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***Original Article***

# 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-4α-phorbol-12-(2,3-dimethyl) butyrate-13-isobutyrate and 17-hydroxy-4-deoxy-4α-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 ± 0.57 and 44.1 ± 3.8 μg/ml, respectively, while compound 1 was relatively inactive with IC50 >50 μg/mL for T-cell proliferation, and >100 μg/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 derivatives 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 [$^3$H] 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 chroma-tography was performed on LiChroprep® Si 60 (25-40 μm), or silica gel 63-200 μm, and size exclusion chromato-graphy 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 taxo-nomist (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 Depart-ment, 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 L × 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 $^1$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 μg/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 37º 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 μg/mL in

duplicates) in supplemented RPMI-1640 along with phytohemagglutinin (PHA) at 37 ºC in $CO_2$ environment for 72 h. Further incubation for 18 h after the addition of thymidine [$^3$H] 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 $IC_{50}$ 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 $^1$H-$^1$H COSY, and HMBC experiments (Fig 1).

*Compound 1*

Colourless oil, UV (CHCl3) $\lambda_{max}$: 239 nm. IR (KBr+$CHCl_3$) $\nu_{max}$ 3733, 3610, 2873, 1714, 1645, 1517, 1456, 1394, 1204, 1160, 1076, 1029 cm$^{-1}$ ; $^1$H NMR ($CDCl_3$, 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); $^{13}$C NMR ($CDCl_3$, 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 $C_{30}H_{44}O_7$ +$H^+$, 517.3159), 383 , 354, 313, 295.

*Compound 2*

Colourless oil, UV ($CHCl_3$) $\lambda_{max}$ (log ε): 232 (3.95), 280 (3.68) nm. IR (KBr+$CHCl_3$) $\nu_{max}$ 3799, 3733 ,3610,3970, 2873,1725,1714, 1680, 1645,1517,1456, 1394,1250, 1204,1160, 1076,1029, 667,408 cm$^{-1}$ ; $^1$H NMR ($CDCl_3$+ $CD_3OD$, 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); $^{13}$C NMR ($CDCl_3$+$CD_3OD$, 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 $C_{30}H_{44}O_8$ +$H^+$, 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 incorpora-tion. Compound 2 showed inhibitory activity against lymphocyte proliferation with $IC_{50}$ of

13.27 ± 0.18 µg/mL whereas compound 1 was reactively inactive ($IC_{50}$ >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 $IC_{50}$ >100 µg/ml, while moderate inhibitory activity with $IC_{50}$ equal to 44.1 ± 3.84 µg/ml was found for compound 2**,** which may be due to the inhibition of PMNs proliferation.

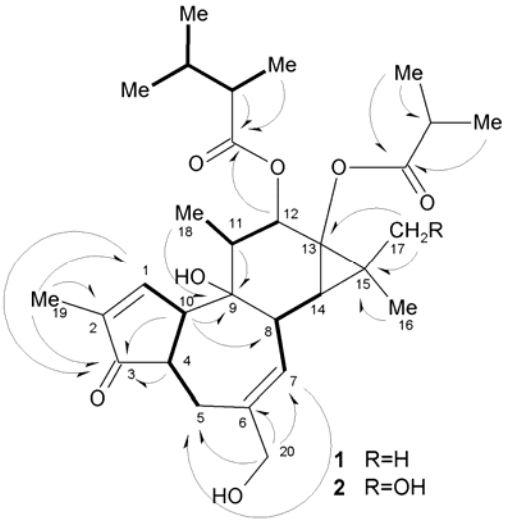


**Fig. 1.** Key DQF-COSY (in bold) and $^{2,3}J$ (H→C) HMBC correlations observed in tigliane-type diterpenes from *Euphorbia aellenii* .

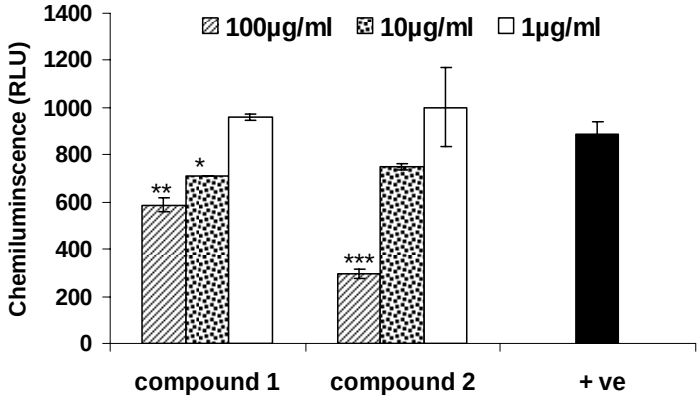


**Fig. 2.** Effects of compounds 1 and 2 on the neutrophils oxidative burst. The luminol dependent chemiluminscence induced by zymosan in the presence of compounds 1 and 2 at three concentrations are compared to those of the positive control (+ve). Data are presented as means ± SD for three measurements. 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 $^1$H-NMR spectrum, four methyl groups $\delta_H$ 1.07 d (6.3), 1.14 s, 1.18 s and 1.76, two geminal oxymethylene protons $\delta_H$ 4.00 (d, *J*=12.3 Hz, H-20b) and 3.89 (d, 12.3 Hz, H-20a), two geminal hydrogens $\delta_H$ 2.43 (dd, *J*= 15.3, 5.1 Hz, H-5a) and 3.42 (br-d, H-5b) together with two olefinic protons $\delta_H$ 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 ~ 9-10 Hz) and at *ca* $\delta_H$=1 (J~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 $\delta_H$ 7.03 of an olefinic group with ketone carbonyl $\delta_C$ 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 $\delta_H$ 1.14 and 1.18 with quaternary carbon $\delta_C$ 25.2 (C-15) confirmed the presence of these two geminal unfunctionalized methyl groups on cyclopropane ring (8, 11). The $^1$H-$^1$H -COSY correlations confirmed the following protons : $CH_3$-CH-CH($CH_3$)-$CH_3$ [$\delta_H$ 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 ($\delta_H$ 5.45) and H-2' ($\delta_H$ 2.20) with ester carbonyl carbon ($\delta_c$ 175.5) confirmed this fragment as 2', 3'-dimethyl-butanoate moiety attached to C-12 (12). Likewise, $^{13}$C- and $^1$H-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" ($\delta_H$ 1.15) with H-14 ($\delta_H$ 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 4α-deoxy tigliane, bearing one –$CH_2$OH group (δc 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-10α 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-8β, as well. The large *J*-coupling constant (10.2 Hz) between H-12α and H-11 revealed their *trans* orientation and consequently the observed NOE of H-11β 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-4α-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

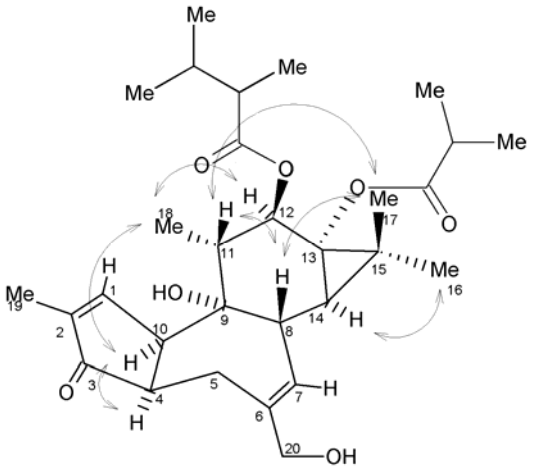


**Fig. 3.** Key correlations observed in the NOESY spectrum of compound 1 from *Euphorbia aellenii*.

hydroxyl group on C-17 ($\delta_c$ 70.4) which proposed compound 2 to be 17-hydroxy-4-deoxy-4α-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 μg/ml, respectively, while compound 1 was relatively inactive with IC50 >50 μg/mL for T-cell proliferation and >100 μg/ml for ROS. Similar studies on closely related phorbol derivatives for stimulation of human mononuclear cells have shown that 4β -deoxyphorbol esters stimulated cell proliferation in a dose-related manner, while 4α -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, 4β -phorbol 12,13-dibutyrate showed activity in a concentration-dependent manner, whereas the structurally related isomer 4α -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 proinflamatory promoters. In the current study and previous reports using related 4α –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-4α-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 polymorphoneutrophils (PMNs) in human whole blood and lymphocyte proliferation. Therefore, *E.aellenii* could be a new source of theses 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 erythematous (14).

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