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Further Terpenoids from *Euphorbia tirucalli*

Thuc-Huy Duong,^{a,b} Mehdi A. Beniddir,^c Grégory Genta-Jouve,^d Huu-Hung Nguyen,^e Dinh-Phuoc Nguyen,^f Thi-Anh-Tuyet Nguyen,^g Dinh-Hung Mac,^h Joël Boustie,ⁱ Kim-Phi-Phung Nguyen,^j Warinthorn Chavasiri,^{f*} and Pierre Le Pogam^{c*}

^a*Department for Management of Science and Technology Development, Ton Duc Thang University, Ho Chi Minh City, Vietnam*

^b*Faculty of Applied Sciences, Ton Duc Thang University, Ho Chi Minh City, Vietnam*

^c*BioCIS, Université Paris-Sud, CNRS, Université Paris-Saclay, 5 Rue J.-B. Clément, 92290 Châtenay-Malabry, France*

^d*Équipe C-TAC, UMR CNRS 8638 COMETE – Université Paris Descartes, 4 Avenue de l'Observatoire, 75006 Paris, France*

^e*Faculty of Hi-Tech Agriculture and Biotechnology, Nguyen Tat Thanh University, 300A Nguyen Tat Thanh Str., Dist. 4, Ho Chi Minh City 748355, Vietnam*

^f*Department of Chemistry, Faculty of Science, Chulalongkorn University, Phayathai Rd., Patumwan, Bangkok 10330, Thailand*

^g*Department of Chemistry, Ho Chi Minh City University of Education, 280 An Duong Vuong Street, District 5, 748342 Ho Chi Minh City, Vietnam*

^h*Department of Organic Chemistry, University of Science, Ha Noi National University, 19 Le Thanh Tong Str., Dist. Hoan Kiem, Ha Noi City 748355, Vietnam Natural Products Research Unit*

ⁱ*Univ Rennes, CNRS, ISCR (Institut des Sciences Chimiques de Rennes) – UMR 6226, F-35000 Rennes*

^j*Department of Organic Chemistry, University of Science, National University – Ho Chi Minh City, 227 Nguyen Van Cu Str., Dist. 5, Ho Chi Minh City 748355, Vietnam*

Abstract

The phytochemical investigation of *Euphorbia tirucalli* L. (Euphorbiaceae) yielded four new compounds, including a rare cadalene-type sesquiterpene (tirucadalenone), two tirucallane triterpenoids, euphorol L and euphorol M, with the latter being described as an epimeric mixture, and a euphane triterpene, namely, euphorol N, together with 7 known compounds. Their structures and absolute configurations were elucidated from analysis of 1D (¹H, J-modulated ¹³C) and 2D NMR (HSQC, HMBC and NOESY), high-resolution mass spectrometry (HRESIMS), optical rotation, and GIAO NMR shift calculation followed by CP3 analysis, along with comparison with literature reports. All these compounds were tested for cytotoxicity against K562, MCF-7 and/or HepG2 tumor cell lines. Only tirucadalenone displayed a mild cytotoxic activity.

Keywords: *Euphorbia tirucalli* L.; *Euphorbiaceae*; Sesquiterpene; Cadalene; Triterpenes; Tirucallane; Euphane.

Graphical abstract

Four previously undescribed terpenoids: a cadalene (**1**), two tirucallanes (**2** and **3a/3b**), and a euphane (**4**) were isolated from *Euphorbia tirucalli* L.

1. Introduction

Euphorbia is among the largest 'giant genera' of flowering plants with close to 2.160 recognized species being renowned for their remarkably diverse growth forms, making it the third largest genus of angiosperms, second only to *Astragalus* (Fabaceae) and *Psychotria* (Rubiaceae) [1,2]. *Euphorbia* plants are easily distinguishable by their irritant milky latex and their specialized inflorescences (cyathia) [3]. The chemical diversity of Euphorbiaceae is mainly related to their isoprenoid constituents. In particular, diterpenoids account for the majority of the metabolites reported in this genus with more than 650 metabolites being recorded, falling into more than twenty core frameworks including casbanes, jatrophanes, daphnanes, tiglianens, and ingenanes series alongside triterpenoids and sesquiterpenoids [4]. As such, several *Euphorbia* terpenes endowed with promising pharmacological properties were reported during the last decade including ingenol-3-angelate that was approved by the FDA in 2012 and by EMA in 2013 for actinic keratosis treatment [5] while other phorbol and ingenane derivatives are currently under clinical investigations [6,7]. In this privileged taxa, *Euphorbia tirucalli* L. is a shrub or small tree endemic to tropical areas with pencil-like branches from which derives its vernacular name of pencil tree [8]. *E. tirucalli* L. is widely distributed in the Pantropical region of Madagascar, The Cape region (South Africa), East Africa, and Mainland Southeast Asia and is grown as garden plant in various tropical countries. Despite being widely regarded as a toxic plant, its various parts are highly valued for their medicinal properties in local medicine [9]. It is used in African folk medicine against warts, cough, sexual impotence, haemorrhoids, epilepsy and cancer [10,11]. In India, this plant is used for the treatment of cancer, asthma, leprosy and leucorrhoea [10]. The bark and the latex of this plant exhibit a variety of significant pharmacological activities such as antibacterial [12,13], antiherpetic [14], and anti-mutagenic [15]. Furthermore, *E. tirucalli* latex exerts pesticidal properties against various pests including mosquitoes [16,17], molluscs [18,19]. Exposure to *Euphorbia tirucalli* has been proposed to be a cofactor in the genesis of endemic Burkitt's lymphoma as suggested by the coincidence between this condition and human exposure to this plant in the lymphoma belt of Africa [20]. *E. tirucalli* is also widely used for poison fishing in tropical Africa [21] as corroborated by its strong piscicidal effects towards the catfish *Heteropneustes fossilis* [22]. Owing to the high amounts of triterpenes and sterols of *E. tirucalli*, it was also suggested that this plant might be used for rubber fractionation so that it was investigated for its diesel oil properties [23].

As a continuation of our research focused on the diversity of bioactive metabolites from Vietnamese medicinal plants [24], we herein report on the structure elucidation of a rare cadalene-type sesquiterpene (**1**), two tirucallane-type triterpenes (**2**, **3a** and **3b**) and an euphane-derived triterpenoid (**4**), along with 7 known compounds.

2. Results and Discussion

Dried whole plants of *Euphorbia tirucalli* were extracted with ethanol. As the crude extract evaporated *in vacuo*, a precipitate was formed, which was filtered off prior to any further phytochemical processing. Both filtered ethanol extract and precipitate were successively extracted with *n*-hexane, EtOAc and *n*-BuOH. Further purifications were performed using a combination of chromatographic techniques including silica gel and preparative thin-layer chromatography. Through this phytochemical study, four undescribed terpenoids (**1–4**) were isolated, elucidated through NMR and mass spectrometric analyses and then assessed for their cytotoxicity against tumor cell lines (Fig. 1).

FIGURE 1

Tirucadalenone (**1**) was isolated as a white amorphous solid and its molecular formula was established as C₁₅H₁₆O₄ (corresponding to an index of hydrogen deficiency of 8) based on the sodiated ion peak at *m/z* 283.0968 ([M+Na]⁺, calcd. 283.0941). The ¹H NMR data of **1** showed two *ortho* aromatic protons (δ_{H} 7.42, 1H, d, *J* = 8.0 Hz and 7.30, 1H, d, *J* = 8.0 Hz), one hydroxy group (δ_{H} 6.67), one olefinic proton (δ_{H} 6.13, q, *J* = 1.0 Hz), four methyl groups [δ_{H} 2.58, 2.08, 1.61, 1.56 (each 3H, s)]. The ¹³C NMR and HSQC spectra revealed 14 different carbon resonances comprising one ketone carbonyl group, four methyl groups, three sp² methine carbons, two oxygenated sp³ tertiary carbons and four sp² quaternary carbons. Long-range heteronuclear correlations led to define a first aromatic ring that comprised the two *ortho*-coupled aromatic protons and a first methyl group. In particular, H-9 revealed key HMBC cross-peaks with C-7 (δ_{C} 137.4), C-8 (δ_{C} 128.6) and C-10 (δ_{C} 138.7). The CH₃-15 moiety was connected to C-10 as evidenced by the HMBC correlations from these protons to C-9 (δ_{C} 133.1) and C-10. A third HMBC cross-peak of equal intensity connected these protons to what seemingly corresponded to C-8, which would stand for a ⁴*J* HMBC correlation. Instead, it could rather be supposed that this correlation might be associated to the missing quaternary carbon C-1 (δ_{C} 128.6). Subsequently, HMBC correlations from both CH₃-13 and CH₃-14 to C-12 and C-7 were diagnostic of the attachment of an isopropyl moiety on this latter carbon. This connectivity is also supported by the H-8/C-12 HMBC cross peak. A second nucleus, the so-called B-ring, could be elucidated from long-range heteronuclear correlations originating from H-3, H₃-11 and OH-5. The CH₃-11 moiety could be located on C-4 based on the HMBC correlation from these protons to C-3, C-4 and C-5. Likewise, the C-5 position of the hydroxy group could be deduced from its HMBC correlations to C-4, C-5 and C-6, consistently with the H-3/C-5 HMBC cross-peak. The structure of the remaining part of this nucleus could be determined based on H-3/C-1 and H₃-11/C-2 HMBC cross-peaks. Most interestingly, the determined position of the ketone moiety is in line with the ⁴*J* HMBC coupling from H₃-15 to C-2. The

downfield chemical shift of C-4 (δ_c 154.0) further backed up the defined α,β -unsaturated ketone moiety. Finally, owing to C-1, C-6 and C-7 being three sp^2 quaternary carbons and due to all the other carbons of the molecules being already assigned at this stage, the C-1-C-6-C-7 connectivity could be deduced to afford a two fused six-membered ring. The spectroscopic features described so far represent seven indices of hydrogen deficiency, still leaving one to be added while two oxygen atoms also have to be incorporated into the structure. The ^{13}C NMR data also indicate that both C-5 and C-12 represent oxygenated sp^3 quaternary carbons. A pertinent way to meet all these requirements is to bridge C-5 and C-12 through a peroxy bridge, resulting in an 1,2-dioxane system. This structure is supported by the chemical shift value of C-12 which is typical of a peroxydated carbon [25–27] while the carbon resonance of C-5 is reminiscent of the value reported from cyclic peroxyhemiketal-containing terpenoids [28]. Therefore, the structure of compound **1** was elucidated as depicted in Fig. 1.

FIGURE 2

TABLE 1

Compound **1** displays the 1,7-dimethyl-4-isopropyldecalin skeleton typical of the so-called cadinane sesquiterpenes *lato sensu*, with further sub skeletons being distinguished according to the stereochemistry of the ring linkage [29]. This archetypal substitution pattern is related to their biogenesis proceeding from either a bisabolane or a germacrane cation [30]. One or both cadinane rings can subsequently aromatize. Calamene-type sesquiterpenes contain one benzenoid ring while cadalenes incorporate the whole naphthalene bicycle [31]. Some tricyclic analogues of cadinane sesquiterpenes also extended the chemical diversity of this structural class, leading to introduce a fused five or six-membered oxygenated heterocycle [32–34] Nevertheless, the 6/6/6-fused tricyclic ring skeleton of tirucadalenone comprising an 1,2-dioxane core is an unprecedented structural feature. The *para*-disposition of the 5-OH group moiety to the carbonyl group at C-2 suggests that **1** might stem from a *para* quinone precursor. A variety of *ortho* [35,36] and *para* quinones [37,38] were indeed reported within cadalene sesquiterpenes. Accordingly, a convenient starting point to access **1** would be a C-12 hydroperoxide of the known cadalenequinone [39]. Even though this specific derivative of cadalenequinone has not been reported so far, terpenes' hydroperoxidations are of considerable generality [40]. Thereafter, an intramolecular nucleophilic attack of the peroxide group onto the putative C-5 carbonyl group could straightforwardly afford the final peroxyhemiketal function, based on a reactivity widely described elsewhere [41,42], and as earlier proposed to step in the biosynthesis of cyclic peroxyketal metabolites [28]. The lack of optical activity hinted the racemic nature of **1**, as further backed up by the lack of any Cotton effect in the ECD spectrum. This would be related to the

proposed biosynthetic scenario according to which the access to the cyclic peroxyhemiketal would merely be driven by chemical reactivity and most likely involve no enzyme in the process, thereby not awaiting any stereoselectivity to be introduced at this stage. Among the scarce number of natural products comprising such peroxyhemiketal or peroxyketal groups, some were reported as epimeric mixtures such as sinugibberosides from the soft coral *Sinularia gibberosa* [28] and xestins from the marine sponge *Xestospongia* [43]. The hemiketal nature of C-5 may render its configuration labile throughout phytochemical processing [44,45]. Cadinane-group sesquiterpenes, *lato sensu*, are being subdivided based on the nature of the ring fusion and the orientation of the isopropyl group at C-7, dividing them into four structural classes : cadinanes, muurolanes, bulgaranes, and amorphanes [46]. Regarding the specific example of tirucadalenone, the subsequent aromatization of the skeleton prevents ascribing it to one of these basic skeletons. This compound was detected unambiguously from the crude *n*-hexane extract, hinting that this compound should be a genuine natural product (see supporting information). This does not formally exclude the possibility of a peroxidation artifact, which may occur during extraction.

Euphorol L (**2**) was isolated as a white gum with a molecular formula of $C_{30}H_{48}O_5$ (7 indices of hydrogen deficiency) as indicated by the $[M-H]^-$ peak at m/z 487.3416 (calcd. for $C_{30}H_{47}O_5$, 487.3429) in the HRESIMS spectrum. The 1H , J-modulated and ^{13}C NMR data exhibited resonances for a disubstituted double-bond (δ_H 5.61 (2H), δ_C 137.3 and 128.7), an α,β -unsaturated ketone (δ_C 199.7, 140.3 and 163.0), a secondary alcohol methine [δ_H 3.27, *dd*, ($J = 11.5, 6.0$); δ_C 78.1], one secondary methyl [δ_H 0.96, *d* ($J = 6.5$)] and seven tertiary methyl groups (δ_H 0.76, 0.91, 0.98, 1.13 and 1.27 (x3)) along with seven methylene carbons, three further methine carbons and five quaternary carbons (including an oxygenated one). Spectroscopic features described so far account for three units of desaturation (two olefinic moieties and one ketone), thus determining the structure to be tetracyclic. The structure of the fused ring moiety was entirely confirmed by HMBC experiments as displayed in Fig. 3. The quaternary carbon C-25 was deduced to bear a hydroperoxy group as evidenced by the value of its chemical shift at δ_C 80.0 [25–27] against *ca.* 70 ppm in the case of an alcohol moiety [47,48]. Accordingly, the signals related to the side chain were superimposable to those of known tetracyclic triterpenes having the $\Delta^{23, 25}$ -hydroperoxide moiety: inoterpene C [49], floralginsenosides B and D–F [50] and floralquinquenosides A and C [51]. The signals related to the olefinic protons resulted in a multiplet at δ_H 5.6 that could not be resolved by obtaining a spectrum of **2** in other deuterated solvents, precluding the straightforward assignment of the geometry of the double bond based on the magnitude of the vicinal coupling constant value. Accordingly, the geometry of the side chain was established as E primarily based on comparison of the ^{13}C NMR spectroscopic data of analogues with similar side chain [52–54] and further backed up by biosynthetic considerations since most related

triterpenes isolated from plants display this configuration [55]. Altogether, these data led to characterize the 2D structure of **2** as displayed in Fig. 1. consistently with NMR data reported elsewhere on related scaffolds [48,56].

FIGURE 3

The large coupling constants of H-3 [δ_{H} 3.27, *dd*, ($J = 11.0, 6.0$)] indicated its axial α -position, thus deducing the equatorial β -position of 3-OH moiety [57–59]. The stereochemistry of the tetracyclic nucleus and the orientation of the side chain could be further inferred from the key NOE correlations emphasized in Fig. 4. These configurations were consistent with either a euphane or a tirucallane triterpene that only differ by the configuration of C-20 [60]. NMR spectral data of tetracyclic triterpenes indicate that carbon resonances are too similar between euphane (2*OR*) and tirucallane (2*S*) triterpenoids to afford a clear-cut discrimination between these structural classes on this sole basis, which led to several erroneous structural reports [61]. A reliable spectroscopic approach to differentiate between these structural classes is the nuclear Overhauser effect of H-21 with H-16 α,β in euphanes and with H-12 α in tirucallanes [62–64,47,48,65]. Compound **2** revealed the key NOESY correlation H-21/H-12 α determining it as a tirucallane triterpenoid, as further suggested by the NOESY cross-peak between H₃-18 and both H-20 and H₃-21 (Fig. 4).

FIGURE 4

At last, the negative optical rotation of **2** (-92.3) was a further clue indicating that **2** pertained to the tirucallane triterpenes rather than to the euphane series [66,67]. Thus, the structure of **2**, namely euphorol L, was determined as indicated in Fig. 1. Both ¹H and ¹³C NMR chemical shift values of **2** were consistent with those reported from the identical tetracyclic scaffold of tirucalla-8,24-diene-3 β ,11 β -diol-7-one [56].

Euphorol M (**3**) was isolated as an optically active white amorphous solid, the molecular formula of which was established as C₃₀H₅₂O₃ based on its negative-ion mode HRESIMS data (m/z 459.3803, calcd for C₃₀H₅₂O₃-H, 459.3844). As for compound **2**, a 3 β -hydroxy- Δ^8 tetracyclic scaffold could be assigned based on 1 and 2D NMR spectral data. Some slight differences could nevertheless be observed between the fused ring structures of **2** and **3**. These could be related to the reduction of the C-7 ketone group to give rise to a further diastereotopic methylene signal, also being in line with the upfield shift of the vicinal carbon C-6 (δ_{C} 19.1) and of the olefinic carbons C-8 and C-9 (δ_{C} 133.7 and 134.2, respectively). The relative configuration of **3** was determined to be similar to that of **2** from both ¹H NMR chemical shift values and NOESY correlations. At first, the large magnitude of the vicinal coupling constant of H-3 established the equatorial position of the 3 β -hydroxy group. Subsequently, the significant NOE correlations: H-3/H-5, H-3/H₃-28, H₃-18/H-20, H₃-18-H-22, H₃-29/H₃-19 and H₃-

30/H-17 defined a similar stereochemistry of the tetracyclic triterpene core. The NOE correlations from the diastereotopic methylenic protons at C-11 ($H_3-18/H-11\alpha$ and $H_3-19/H-11\beta$) revealed the antifacial orientations of the methyl groups CH_3-18 and CH_3-19 , ruling out the possibility of a lanostane triterpene. As to the side chain, NMR data revealed that the double bond was reduced and that a hydroxy substituent was introduced on C-24. At last, the chemical shift of C-25 (δ_C 73.3) indicates that its hydroperoxy substituent was replaced by an alcohol group. Compound **3** exhibited NOE correlations between H_3-18 and both H-20 and H_3-21 as well as between H-12 and H_3-21 that are possible only in case of (*S*)-configuration at C-20, leading to assign **3** a tirucallol skeleton as well, as further backed up by the negative optical rotation of **3** (-121.0). Having determined the planar structure of **3**, a detailed inspection of the 1H and ^{13}C NMR data revealed noticeable shifts in C-24 chemical shift value (δ_H 3.35 and 3.29; δ_C 79.8 and 78.9) leading to the surmise that **3** might indeed represent a mixture of C-24 epimers. Doubling of carbon resonances of adjacent carbons is in line with this inference. For further verification, Smith and Goodman's CP3 parameter method was undertaken to demonstrate the epimeric relationship of **3a** and **3b** and individually assign NMR data. This strategy has been extensively applied in structural validation and revision in cases when the NMR data of a pair of diastereomers are available [68,69]. Taking into account both 1H and ^{13}C NMR spectroscopic data for CP3 probability analysis led to assign the configuration of C-24 as *S* for the major form **3a** (see supporting information) since the CP3 parameter results in respective values of 0.46 and -0.37 for the correct and incorrect assignments. Jointly with Bayes' theorem, this leads to define a quantifiable confidence of 67.9%. Compound **3** was therefore determined to be a mixture of diastereoisomers with the major form (24*S*) and the minor form (24*R*) in a 6:4 ratio. Although the determined side chain is fairly common among various triterpene series such as lanostanes [70] or cycloartanes [71], it is herein first being reported on a tirucallane scaffold.

Euphorol N (**4**), isolated as a white amorphous solid, was determined as $C_{30}H_{52}O_3$ from the HRESIMS deprotonated molecular peak at m/z 459.3809 (calcd for $C_{30}H_{52}O_3-H$, 459.3844), making it an isomer of **3**. The comparison of NMR data indeed supported that these two metabolites were very closely related to each other, with a common planar structure and identical configurations, except for the side chain moiety. The key NOE cross-peak between $H_3-18/H-11\alpha$, $H_3-19/H-11\beta$, and $H_3-21/H-16\alpha$ defined a euphane-type triterpene [63,72].

The 1H NMR chemical shift of CH_3-21 (0.85, *d*, 5.5 Hz), along with the positive optical rotation of **3** (+ 17.0) further confirmed that this metabolite pertained to the euphane rather than the tirucallane series [73–75]. Such euphane derivatives were formerly reported to occur within *Clusia columnaris* but were isolated as a mixture of C-24 epimers [76]. The structure elucidation of this compound by Compagnone *et al.* can be regarded as dubious since it was determined based on the

sole basis of chemical shift values which usually do not afford a clear-cut distinction between euphane and tirucallane series, as aforementioned. Thus, neither the diagnostic optical rotation value nor the key NOESY correlations to reliably discriminate between these two structural series were discussed, making the structural assignment rather uncertain. Likewise, the co-isolation of euphol from *Clusia columnaris* is not a relevant argument since both euphane and tirucallane series can arise from the same enzymatic system [60], making these structural series often associated within a single plant, as reported here. The few NMR chemical shift values discrepancies between the currently reported structure and those appearing in Compagnone et al. may indicate that this euphane is herein first described due to a former misidentification and reported as a single molecule with fully assigned spectroscopic data. The chemical shifts of **4** were in excellent agreement with those of **3a**, thus determining a (24S) stereochemistry for this former compound as well. Altogether, these spectroscopic data determined **4** as shown in Fig. 1.

Seven known compounds were also isolated from the ethanol extract of *E. tirucalli* and identified as the triterpenes euphorol D [59], euphol [77], and lupanone [78], the sterol ergosterol peroxide [79], the terpene alcohol vomifoliol [80], the coumarin scopoletin [81] and the anthraquinone aloe-emodin [82]. The structures of these compounds were determined based on the thorough analysis of their 1D and 2D NMR spectra, mass spectrometry data, alpha D values and GIAO NMR shift calculations with subsequent CP3 probabilities; as well as on comparisons with literature data.

In this study, compounds **1-4** were evaluated for their cytotoxicity against K562 (chronic myelogenous leukemia), MCF-7 (breast cancer) and/or HepG2 (liver hepatocellular carcinoma) cell lines. Compound **1** exerted a mild activity against both K562 and HepG2 cell lines with respective IC₅₀ values of 22 (± 1.4) and 89 (± 1.2) µg/mL (*i. e.* respective values of 84.6 (± 5.4) and 342 (± 4.6) µM). Other compounds failed to reveal any cytotoxicity on HepG2 and MCF-7 cell lines.

3. Conclusions

The present study reports the isolation and identification of four new terpenic compounds with cadalene, tirucallane and euphane skeletons from whole plant extracts of *Euphorbia tirucalli*. As far as can be ascertained, the 6/6/6-fused tricyclic ring of the cadalene sesquiterpene tirucadalenone (**1**) incorporating a 1,2-dioxane ring is unprecedented in nature. The complexity of the studied extract might stem from this phytochemical investigation being conducted on whole plants rather than specific organs.

4. Experimental section

4.1 General experimental procedures

Optical rotations were measured on a Jasco DIP-370 digital polarimeter at 293 K. Electronic circular dichroism were recorded using a Jasco J-815 ECD spectrometer. UV spectra were obtained using a Perkin Elmer Lambda 25 UV-Vis spectrometer. The IR spectra were acquired using a Shimadzu FTIR-8200 infrared spectrophotometer. NMR spectra were measured on a Bruker Avance III (500 MHz for ¹H NMR and 125 MHz for ¹³C NMR) spectrometer with TMS as internal standard. Proton chemical shifts were referenced to the solvent residual signal of CD₃COCD₃ at δ_H 2.05 or of CDCl₃ at δ_H 7.18. The ¹³C-NMR spectra were referenced to the central peak of CD₃COCD₃ at δ_C 29.4 or of CDCl₃ at δ_C 77.8. The HR-ESI-MS were recorded on a HR-ESI-MS Bruker microTOF Q-II. TLC analyses were carried out on precoated silica gel 60 F₂₅₄ (Merck) and spots were visualized by spraying with 30% H₂SO₄ solution followed by heating. Gravity column chromatography was performed with Silica gel 60 (0.040–0.063 mm, Himedia). Samples were analysed using an Agilent LC-MS system comprising an Agilent 1260 Infinity HPLC hyphenated with an Agilent 6530 Q-TOF-MS fitted with an ESI source operating in

positive-ion mode. A Sunfire analytical C₁₈ column (150 x 2.1mm; i.d. 3.5 μm, Waters) was used, with a flow rate of 250 μL/min. HPLC analyses were performed by elution gradient using the following parameters: A (0.1% formic acid in water) and B (acetonitrile); T, 0-2 min 5% B; 2-20 min, 100% B linear; 20-30 min, 100 % B; 30-31 min, 5% B linear. ESI conditions were set with the capillary temperature at 320°C, source voltage at 3.5 kV, and a sheath gas flow rate of 10 L/min.

4.2 Plant material

Whole plants of *Euphorbia tirucalli* L. (Euphorbiaceae) were collected from Hong Son village, Ham Thuan Bac, in Binh Thuan province (11.129754° N, 108.143550° E) in July 2014 (wet season). The botanical sample was identified by Dr. Pham Van Ngot, Department of Botany, Faculty of Biology, Ho Chi Minh University of Pedagogy. A voucher specimen (No UP002) is deposited in the herbarium of the Department of Organic Chemistry, University of Science, Ho Chi Minh City.

4.3 Extraction and isolation

The clean, air-dried and ground material (3.5 kg) was extracted by maceration with EtOH (10 L x 2) at 70°C. A precipitate occurred as the crude extract was being evaporated under reduced pressure, which was filtered off to be further processed separately, affording 250.4 g of precipitate **P**. The filtered solution was evaporated to dryness to yield 290.3 g of crude ethanol extract. The dry residue of this latter extract was subsequently dissolved using solvents of increasing polarities: *n*-hexane (**H**, 94.2 g), EtOAc (**EA**, 61.8 g) and *n*-BuOH (**B**, 27.0 g). Extract **H** (94.2 g) was applied to silica gel CC using a *n*-hexane/EtOAc/Acetone gradient system (12:1:1 to 5:1:1; v/v/v) to afford three fractions. Among these, fraction **H2** (15.7 g) was fractionated by Sephadex LH-20 CC using MeOH to yield three subfractions (**H2.1-H2.3**). Sub-fraction **H2.1** (4.1 g) was applied to normal phase silica gel CC and eluted isocratically with the solvent system *n*-hexane/EtOAc/EtOH/Acetic acid (9:2:1:0.2; v/v/v/v) to give eight subfractions **H2.1.1-H2.1.8**. Fraction **H2.1.1** (1.8 g) was subjected to silica gel CC using *n*-hexane/EtOAc/acetone (12:1:1; v/v/v) to isolate **7** (21 mg) and **12** (25 mg). Fraction **H2.1.2** (388 mg) was submitted to reverse phase C₁₈ silica gel CC, using MeOH/Acetone/H₂O (2:1:1; v/v/v) as mobile phase to purify compound **1** (3 mg). Fraction **H2.1.3** (489.0 mg) was further chromatographed by reverse phase C₁₈ silica gel CC and isocratically eluted with a MeOH/Acetone/H₂O (1:3:1; v/v/v) solvent system to obtain three subfractions **H2.1.3.1-H2.1.3.3**. Isocratic elution of **H2.1.3.1** (121 mg) on silica gel CC by the solvent system *n*-hexane/chloroform/EtOAc/Acetone (100:40:24:10) afforded compounds **2** (2.7 mg), **3** (3.1 mg) and **4** (3.4 mg). Likewise, fraction **H2.1.3.3** was fractionated using the same solvent system to yield **6** (2.1 mg), **8** (3.2 mg) and **11** (2.1 mg). Fraction **H2.3** (3.7 g) was

fractionated by normal phase silica gel CC using *n*-hexane/EtOAc/Acetone (7:1:1; *v/v/v*) as mobile phase to obtain three fractions **H2.3.1-H2.3.3**. Subfraction **H2.3.1** (241 mg) was further purified using the same chromatographic procedure to afford **9** (11 mg) and **10** (8 mg).

4.4 Spectroscopic data

4.4.1. Tirucadalenone (**1**)

Light-yellow amorphous solid; $[\alpha]_D^{20}$ 0 (*c* 0.1, MeCN); UV (CHCl₃) λ_{\max} (log ϵ) 229 (3.30), 276 (2.86), 308 (2.48) nm; IR (KBr) ν_{\max} 3400, 1655, 1604, 1519, 828, 777 cm⁻¹; HRESIMS *m/z* 283.0968 [M+Na]⁺ (calcd for C₁₅H₁₆O₄ + Na, 283.0941). For ¹H and ¹³C NMR (acetone-*d*₆) spectroscopic data, see Table 1.

4.4.2. Euphorol L (**2**)

White gum; $[\alpha]_D^{20}$ -92.3 (*c* 0.1, MeOH); UV (CHCl₃) λ_{\max} (log ϵ) 246 (4.03), 277 (3.46) nm; IR (KBr) ν_{\max} 3368, 2974, 2863, 1645, 1456, 1379, 1037, 622 cm⁻¹; HRESIMS *m/z* 487.3416 [M-H]⁻ (calcd for C₃₀H₄₇O₅, 487.34290). For ¹H and ¹³C NMR (acetone-*d*₆) spectroscopic data, see Table 2.

4.4.3. Euphorol M (**3**)

White amorphous solid; $[\alpha]_D^{20}$ -121 (*c* 0.1, MeOH); UV (CHCl₃) λ_{\max} (log ϵ) 228 (3.55) nm; ν_{\max} 3438, 2940, 1645, 1070, 1060, 1030 cm⁻¹; HRESIMS *m/z* 459.3803 [M-H]⁻ (calcd for C₃₀H₅₁O₃, 459.3844). For ¹H and ¹³C NMR (chloroform-*d*₁) spectroscopic data, see Table 2.

4.4.4. Euphorol N (**4**)

White amorphous solid; $[\alpha]_D^{20}$ +17 (*c* 0.1, MeOH); UV (CHCl₃) λ_{\max} (log ϵ) 227 (3.63) nm; ν_{\max} 3438, 2940, 1645, 1070, 1060, 1030 cm⁻¹; HRESIMS *m/z* 459.3809 [M-H]⁻ (calcd for C₃₀H₅₁O₃, 459.3844). For ¹H and ¹³C NMR (chloroform-*d*₁) spectroscopic data, see Table 2.

4.5 Biological assays

Cytotoxic activities of the formerly unreported metabolites against the HepG2 (liver hepatocellular carcinoma) and K562 (chronic myelogenous leukemia) tumor cell lines. These two cell lines were cultured in RPMI 1640 medium or in DMEM medium, respectively, supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μ g/mL streptomycin and maintained at 37 °C and 5% CO₂ with 95% humidity. Viable cells were counted and inoculated in 96-well plate with density of 10⁴ cells/100 μ L/well for HepG2 and 10⁵ cells/100 μ L/well for K562. After 24 hours, the cells were treated with the compounds and doxorubicin (positive control) diluted in culture media at 100, 50, 25, 12.5, 6.25, 3.125 and 0 μ g/mL concentration containing 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0% dimethyl sulfoxide (DMSO), respectively. DMSO in culture media was used as negative control. In addition, culture medium without cells was used as blank. All experiments were done in triplicate. The plates were incubated in 5% CO₂, 95% humidity at 37 °C for 72 h. 10 μ L of 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml stock solution) was added into each well and incubated in 37 °C in 5% CO₂ for 3.5 hours. 70 µL of Detergent Reagent (10% SDS) was added into each well and the plate was maintained in 37 °C for 16 h. The optical density of each well was read by using a scanning multiwell spectrophotometer (Sunrise) at wavelength of 595 nm. Cell survival was measured as the percentage absorbance compared to the negative control (DMSO-treated cells).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at

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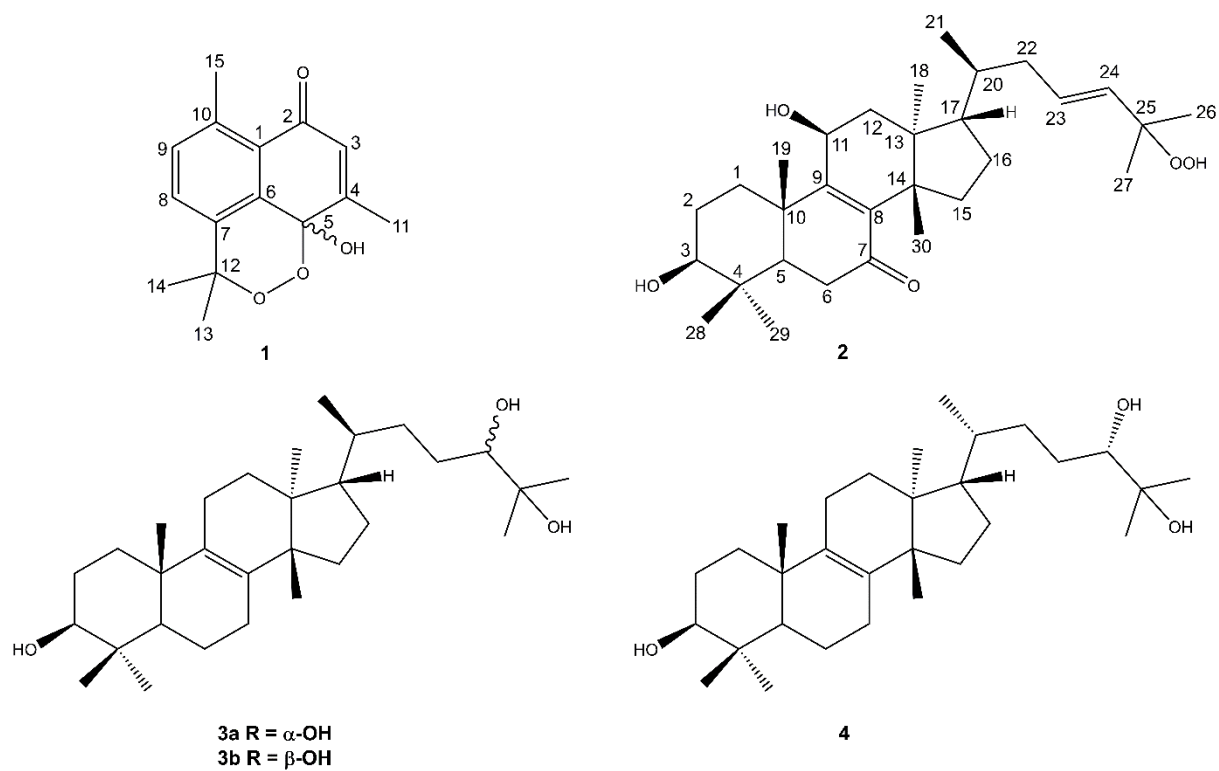


Fig. 1. Structures of compounds 1–4.

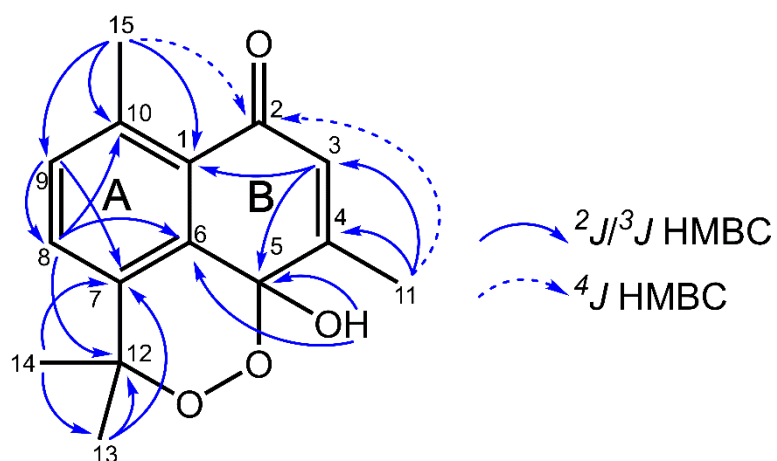


Fig. 2. Key HMBC correlations of compound 1.

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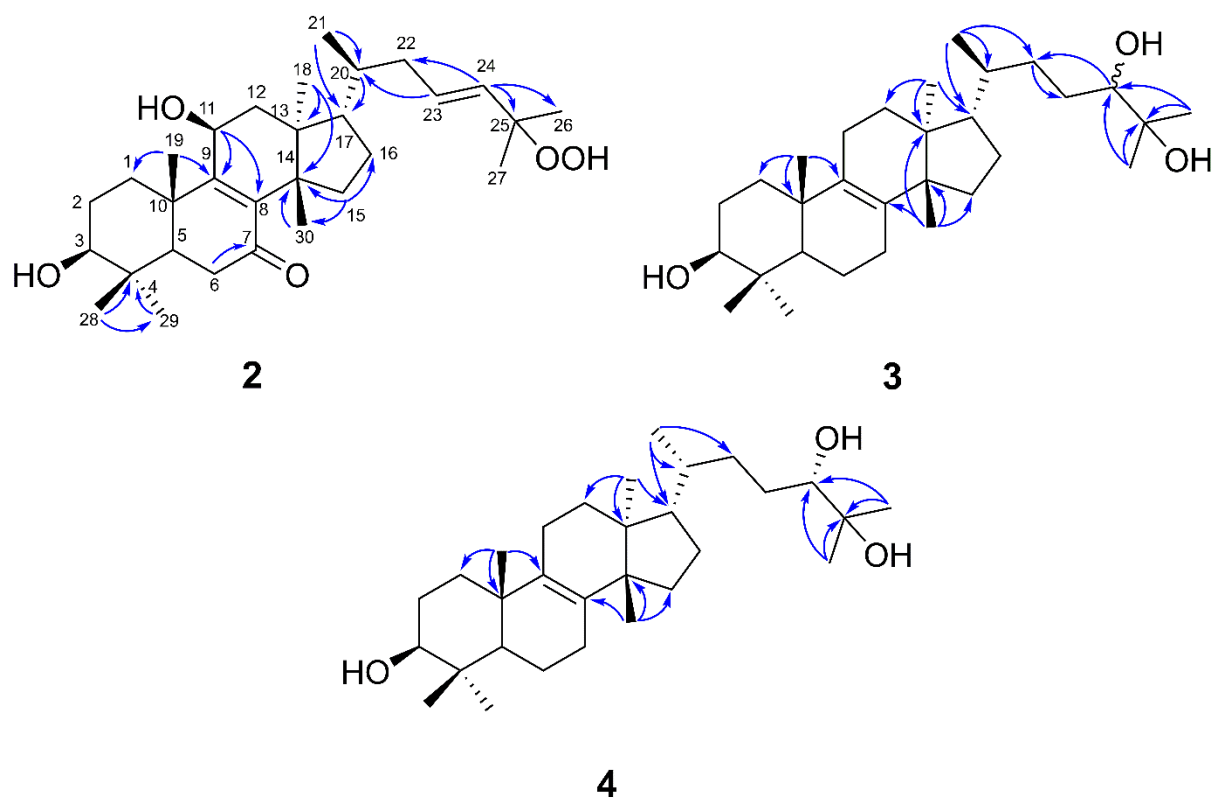


Fig. 3. Key HMBC correlations of compounds 2–4.

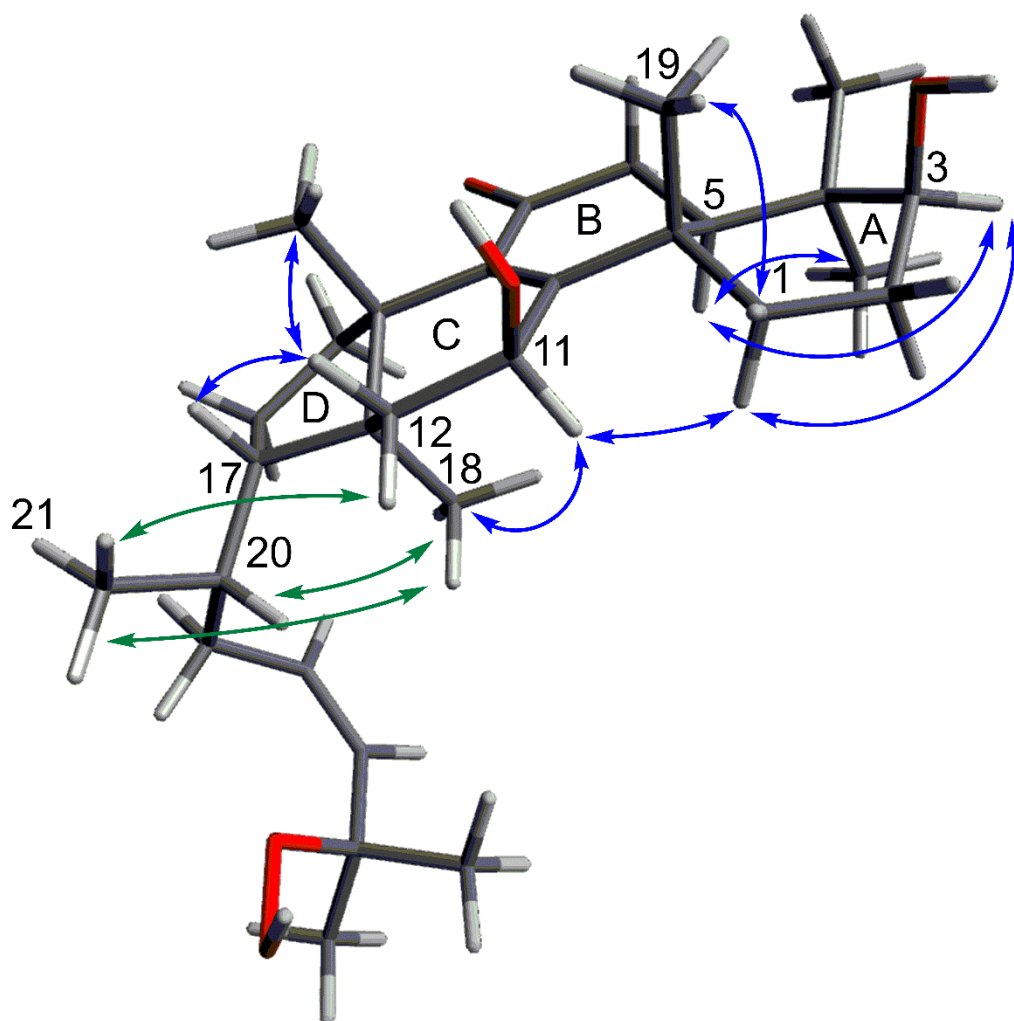


Fig. 4. Main NOESY correlations of compound 2. Key correlations indicating the pertaining to the tirucallane series are indicated as green arrows.

Table 1

^1H (500 MHz) and ^{13}C (125 MHz) NMR data for **1** (acetone- d_6).

Position	δ_c	δ_H mult. (J in Hz)
1	128.6	
2	186.8	
3	131.5	6.13, q (1.0)
4	154.0	
5	94.1	
6	137.8	
7	137.4	
8	128.6	7.42, d (8.0)
9	133.1	7.30, d (8.0)
10	138.7	
11	16.1	2.08, s
12	80.9	
13	28.8	1.56, s
14	25.0	1.61, s
15	21.6	2.58, s
5-OH		6.67, br s

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Table 2

¹H (500 MHz) and ¹³C (125 MHz) NMR data for **2-4**.

N	2^a		3^a^b		3^b^b		4^b	
	δ_{H} mult. (<i>J</i> in Hz)	δ_{C}	δ_{H} mult. (<i>J</i> in Hz)	δ_{C}	δ_{H} mult. (<i>J</i> in Hz)	δ_{C}	δ_{H} mult. (<i>J</i> in Hz)	δ_{C}
1 α	1.66, m	34.2	1.20, m	35.4	1.20, m	35.4	1.19, m	35.4
1 β	2.54, m		1.77, m		1.77, m		1.75, m	
2	1.72, m 1.68, m	28.4	1.61-1.69, m	27.8	1.61-1.69, m	27.8	1.62-1.68, m 1.62-1.68, m	27.8
3 α	3.27, dd (11.5, 4.5)	78.1	3.23, dd (11.5, 4.5)	79.2	3.23, dd (11.5, 4.5)	79.2	3.23, dd (12.0, 4.5)	79.2
4		39.8		39.1		39.1		39.1
5	1.72, m	50.2	1.11, m	51.1	1.11, m	51.1	1.11, m	51.1
6 α	2.38-2.41, m	36.4	1.67, m	19.1	1.67, m	19.1	1.66, m	19.1
6 β	2.38-2.41, m		1.41, m		1.41, m		1.40, m	
7 α		199.7	1.93, m	28.1	1.93, m	28.1	1.91, m	28.1
7 β			2.08, m		2.08, m		2.08, m	
8		140.3		133.7		133.7		133.6
9		163.0		134.2		134.2		134.2
10		40.5		37.6		37.6		37.4
11 α	4.70 t (6.0)	67.9	1.94, m	21.6	1.94, m	21.6	1.94, m	21.7
11 β			2.06, m		2.06, m		2.05, m	
12 α	1.86, m	43.2	1.62 m/1.75, m	30.9	1.62, m/1.75, m	30.9	1.62, m/1.72, m	31.1
12 β	2.33, m		1.62 m/1.75, m		1.62, m/1.75, m		1.62, m/1.72, m	
13		46.9		44.3		44.3		44.3

14		48.8		50.3		50.3		50.1
15 α	2.08, m	32.9	1.19, m	30.0	1.19, m	30.0	1.19, m	29.9
15 β	1.42, m		1.50, m		1.50, m		1.51, m	
16 α	2.05, m	28.3	2.05, m	28.3	2.05, m	28.3	1.35, m	28.3
16 β	1.35, m		1.35, m		1.35, m		1.89, m	
17	1.58, m	49.7	1.42, m	50.6	1.49, m	50.4	1.51, m	50.0
18	0.76, s	16.5	0.76, s	15.6	0.77, s	15.6	0.77, s	15.7
19	1.27, s	19.8	0.95, s	20.3	0.95, s	20.3	0.95, s	20.3
20	1.48, s	37.5	1.39-1.41, m	36.5	1.39-1.41, m	37.0	1.49, m	35.9
21	0.96, d (6.5)	19.1	0.91, d (6.5)	19.0	0.92, d (6.5)	18.7	0.85, d (5.5)	19.0
22	2.20, m	40.1	1.48, m	33.2	1.75, m	33.7	1.67, m	32.3
	1.80, m		1.25, m		1.01, m		1.29, m	
23	5.61, m ^c	128.7	1.56, m/1.37, m	28.6	1.56, m/1.37, m	28.8	1.61, m/1.37, m	28.3
24	5.61, m ^c	137.3	3.35, dd (7.0, 6.5)	78.9	3.29, d (9.5)	79.8	3.35, dd (9.0, 3.5)	78.9
25		80.0 ^d		73.3		73.4		73.4
26	1.27, s	25.6	1.16, s	26.7	1.16, s	26.7	1.16, s	26.7
27	1.27, s	25.1	1.22, s	23.3	1.22, s	23.4	1.22, s	23.4
28	0.91, s	15.8	1.00, s	28.2	1.00, s	28.2	1.00, s	28.2
29	0.98, s	28.1	0.80, s	15.7	0.80, s	15.7	0.80, s	15.8
30	1.13, s	25.0	0.87, s	24.5	0.87, s	24.5	0.88, s	24.6

All spectra were recorded ^a in acetone-*d*₆; ^b in chloroform-*d*₁; ^c Overlapped; ^d Inverse detection by HMBC.

Fig. 1. Structures of compounds **1–4**.

Fig. 2. Key HMBC correlations of compound **1**.

Fig. 3. Key HMBC correlations of compounds **2–4**.

Fig. 4. Main NOESY correlations of compound **2**. Key correlations indicating the pertaining to the tirucallane series are indicated as green arrows.

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