes and myrosinol type skeletons related to lathyranes have been isolated from this plant (4-5). In the present study, two new tigliane type deriva-

tives were isolated and their structures were elucidated by NMR and other spectroscopic methods. Immunomodulating potentials of the isolated compounds were tested by chemiluminescence assay using neutrophils of human whole blood.

MATERIALS AND METHODS

Materials

Calcium chloride and magnesium sulphate purchased from Sigma-aldrich (USA), luminol (3-aminophthalhydrazide) from Research Organics (USA), serum opsonized zymosan (*Saccharomyces cerevisiae* origin) from Fluka (Switzerland) and thymidine [³H] from Amersham (UK). All solvents used were of analytical grade and purchased from Merck (Germany).

NMR and IR spectroscopy

NMR spectra were recorded on a Bruker

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Avance AV 300 and AV 600 NMR instrument (Switzerland) using CDCl_3 as solvent. The IR spectra were recorded on a JASCO 302-A spectrophotometer (Japan), EI-MS spectra were measured in an electron impact mode on Varian MAT 112 or MAT 312 spectrometers.

Chromatographic condition

Recycling preparative HPLC was carried out on an LC-908 (Hitachi Co., Japan) equipped with UV and RI detectors using a YMC-Pack-Sil column (250×20 mm i.d.). Flash chromatography was performed on LiChroprep® Si 60 (25-40 μm), or silica gel 63-200 μm , and size exclusion chromatography on *Sephadex*® LH-20 (*Sigma-Aldrich*). HPTLC was conducted on precoated silica gel GF-254 plates (20×20 cm, 0.5 mm thick) (Merck, Germany) and visualization of the plates was achieved at 254 /366 nm.

Plant materials

The aerial flowering parts of the *Euphorbia aellenii* Rech. f. (Euphorbiaceae) were collected in August 2007 from populations growing in Galil-e-Shirvan (Alt. 1600 m), Northern Khorasan province (Iran) and identified by Mrs. Yasamin Naseh, plant taxonomist (Department of Botany, Herbaceous Sciences Research Center at the Ferdowsi University of Mashhad). A voucher specimen (no. 2024) of the plant has been deposited in the herbarium of the Pharmacognosy Department, School of Pharmacy and Pharmaceutical sciences at the Isfahan University of Medical Sciences (Iran).

Extraction and isolation

Air-dried plant was ground to fine powder (7 kg) and macerated for 4 days with MeOH ($20 \text{ L} \times 3$) at room temperature. Filtration and *in vacuo* evaporation resulted in a green gum (500 g) which was partitioned between methanol and *n*-hexane. The defatted extract was concentrated, dissolved in water, and successively extracted with chloroform, ethyl acetate and *n*-butanol, respectively. The obtained fractions were compared *in vitro* for their cytotoxic activities against brine-shrimp eggs (6). Consequently, the chloroform fraction (240 g) with potential of cytotoxic activity was subjected to column chroma-

tography over normal silica gel, using Hexane/ CHCl_3 mixtures of increasing polarity up to 100%. Hexane/ CHCl_3 (20:80) was further purified using gradient mixtures of Hexane/EtOAc (0→50) on silica. Next, the diethyl ether soluble part of Hexane/EtOAc (70:30) fraction was purified on sephadex LH-20 (DCM/MeOH, 1:2) followed by RP-18 CC (MeOH/ H_2O , 70:30) to remove chlorophylls and pigments. Finally, fractions containing diterpenes (inferred from $^1\text{H-NMR}$ spectra) were subjected to recycling HPLC (Hexane/EtOAc, 70:30) to obtain compounds 1 (11.0 mg) and 2 (8.0 mg).

Phagocyte chemiluminescence assay

In stimulated polymorphonuclear cells (PMNs), inhibition of chemiluminescence may be mediated by three main mechanisms including cell death, scavenging of ROS and inhibition of enzymes involved in the signal transduction pathways of the ROS generation process. In this assay, formation of the reactive oxidants in whole blood during the oxidative burst was measured by the luminol-enhanced chemiluminescence assay procedure (7,8). In brief, three concentrations (1, 10 and 100 $\mu\text{g/mL}$) of each compound were prepared in 25 μL of Hank's Buffered Salt Solution (HBSS⁺⁺) in 96 well flat bottomed plates for a final incubation volume of 100 μL . Then 25 μL of whole blood diluted 1:50 in suspension of HBSS⁺⁺ with calcium chloride and magnesium sulphate was added. Positive control, negative control and blank wells were included in the assay. Cells and compounds were incubated for 30 min at 37° C. 25 μL luminol (3-aminophthalhydrazide) was then added into each well and 25 μL serum opsonized zymosan (*Saccharomyces cerevisiae* origin) was added except for negative and blank wells. The phagocytosis kinetic was monitored with luminometer (Labsystems Luminoskan, Finland) for 50 min in the repeated scan mode. Peak and total integral chemiluminescence readings were expressed in terms of relative light unit (RLU).

T-Cell proliferation assay

Peripheral human blood lymphocytes were incubated with different concentrations of the test compounds (0.5, 5, and 50 $\mu\text{g/mL}$ in

duplicates) in supplemented RPMI-1640 along with phytohemagglutinin (PHA) at 37 °C in CO₂ environment for 72 h. Further incubation for 18 h after the addition of thymidine [³H] was done and cells were harvested using a cell harvester (Inotech Dottikon, Switzerland). Finally, proliferation level was determined by the radioactivity count as CPM reading recorded from the Beta-scintillation counter (5).

Statistical analysis

All samples were presented as mean ± SD for three measurements. One-way ANOVA was used to calculate P<0.05 for each compound against the control (+ve) and the IC₅₀ values were calculated using Excel 2007.

RESULTS

Chemistry

Two new compounds (1-2) were obtained with the following NMR and spectroscopic properties and assigned aided by the ¹H-¹H COSY, and HMBC experiments (Fig 1).

Compound 1

Colourless oil, UV (CHCl₃) λ_{max}: 239 nm. IR (KBr+CHCl₃) ν_{max} 3733, 3610, 2873, 1714, 1645, 1517, 1456, 1394, 1204, 1160, 1076, 1029 cm⁻¹; ¹H NMR (CDCl₃, 300 MHz, *J* in Hz): δ 7.03 (1H, bs, H-1), 5.45 (1H, d, *J*=10.02, H-12), 5.09 (1H, bs, H-7), 4.00 (1H, d, *J*=12.3, H-20b), 3.89 (1H, d, *J*=12.3, H-20a), 3.46 (1H, bd, H-10), 3.42 (1H, bd, H-5a), 2.75 (1H, m, H-4), 2.51 (1H, overlapped, iBu-2''), 2.43 (1H, dd, *J*=15.3, 5.1, H-5b), 2.20 (1H, dq, *J*=7.2, 7.2, diMeBu-2'), 1.94 (1H, overlapped, H-8), 1.88 (1H, overlapped, diMeBu-3'), 1.76 (3H, s, H-19), 1.67 (1H, dq, *J*=10.8, 6.3, H-11), 1.18 (3H, s, H-17), 1.15 (3H, d, *J*=7.2, iBu-3''), 1.14 (3H, s, H-16), 1.13 (3H, d, *J*=7.2, diMeBu-5'), 1.11 (3H, d, *J*=7.2, iBu-4''), 1.07 (3H, d, *J*=6.3, H-18), 0.96 (3H, d, *J*=7.2, diMeBu-4'), 0.92 (3H, d, *J*=7.2, diMeBu-6'), 0.75 (1H, d, *J*=5.1, H-14); ¹³C NMR (CDCl₃, 125 MHz): δ 213.1 (C-3), 178.9 (iBu-C-1''), 175.6 (diMeBu-C-1'), 156.1 (C-1), 143.2 (C-2), 137.1 (C-6), 126.4 (C-7), 78.0 (C-9), 74.8 (C-12), 69.3 (C-20), 64.8 (C-13), 49.6 (C-4), 47.3 (diMeBu-C-2'), 47.3 (C-10), 43.1 (C-11), 40.7 (C-8), 37.1 (C-14), 34.3 (iBu-C-

2''), 31.1 (diMeBu-C-3'), 25.2 (C-15), 25.2 (C-5), 24.1 (C-16), 20.8 (diMeBu-C-6'), 19.3 (diMeBu-C-4'), 18.5 (iBu-C-3''), 18.5 (iBu-C-4''), 16.4 (C-17), 14.5 (diMeBu-C-5'), 11.8 (C-18), 10.4 (C-19); HRESI-MS, Positive mode: *m/z* 517.3207 (calc. for C₃₀H₄₄O₇ +H⁺, 517.3159), 383, 354, 313, 295.

Compound 2

Colourless oil, UV (CHCl₃) λ_{max} (log ε): 232 (3.95), 280 (3.68) nm. IR (KBr+CHCl₃) ν_{max} 3799, 3733, 3610, 3970, 2873, 1725, 1714, 1680, 1645, 1517, 1456, 1394, 1250, 1204, 1160, 1076, 1029, 667, 408 cm⁻¹; ¹H NMR (CDCl₃+CD₃OD, 300 MHz, *J* in Hz): δ 7.00 (1H, bs, H-1), 5.45 (1H, d, *J*=10.5, H-12), 5.06 (1H, bs, H-7), 3.90 (1H, d, *J*=13.1, H-20b), 3.82 (1H, d, *J*=13.1, H-20a), 3.57 (1H, br-s, H-17), 3.43 (1H, bd, H-10), 3.33 (1H, overlapped, H-5a), 2.75 (1H, m, H-4), 2.45 (1H, m, iBu-2''), 2.33 (1H, dd, *J*=15.3, 4.8, H-5b), 2.17 (1H, dq, *J*=6.9, 6.99, diMeBu-2'), 1.89 (1H, bd, H-8), 1.84 (1H, m, diMeBu-3'), 1.71 (3H, s, H-19), 1.64 (1H, dq, *J*=10.8, 6.3, H-11), 1.15 (3H, s, H-16), 1.15 (3H, d, *J*=7.2, diMeBu-6'), 1.10 (3H, overlapped, diMeBu-5'), 1.08 (3H, d, *J*=6.9, iBu-3''), 1.06 (3H, d, *J*=6.9, iBu-4''), 1.01 (3H, d, *J*=6.3, H-18), 0.93 (3H, d, *J*=6.9, diMeBu-4'), 0.72 (1H, d, *J*=4.8, H-14); ¹³C NMR (CDCl₃+CD₃OD, 125 MHz): δ 213.0 (C-3), 179.1 (iBu-C-1''), 175.1 (diMeBu-C-1'), 156.5 (C-1), 143.3 (C-2), 136.0 (C-6), 125.2 (C-7), 78.0 (C-9), 74.9 (C-12), 70.4 (C-17), 68.8 (C-20), 65.7 (C-13), 49.8 (C-4), 47.3 (diMeBu-C-2'), 47.2 (C-10), 42.9 (C-11), 40.7 (C-8), 37.0 (C-14), 34.2 (iBu-C-2''), 31.1 (diMeBu-C-3'), 25.3 (C-15), 25.3 (C-5), 24.0 (C-16), 20.7 (diMeBu-C-6'), 19.2 (diMeBu-C-4'), 18.4 (iBu-C-3''), 18.4 (iBu-C-4''), 14.5 (diMeBu-C-5'), 11.7 (C-18), 10.3 (C-19); HRESI-MS, Positive mode: *m/z* 533.3165 (calc. for C₃₀H₄₄O₈ +H⁺, 533.3109), 515, 417, 399, 354, 329, 301, 217.

Lymphocyte proliferation assay

The anti-proliferation effect of the test compounds was determined by measuring the PHA-induced T-cell proliferation by determining radioactive thymidine incorporation. Compound 2 showed inhibitory activity against lymphocyte proliferation with IC₅₀ of

13.27 ± 0.18 µg/mL whereas compound 1 was reactively inactive (IC₅₀ >50 µg/mL).

Chemiluminescence assay

The concentration effects of the tested compounds on human whole blood employing luminol and zymosan for the intracellular oxidative burst studies are presented in Fig. 2. The inhibitory effect of compound 1 at the concentrations of 100 (P<0.001) and 10 µg/ml (P<0.01) was significantly greater than positive control. This effect, however, did not

reach to a significant level at concentration of 1 µg/ml. The inhibitory effect of compound 2 at the concentration of 100 µg/ml was markedly larger than that of the control (p<0.0001), but not at concentrations of 10 and 1 µg/ml.

Weak inhibition activity was observed for compound 1 with the IC₅₀ >100 µg/ml, while moderate inhibitory activity with IC₅₀ equal to 44.1 ± 3.84 µg/ml was found for compound 2, which may be due to the inhibition of PMNs proliferation.

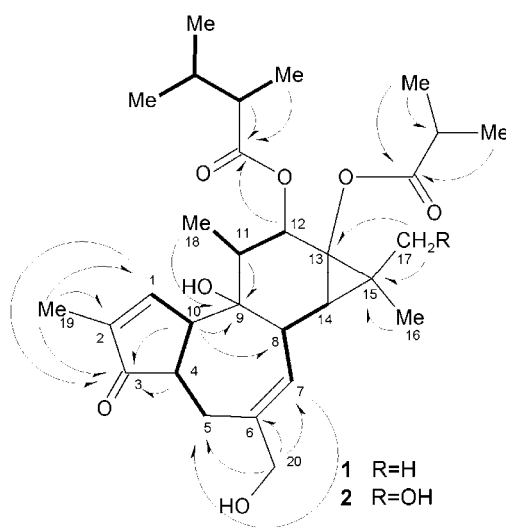


Fig. 1. Key DQF-COSY (in bold) and ^{2,3}J(H→C) HMBC correlations observed in tiglane-type diterpenes from *Euphorbia aellenii*.

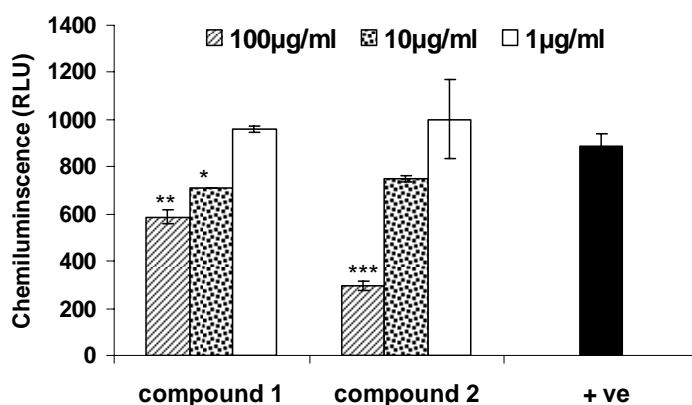


Fig. 2. Effects of compounds 1 and 2 on the neutrophils oxidative burst. The luminol dependent chemiluminescence induced by zymosan in the presence of compounds 1 and 2 at three concentrations are compared to those of the positive control (+ve). Data are presented as means ± SD for three measurements. One-way ANOVA was used to analyze the differences between the inhibitory effects of each compound and positive control. Stars show statistically significant differences between the test and control. *P<0.01, **P<0.001, ***P<0.0001 (ANOVA).

DISCUSSION

Compound 1 obtained as colourless oily mass was assigned the molecular formula of $C_{30}H_{44}O_7$ on the basis of positive HRESI-MS, m/z 517.3207 (calc. for $C_{30}H_{44}O_7 + H^+$, 517.3159), in accordance with number of carbons and hydrogens counted in NMR data. The IR spectrum confirmed the presence of carbonyls (1675 - 1750 cm^{-1}), C-O (1020 - 1250 cm^{-1}), double bond absorption (1645 , 1517 cm^{-1}) and free hydroxyls (3733 , 3610 cm^{-1}). According to the nine degrees of unsaturation derived from molecular formula, ^{13}C -NMR and DEPT spectral data, one ketone group, two ester carbonyls, two double bonds, four rings have been deduced in the molecule. In the 1H -NMR spectrum, four methyl groups δ_H 1.07 d (6.3), 1.14 s, 1.18 s and 1.76, two geminal oxymethylene protons δ_H 4.00 (d, $J=12.3$ Hz, H-20b) and 3.89 (d, $J=12.3$ Hz, H-20a), two geminal hydrogens δ_H 2.43 (dd, $J=15.3$, 5.1 Hz, H-5a) and 3.42 (br-d, H-5b) together with two olefinic protons δ_H 7.03 (br-s, H-1) and 5.09 (br-s, H-7) as well as the methine doublets at *ca* $\delta_H=5.4$ ($J \sim 9$ - 10 Hz) and at *ca* $\delta_H=1$ ($J \sim 5$ Hz) attributed to H-12 (5.45, d, $J=10.2$ Hz) and H-14 (0.74, d, $J=5.1$ Hz), respectively showed typical signals of phorbol esters (8-10). HMBC correlation of δ_H 7.03 of an olefinic group with ketone carbonyl δ_C 213.1 along with IR absorption (1675 cm^{-1}) indicated one α , β -unsaturated ketone system. Moreover, signals of the cyclopropane moiety, C-13 ($\delta_C=64.8$) and C-15 ($\delta_C=25.3$) together with HMBC correlations with two singlet methyl groups at δ_H 1.14 and 1.18 with quaternary carbon δ_C 25.2 (C-15) confirmed the presence of these two geminal unfunctionalized methyl groups on cyclopropane ring (8, 11). The 1H - 1H -COSY correlations confirmed the following protons: CH_3 -CH-CH(CH_3)- CH_3 [δ_H 1.13 d (7.2), 2.20 dq (7.2; 7.2), 1.88 m) and 0.92 d (7.2)] to be in one spin system and presence of ion peak, m/z 400 in MS spectrum [M-116], as well as HMBC of H-12 (δ_H 5.45) and H-2' (δ_H 2.20) with ester carbonyl carbon (δ_C 175.5) confirmed this fragment as 2', 3'-dimethylbutanoate moiety attached to C-12 (12). Likewise, ^{13}C - and 1H -NMR data δ 178.9, 34.3

(2.51, m), 18.5 (1.15, d, $J=7.2$ Hz) and 18.5 (1.11, d, $J=7.2$ Hz) exhibited typical signals of isobutanoate group (8). Lack of HMBC correlations of isobutanoate group with any oxygenated carbons, suggested its position on a quaternary C-O (C-13) and it was confirmed by NOESY effects of Me-3" (δ_H 1.15) with H-14 (δ_H 0.74) denying its position on C-9 (8, 13). Therefore, in light of above observations, accordance to NMR data with literature and HMBC and DQF-COSY spectra (Fig. 1), compound 1 recognized as a 4 α -deoxy tiglane, bearing one $-CH_2OH$ group (δ_C 68.8) on C-6.

The stereochemistry of compound 1 was obtained by the analysis of NOESY spectra and J -coupling constants. According to the literature, all up to now have been discovered from plants, have shown β configuration for H-8 and α -orientation for C-9-OH and H-10 (10,11). Therefore, with considering those as references, the NOE effects of H-10 α with Me-18; Me-18 with H-12 and H-4 supported α position for these protons, in which the latter is confirmed by lack of NOE between H-4 and H-8 β , as well. The large J -coupling constant (10.2 Hz) between H-12 α and H-11 revealed their *trans* orientation and consequently the observed NOE of H-11 β with Me-17 disclosed that gem-dimethyl cyclopropane moiety were on the plane and the three-membered ring *cis* joined to seven-membered ring (Fig. 3). Therefore, based on above explanations, compound 1 was identified as 4-deoxy-4 α -phorbol-12-(2,3-dimethyl)butyrate-13-isobutyrate.

The molecular formula of compound 2 was assigned as $C_{30}H_{44}O_8$ by the positive HRESI-MS, m/z 533.3165 (calc. for $C_{30}H_{44}O_8 + H^+$, 533.3109), according to the number of carbons and hydrogens counted in NMR data. IR spectrum showed a prominent peak of carbonyls (1680 - 1725 cm^{-1}), olefinic group (1645 cm^{-1}), C-O functions (1029 - 1250 cm^{-1}) and hydroxyl groups (3610 - 3733 cm^{-1}). The nine degree of unsaturation, ^{13}C -NMR and DEPT spectral data supported the presence of three carbonyls (one ketonic and two esteric), two double bonds and consequently, four rings in the molecule. More observation in NMR data showed close similarity with that of compound 1, except for an additional free

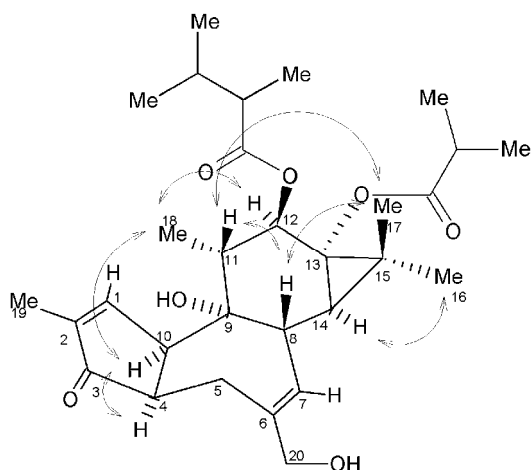


Fig. 3. Key correlations observed in the NOESY spectrum of compound 1 from *Euphorbia aellenii*.

hydroxyl group on C-17 (δ_c 70.4) which proposed compound 2 to be 17-hydroxy-4-deoxy-4 α -phorbol-12-(2,3-dimethyl) butyrate-13-isobutyrate diterpenoid.

Immunomodulating potential of the isolated compounds tested in standard proliferation and chemiluminescence assays showed moderate inhibitory activity against both T-cell proliferation and ROS production in whole blood for compound 2 with IC₅₀ of 14.0 ± 0.57 and 44.1 ± 3.8 $\mu\text{g/ml}$, respectively, while compound 1 was relatively inactive with IC₅₀ >50 $\mu\text{g/ml}$ for T-cell proliferation and >100 $\mu\text{g/ml}$ for ROS. Similar studies on closely related phorbol derivatives for stimulation of human mononuclear cells have shown that 4 β -deoxyphorbol esters stimulated cell proliferation in a dose-related manner, while 4 α -deoxyphorbol esters had no effects which is in agreement with the results of the present study (15). In another study on the effects of various phorbol-based protein kinase C (PKC) activators which resulted in proliferation of T cells, 4 β -phorbol 12,13-dibutyrate showed activity in a concentration-dependent manner, whereas the structurally related isomer 4 α -phorbol 12,13-dibutyrate was inactive (16). Indeed, there is conflicting data as to whether 4-deoxy-phorbol esters are lymphocyte mitogens. This issue is important as phorbol esters are often regarded as proinflammatory promoters. In the current study and previous reports using related 4 α -phorbol esters either no response or weak response to lymphocyte

proliferation has been observed indicating that H-4 orientation is crucial to impart this effect.

CONCLUSION

Using size exclusion chromatography on Sephadex LH 20 and recycling HPLC with normal-phase column as powerful means for isolating phorbol esters, two new 4-deoxy-4 α -phorbol esters were isolated from *Euphorbia aellenii* Rech. F., and their structures were elucidated by NMR and other spectroscopic methods. Immunomodulating potential of the isolated compounds was tested by phagocyte chemiluminescence and T-cell proliferation assays. These compounds showed moderate inhibitory activity on phagocytosis oxidative burst on polymorphonuclear neutrophils (PMNs) in human whole blood and lymphocyte proliferation. Therefore, *E. aellenii* could be a new source of these chemo-type lead-compounds as starting material for semi-synthetic tiglians for discovering immunomodulating agents which are currently used to modulate the host natural defense and immune function in various conditions such as treatment of infections, organ rejection, rheumatoid arthritis and systemic lupus erythematosus (14).

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