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# The first evidence of lumiphorbol as a metabolite of the *Euphorbia* species

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*Abstract: Euphorbia nicaeensis* All. belongs to the flowering plant family Euphorbiaceae. Our previous research has shown that the latex of this species is a rich source of jatrophane-type diterpenes. Due to the exceptional biological activities exhibited by these compounds, the research was extended to the root of this plant species. From the roots of *E. nicaeensis*, collected in Deliblato Sand (Serbia), a diterpene from an extremely rare class of lumiphorbol was isolated for the first time from *Euphorbia* species. The structure of the isolated compound was determined by spectroscopic techniques (1D and 2D NMR), as well as HRESIMS data.

*Keywords: Euphorbia nicaeensis*; Euphorbiaceae; diterpenes; lumiphorbol.

## INTRODUCTION

Species of the *Euphorbia* genus are well known for their impressive structural diversity and adaptability to different environments.[1] These plants produce a wide range of secondary metabolites, among which diterpenes stand out due to their bioactivity and potential application in modern medicine.[2] Diterpenes from *Euphorbia* species are structurally diverse, including compounds such as phorbol esters, ingenane derivatives, jatrophane diterpenoids, and many others.[3-5] These metabolites often have protective roles in plants, such as deterring herbivores and protecting themselves from pathogens.[5-8] However, their importance goes beyond approved protective role in plants, as studies have shown promising pharmacological properties of these metabolites. A well-known group of diterpenes derived from *Euphorbia* are phorbol esters. These compounds are

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known for their ability to activate protein kinase C (PKC), an enzyme involved in cellular signalling pathways that regulate various cellular functions, including proliferation, differentiation, and apoptosis.[9] Although phorbol esters are associated with tumour-promoting activity, their mechanism of action has been crucial in cancer research, helping to develop therapeutic strategies targeting PKC.[9] On the other hand, many phorbol derivatives have been shown not to have tumour-promoting effects, but rather exhibit anticancer activity against certain human cancer cell lines.[10-12] Additionally, tigliane derivatives have demonstrated strong inhibitory activity against HIV-1 and HIV-2.[13] Ingenane derivatives play a significant role in modern medicine. Ingenol-mebutate, derived from *Euphorbia peplus*, was approved for the topical treatment of actinic keratosis, a precursor of skin cancer.[14] Its mechanism involves inducing rapid cell death in abnormal cells while minimizing damage to healthy tissue.[14] However, it was withdrawn from use in the European Union in 2019. due to safety concerns.[15] Research indicated a potential link between long-term use of ingenol-mebutate and an increased risk of skin cancer, including basal cell carcinoma and squamous cell carcinoma, and that the drug's risks outweigh its benefits.[15] The most specific and rare class of diterpenes isolated from the *Euphorbia* genus are lumiphorbols, which are considered phorbol derivatives. It has been experimentally proven that lumiphorbols are derived from tigliane esters, where rings A and B are *cis* connected. The [2+2] cycloaddition, involving the double bonds at positions C1(2) and C6(7), occurs in the presence of light with a wavelength of 254 nm under laboratory conditions, while the mechanism of their formation in plants, particularly in the root, which is not exposed to UV radiation, remains unknown. A notable example is lumiphorbol triacetate, a cage derivative of 4α-phorbol, which was first described by E. Hecker and his colleagues in 1968.[16] In their study, Hecker et al. detailed the synthesis of lumiphorbol triacetate via ultraviolet (UV) irradiation of 4α-phorbol-12,13,20-triacetate. This process induces an intramolecular cycloaddition, resulting in the unique cage-like structure of lumiphorbol.[17] Due to its extremely specific structure, the biological activity of lumiphorbol derivatives was also investigated. For example, the ability of lumiphorbol-12,13,20-triacetate to activate PKC was evaluated. This compound was found to activate PKC by 93% at high concentrations, while no activation was observed at standard concentration.[18]

Our previous study,[19] where the subject of investigation was the latex of *E. nicaeensis*, showed that the species is a rich source of jatrophane derivatives that showed a strong inhibition of P-gp, so after the initial investigation, the research was extended to the root of the species.

## EXPERIMENTAL

### *Apparatus and Reagents*

Optical rotations were measured on an Autopol IV (Rudolph Research Analytical) polarimeter equipped with a sodium lamp (589 nm) and 10 cm microcell. All NMR data were acquired on Bruker Avance III 500 NMR spectrometer (500 MHz for $^{1}$H and 125 MHz for $^{13}$C NMR, in $CDCl_3$, with TMS as internal standard). The NMR spectra were analysed using TopSpin 3.6.2 software. High-resolution LC/ESI positive TOF mass spectra were measured on a HPLC instrument (Agilent 1200 Series) coupled with a 6210 Time-of-Flight LC/MS system (Agilent Technologies). NP-HPLC-DAD: Agilent Technologies 1260 Series liquid chromatograph equipped with diode-array detector, autosampler, and collector; Zorbax RX-Sil (250 × 9.4 mm; 5 μm) column. RP-HPLC-DAD: Agilent Technologies 1100 Series liquid chromatograph equipped with diode-array detector, autosampler, and collector; Zorbax XDB-C18 column (250 × 9.4 mm; 5 μm). Dry-column flash chromatography (DCFC) (Shusterman et al., 1997) was performed on silica gel (ICN Silica 12–26 60 Å, Merck). Silica gel 60 F254 precoated aluminium sheets (0.25 mm, Merck) for TLC control were used. The TLC plates were visualized under a UV lamp at 254 nm and detected by spraying with solution of cerium molybdate in sulphuric acid, followed by heating. All solvents used for HPLC were HPLC grade, while all used solvents for DFCC and TLC were at least of analytical grade.

### *Plant material*

The roots of *Euphorbia nicaeensis* were collected at Deliblato Sands (Serbia), collection site at latitude: 44°56'57" N and longitude: 21°11'13" E, in May 2018. The plant was identified by Professor Petar Marin, University of Belgrade – Faculty of Biology, Institute of Botany. Voucher specimen (No. 16855) has been deposited at the Herbarium of Botanical Garden "Jevremovac" University of Belgrade, Belgrade (Serbia).

### *Isolation and purification*

The process of isolating lumiphorbol involved the preparation of a root extract through continuous extraction with a 96% aqueous ethanol solution. One hundred fifty-two grams of ground *E. nicaeensis* roots were packed into a Soxhlet extraction flask and 250 g of 96% ethanol was added for continuous extraction with heating for 2 h, and then left overnight in the solvent. After removal of the solvent by evaporation on a rotary vacuum evaporator, the obtained extract (25 g) was fractionated by DCFC using silica gel as the stationary phase and a mixture of petroleum ether and acetone in varying volume ratios as the mobile phase. Fraction F3 (2.1 g), obtained by elution with 15% acetone in petroleum ether, was further fractionated by DCFC on silica gel using a mixture of petroleum ether and acetone in different ratios (98:2, 97.5:2.5, 95:5, 90:10, 80:20) as the mobile phase. The collected fractions were monitored by TLC, and similar fractions were combined, resulting in thirteen fractions (1–13). Fraction F3/10 (367.5 mg) was subjected to separation by NP-HPLC on silica gel (Zorbax Rx-SIL column, 250 × 9.4 mm, 5 μm) with *n*-hexane and acetone (95:5, isocratic mode, flow 3 mL/min, 25 °C, 227 nm, stop time 30 min, post time 1 min), yielding six subfractions, F3/10/I to F3/10/VI. The final separation of fraction F3/10/VI was carried out on RP-HPLC using a Zorbax XDB C18 column (250 × 9.4 mm, 5 μm) and a mixture of water and acetonitrile (ACN) in gradient mode as the mobile phase (50–80% ACN (0–10 min), 80–90% ACN (10–15 min), 90–100% ACN (15–21 min), flow 4 mL/min, 25 °C, 227 nm, stop time 21 min, post time 2 min). As a result of this purification, the lumiphorbol (0.7 mg) was isolated. The NMR spectra and HRMS data are available as supplementary material.

## RESULTS AND DISCUSSION

Luminicaeenin A (**1**) was isolated as a colourless amorphous substance. The ion $[M+H]^+$ at *m/z* 479.2424 in HRESIMS indicated the molecular formula $C_{29}H_{34}O_6$, (calcd. for $C_{29}H_{35}O_6$, 479.2428), corresponding to 13 degrees of unsaturation. NMR spectra revealed the functional groups, such as a 3-keto group ($\delta_C$ 218.1), a tertiary OH ($\delta_H$ ca. 5.2, brs; $\delta_C$ 82.6, C-9), and two ester residues, 12-benzoate ($\delta_H$ 8.03, d, 8, 2H, $\delta_H$ 7.46, t, 8 2H and $\delta_H$ 7.58 t, 1H; $\delta_C$ 166.2 and $\delta_C$ 130.3, 129.9, 128.6 and 133.3), and 13-acetate ($\delta_H$ 2.12, s, 3H; $\delta_C$ 173.0 and 21.2). When the saturations originating from the mentioned functional groups are subtracted from the total number of saturations, six more unsaturations remain in the skeleton, which must be distributed through six rings given that in the NMR spectra there are no signals originating from carbon-carbon double bonds. The clue for a cage structure containing six rings was mostly obtained from HMBC, COSY and NOESY correlations shown in fig. 1, as well as by comparison of $^1$H NMR spectral data with those of the related lumiphorbols obtained by intramolecular [2+2] photocycloaddition of 4*α*-phorbol derivatives.[17]

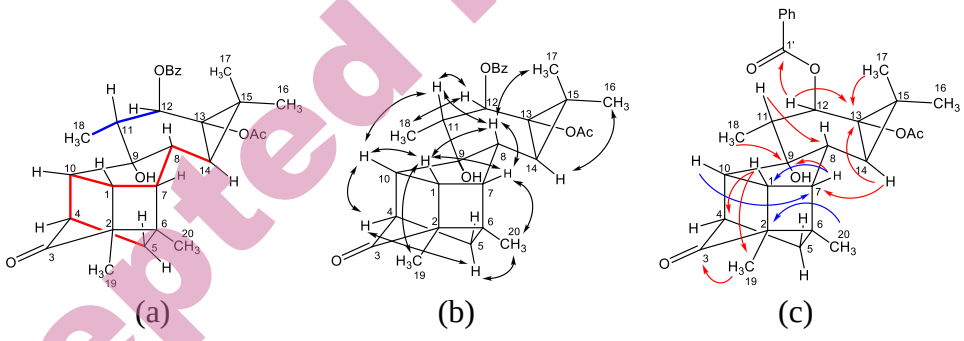


Fig. 1. 2D correlations of **1**; (a) COSY correlations; spin system A (red), spin system B (blue), (b) NOESY correlations and (c) HMBC (H→C) correlations (the correlations occurring across four-membered ring marked blue)

The COSY spectrum of **1** has shown two independent spin systems A and B. The first one (A) included H-1, H-4, H-5, H-7, H-8, H-10 and H-14, while the second (B) included protons H-11, H-12, and H-18. The COSY correlation H-1/H-7 indicated connectivity C(1)-C(7), which is also supported by the HMBC correlation H-7/C-1. At the same time, the HMBC correlations H3-20/C-2 and H-10/C-7 (Fig. 1, c, marked blue) were in accordance with the C(1)-C(7) connectivity in the four-membered ring. The NOEs (Fig. 1, b) also supported the proposed cage structure of **1**. The structure of **1** was elucidated as 4,20-deoxy-4*α*-lumiphorbol-12-benzoate-13-acetate.

Table 1. $^{1}H$ and $^{13}C$ NMR data of **1** (500 MHz for $^{1}H$, and 125 MHz for $^{13}C$, $CDCl_3$, TMS)

| | $^{1}H$ (δ (ppm), *J* (Hz)) | $^{13}C$ (δ (ppm)) |
|---|---|---|
| 1 | 2.30, t, 6 | 43.4 |
| 2 | | 42.9 |
| 3 | | 218.1 |
| 4 | 2.43, brs | 46.48 |
| 5α | 3.24, d, 12 | 38.7 |
| 5β | 1.59, brd, 13 | |
| 6 | | 46.50 |
| 7 | 2.75, t, 5 | 52.2 |
| 8 | 1.76, dd, 10, 5 | 55.0 |
| 9 | | 82.6 |
| 10 | 2.72, t, 5 | 44.2 |
| 11 | 1.67, qui, 7 | 51.5 |
| 12 | 5.78, d, 8 | 81.2 |
| 13 | | 68.2 |
| 14 | 1.49, d, 10 | 30.3 |
| 15 | | 28.5 |
| 16 | 1.20, s | 24.1 |
| 17 | 1.39, s | 18.0 |
| 18 | 1.07, d | 14.4 |
| 19 | 1.12, s | 7.2 |
| 20 | 1.02, s | 23.3 |
| 12-OR | | |
| 1' | | 166.2 |
| 2' | 8.03, d, 8 | 130.3 |
| 3' | 7.46, t, 8 | 129.9 |
| 4' | 7.58, t, 8 | 128.6 |
| 5' | | 133.3 |
| 13-OR | | |
| 1" | | 173.0 |
| 2" | 2.12, s | 21.2 |

Unlike the tigliane core, which is commonly found in Euphorbiaceae, lumiphorbol derivatives are extremely rare. To the best of our knowledge, only one diterpene with the lumiphorbol structure, i.e., 12-*O*-palmitoyl-4-deoxy-16-hydroxylumiphorbol-13-acetate isolated from *Aleuritis fordii* (Euphorbiaceae) from Japan has been described in the literature so far.[20] Otherwise, lumiphorbol derivatives reported in the literature are the products of the intramolecular [2+2] photocycloaddition reaction of 4α-phorbols carried out by irradiating with UV light (λ = 254 nm).[16,17]

## CONCLUSION

This study identified the roots of *E. nicaeensis* as a source of an extremely rare lumiphorbol diterpene skeleton. Considering all the facts presented in the

previous sections, it can be concluded that lumiphorbols represent a fascinating class of compounds with unique structural features and still unexplored biological activities due to their rarity, which indicates the need for further chemical and pharmacological research.

## SUPPLEMENTARY MATERIAL

Additional data are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/13212, or from the corresponding author on request.

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## И З В О Д

### ПРВИ ДОКАЗ ЛУМИФОРБОЛА КАО МЕТАБОЛИТА ВРСТЕ РОДА *EUPHORBIA*

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*Euphorbia nicaeensis* All. је цветница из породице Euphorbiaceae. Наше претходно истраживање је показало да је латекс ове врсте богат извор јатрофанских дитерпена. Због изузетних биолошких активности које ови дитерпени показују, истраживање је проширено на корен врсте у потрази за новим дитерпенима. Из корена *E. nicaeensis*, прикупљеног у Делиблатској пешчари (Србија), изолован је по први пут из врста рода *Euphorbia* дитерпен који припада изузетно реткој класи лумифорбола. Структура изолованог једињења одређена је применом спектроскопских техника (1D и 2D NMR), као и масене спектрометрије високог разлагања (HRESIMS).

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