TETRACYCLIC TRITERPENOIDS FROM *Euphorbia nicaeensis* All.

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In this study, three tetracyclic triterpenes: (*3S,24S*)-tirucall-7-ene-3,24,25-triol (1), (*3S,24R*)-tirucall-7-ene-3,24,25-triol (2) and inoterpene C (3), were isolated from the milkweed *Euphorbia nicaeensis* All. using dry-column flash silica gel chromatography and semipreparative normal-phase HPLC. Their structures were determined on the basis of 1D and 2D NMR spectra and literature review. Although these three compounds have previously been isolated from other plant species, this is the first time that they have been isolated from *E. nicaeensis*.

Keywords: tetracyclic triterpenes, *Euphorbia nicaeensis*, latex, dry-column flash chromatography, NP HPLC, NMR analysis

Introduction

The genus of *Euphorbia* is one of the largest and the most diverse genus with more than 2000 species. Plants of the genus of *Euphorbia* are a rich source of biologically active compounds. Among thousands of compounds derived from *Euphorbia* plants, diterpenes and triterpenes have been most commonly isolated ones. There are special plant cells in the stems of this plant - cells of laticifera, specialized in the production and accumulation of latex. Latex is often white or pale yellow in color and has a protective role - it repels herbivores and can have antifungal effects. The chemical composition of latex is highly variable, in fact, it represents a mixture of terpenoids. Triterpene alcohols do not show any significant biological activities, but they have proven to be very important chemotaxonomic markers. The most common triterpene alcohols of the *Euphorbia* plants latex are cycloartenol, lanosterol, butyrospermol and 24-methylene-cycloartanol [1]. In the previous investigations of latex of *Euphorbia nicaeensis* fifteen jatrophanes were isolated and chemically characterized, seven of which were new compounds [2].

Experimental

General experimental procedures

LC-DAD: Agilent Technologies 1260 Series liquid chromatograph equipped with diode-array detector (λ=210 nm) and autosampler; Program 1 (NP-HPLC), LC conditions: Injection volume 1000 µL (c~10 mg/mL, acetone), Zorbax RX-Sil (250 × 9.4 mm; 5 µm), column temp. 24 °C, mobile phase 3.00 mL/min: A (acetone) and B (petroleum ether), isocratic mode of elution 18% A/82% B. Program 2 (NP-HPLC), LC conditions: Injection volume 1000 µL (c~10 mg/mL, acetone), Zorbax RX-Sil (250 × 9.4 mm; 5 µm), column temp. 24 °C, mobile phase 3.00 mL/min: A (acetone) and B (petroleum ether), isocratic mode of elution with 10% A/90% B. HRESIMS data were obtained on an Agilent 6210 time-of-flight LC/MS system equipped with an ESI interface (ESITOFMS). The solvent was methanol, and the mobile phase was 0.2% HCOOH/CH_3CN, 1:1, 0.2 mL/min. The ESI was operated in a positive mode and nitrogen was used as the drying gas (12 L/min) and nebulizing gas at 350 °C (45 psi). The OCT RF voltage was set to 250 V, and the capillary voltage was set to 4.0 kV. The voltages applied to the fragmentor and skimmer were 140 and 60 V, respectively. The scanning was performed from *m/z* 100 to 1500.

Dry-column flash chromatography (DCFC) and column chromatography (CC) were performed on silica gel (ICN Silica 12 × 26 60 Å and 70 × 230 mesh, ASTM, Merck, respectively). Silica gel 60 F254 precoated aluminum sheets (0.25 mm, Merck) for TLC control were used.

Optical rotations were determined on an Autopol IV (Rudolph Research Analytical) polarimeter equipped with a sodium lamp (589 nm) and 10 cm microcell. ^1H and ^{13}C NMR data were measured on Bruker Avance III 500 NMR spectrometer (500 MHz for ^1H and 125 MHz for ^{13}C NMR, in $CDCl_3$ with TMS).

Plant material

The latex and the root of *E. nicaeensis* were collected from wild stock at Deliblato sands (Serbia) in June 2014 (latex) and in June 2018 (root) at latitude 44,93,008° N and longitude 21,17,769° E. The plant was identified by Prof. Petar Marin, Institut of Botany, Faculty of Biology, University of Belgrade. A vaucher specimen (No 16855) was deposited at the Herbarium of Botanical Garden "Jevremovac" University of Belgrade, Belgrade (Serbia).

Extraction and isolation

The latex (51.2 g) of *E. nicaeensis* was lyophilized at -70 °C to obtain dry material (22.5 g) which was extracted

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twice with n-hexane (250 mL). The obtained extract (18 g) was subjected to dry-column flash chromatography on silica gel using n-hexane/ethyl acetate of different proportions. The elution progress was followed by TLC and ^{1}H NMR. The fraction that contained triterpenes was elueted with 80% EtOAc. This fraction was subjected to isocratic column chromatography on silica gel using n-hexane and isopropanol (9/1, V/V) as eluent, to obtain 4 subfractions. Subfraction 2 (52.1 mg) was further purified by preparative NP-HPLC (program 1) to obtain compounds 1 (1.5 mg) and 2 (2.2 mg).

The root of *E. nicaeensis* was first grounded and extracted with 96% ethanol. The obtained extract (25 g) was subjected to dry-column flash chromatography on silica gel using n-hexane and acetone in different proportion as eluent. The selected fraction (2.1 g), which was eluted with 15% acetone, was subjected to another dry-column flash chromatography on silica gel using n-hexane and acetone of different proportion (2/98 to 50/50) to obtain 4 subfractions. Subfraction 2 (800 mg) was further purified by dry-column flash chromatography on silica gel using isocratic program with 8% acetone in n-hexane. The final purification was done using preparative NP-HPLC (Program 2) yielding compound 3 (2.8 mg).

Results and discussion

Compounds 1 and 2 were isolated from the latex of *E. nicaeensis*. Their ^{1}H and ^{13}C NMR spectra (Figures 2-5) were very mutually similar and revealed the pattern of tetracyclic triterpene skeleton of tirucallane type with the hydroxyl groups at positions C-3, C-24, and C-25, as well as $\Delta^{7,8}$ double bond (Figure 1). The differences in the NMR data of 1 and 2 were noted for the chemical shifts of H-23 and H-24, as well as C-23 and C-24 (Table 1). For both compounds the position of 3-OH was confirmed by the HMBC correlations H-3/C-1,C-2,C-4 and 28-CH_3,29-CH_3/C-3. The 24-OH group was determined according to the HMBC correlations H-22,H-23/C-24 and the 25-OH group according to the HMBC correlations 26-CH_3,27-CH_3/C-25. The position of the $\Delta^{7,8}$ double bond was deduced from the HMBC correlations H-5/C-7, H-9/C-7. Using the comparison with literature NMR data compound 1 was determined as (*3S,24S*)-tirucall-7-ene-3,24,25-triol known from *Ailanthus excelsa* [3], while compound 2 was its 24*R* diastereoisomer known from *Celastrus stylosus* [4]. HRESIMS confirmed molecular formula $C_{30}H_{52}O_3$ for both structures – for compound 1 molecular ion $[M+H]^+$ was at *m/z* 461.3980 while for compound 2 it was at *m/z* 461.3972 (theoretical value for $C_{30}H_{52}O_3$+H is 461.3989).

Compound 3 was isolated from the root of *E. nicaeensis*. It belongs to the lanostane type of triterpenes, possessing 3-OH, $\Delta^{8,9}$, $\Delta^{23,24}$ double bonds and hydroperoxyl group at C-25 (Figure 1). In the ^{13}C NMR spectrum characteristic chemical shifts of the double bonds were found at 131-134 ppm (four carbons) and oxygenated carbons at 79.2 and 82.5 ppm (Figure 7). The position of 3-OH was confirmed by the HMBC correlations H-3/C-1,C-2,C-4 and 28-CH_3,29-CH_3/C-3. The 24-OH group was determined according to the HMBC correlations H-22,H-23/C-24 and the 25-OH group according to the HMBC correlations 26-CH_3,27-CH_3/C-25. The position of the $\Delta^{8,9}$ double bond was confirmed by the HMBC correlations H-7,H-11/C-9, H-6/C-8, while the position of the $\Delta^{23,24}$ double bond was confirmed by the HMBC correlations H-22/C-23,C-24 and H-20/C-23. Chemical shifts of the oxygenated carbons in the ^{13}C NMR spectrum of 3 at 79.2 and 82.5 ppm were in accordance with the literature data for C-3 (and hydroxyl group in this position) and C-25 (hydroperoxyl group at C-25), respectively (Table 1). All the other chemical shifts were also in accordance with the literature data for inoterpene C isolated from *Inonotus obliquus* [5]. Chemical shift for C-25 alcohol [6] differs for 11.7 ppm in comparison with our experimental C-25 value and this was the reason why peroxyl group was proposed and later confirmed by the literature data. All NMR data are in agreement with inoterpene C (C-25 peroxyl derivative) [5].

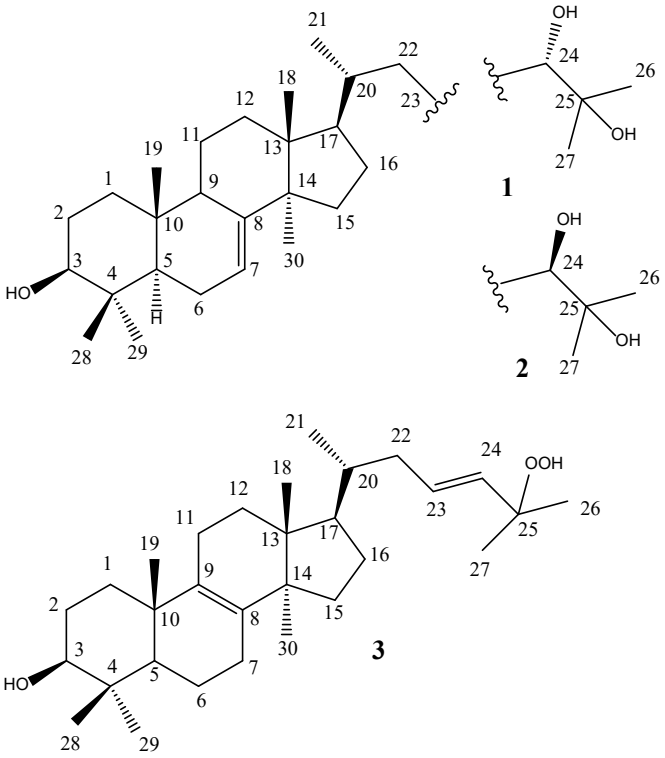


Figure 1. Tetracyclic triterpenes from *E. nicaeensis*

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for compounds 1 and 2 (C_6D_6), 3 ($CDCl_3$) (δ (ppm), *J* (Hz))

	1		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1*α*	0.96 m		0.95 m		1.77 m	
1*β*	1.53 m		1.52 m		1.21 m	
2*α*			1.48 m		1.61 m	
2*β*			0.96 m		1.68 m	
3*α*	3.03 dd (11; 5)	78.8	3.03 dd (11; 5)	78.8	3.24 dd (12; 5)	79.2
4	-	39.1	-	39.1	-	39.2
5	1.27 m	51.1	1.26 m	51.0	1.12 m	51.2
6*α*	2.12 m		2.09 m		1.42 m	
6*β*	1.90 m		1.93 m		1.69 m	
7*α*	5.35 dd (7; 3)		5.34 dd (7; 3)		2.07 m	
7*β*	-		-		1.97 m	
8	-	145.9	-	145.8	-	133.7
9	2.25 m	49.4	2.24 m	49.3	-	134.6
10	-	35.2	-	35.2	-	37.5
11*α*					1.59 m	
11*β*					1.37 m	
12*α*	1.51 m		1.52 m		1.33 m	
12*β*	1.67 m		1.66 m		1.55 m	
13	-	43.9	-	44.0	-	44.4
14	-	51.6	-	51.6	-	50.2
15*α*	1.88 m		1.88 m		1.68 m	
15*β*	1.80 m		1.83 m		1.78 m	
16*α*	2.00 m		1.98 m		1.21 m	
16*β*	1.32 m		1.33 m		1.53 m	
17	1.55 m	53.9	1.56 m	53.7	1.53 m	49.8
18	0.99 s	22.4	0,98 s	22.4	0.80 s	16.0
19	0.78 s	13.3	0.77 s	13.3	0.96 s	20.4
20	1.48 m	35.8	1,44 m	37.0	1.55 m	36.3
21	0.90 d (6.5)	18.7	0.92 d (6.5)	19.1	0.83 d (6)	19.4
22*α*	1.82 m		2.13 m		1.84	
22*β*	1.23 m		0.98 m		2.41	
23*α*	1.43 m		1.57 m			
23*β*	1.37 m		1.15 m			
24	3.28 dd (10; 2)	78.2	3.18 dd (10; 2)	79.5	5.51 d (16)	134.3
25	-	72.7	-	72.8	-	82.5
26	1.04 s	23.4	1.04 s	23.4	1.34 s	24.6
27	1.04 s	26.6	1.04 s	26.6	1.34 s	24.7
28	0.89 s	15.0	0.88 s	15.0	1.00 s	28.3
29	0.95 s	27.8	0.96 s	27.8	0.80 s	15.7
30	1.06 s	27.5	1.06 s	27.5	0.88 s	24.6

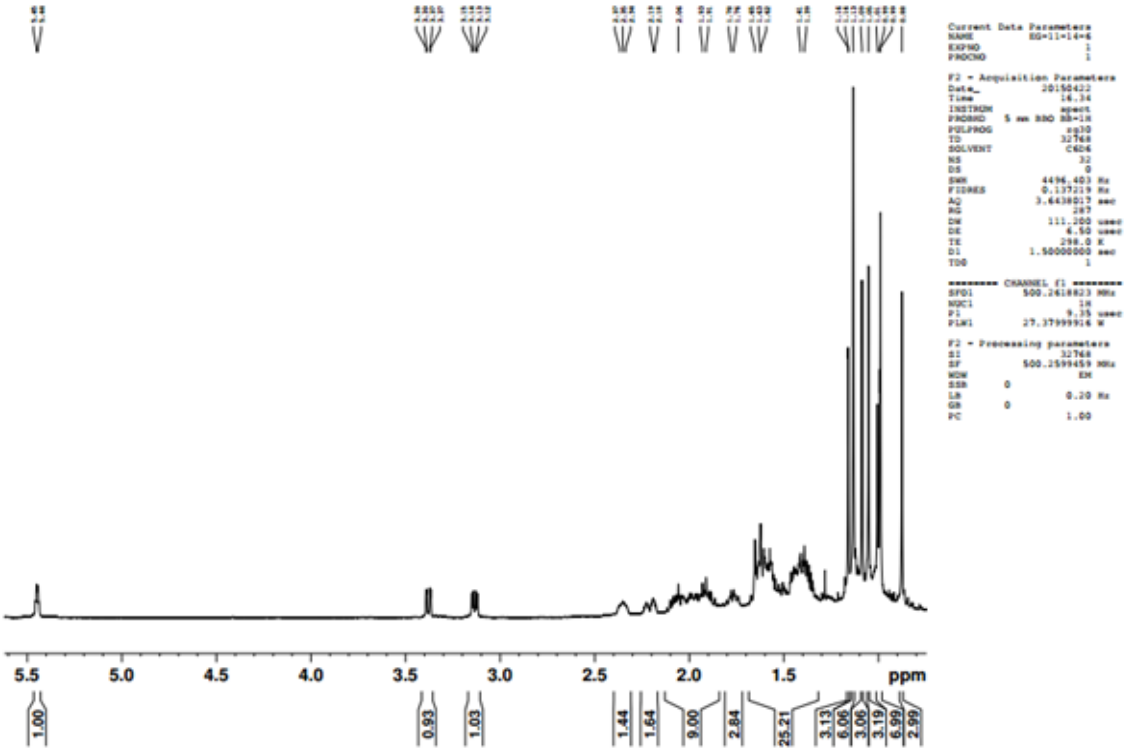


Figure 2. ^{1}H NMR spectrum of compound 1

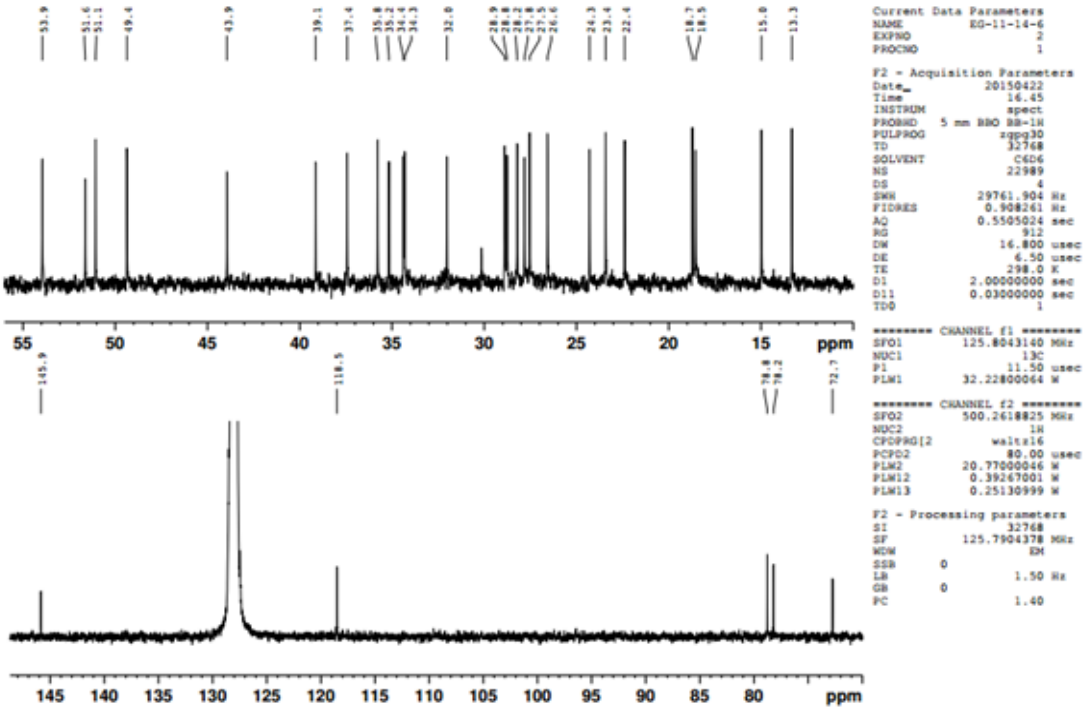


Figure 3. ^{13}C NMR spectrum of compound 1

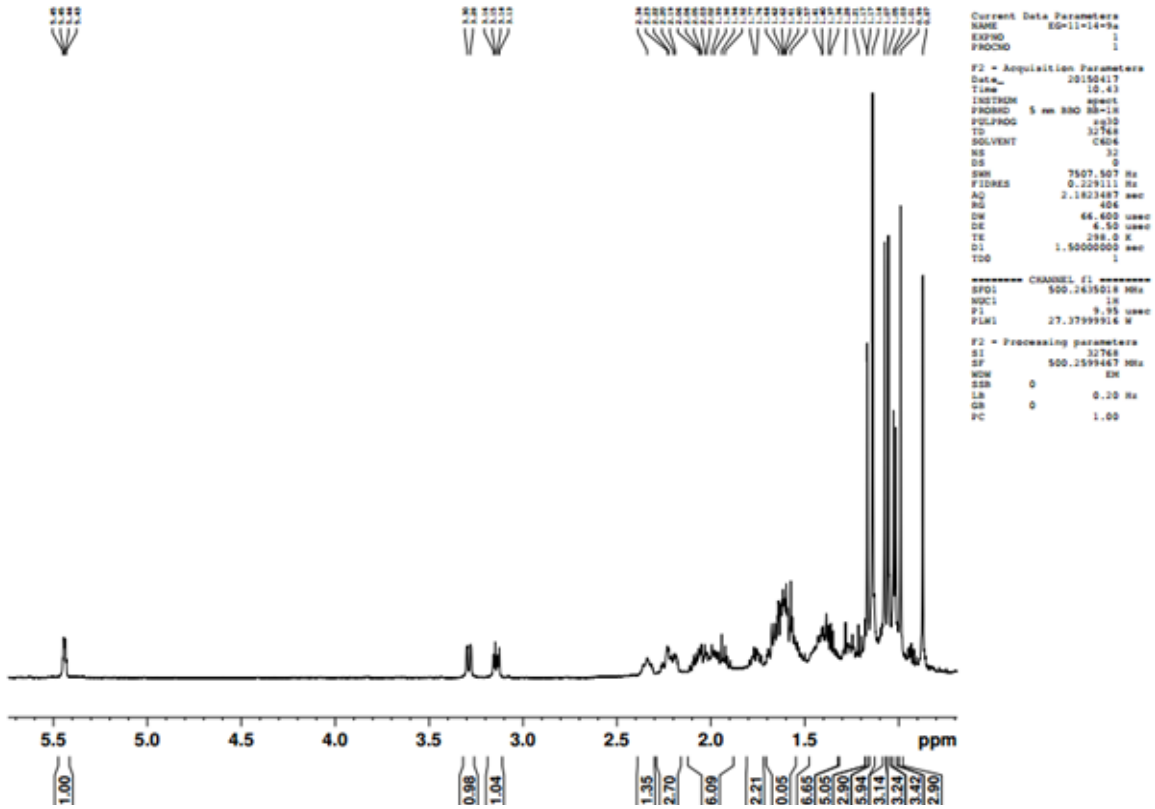


Figure 4.[1]H NMR spectrum of compound 2

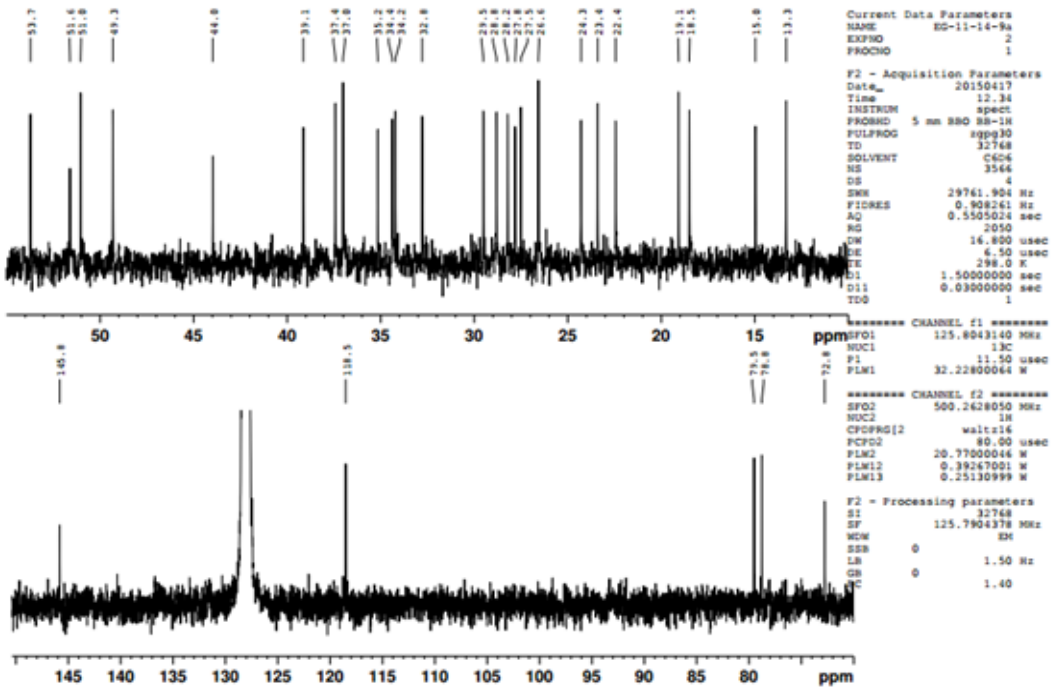


Figure 5. [13]C NMR spectrum of compound 2

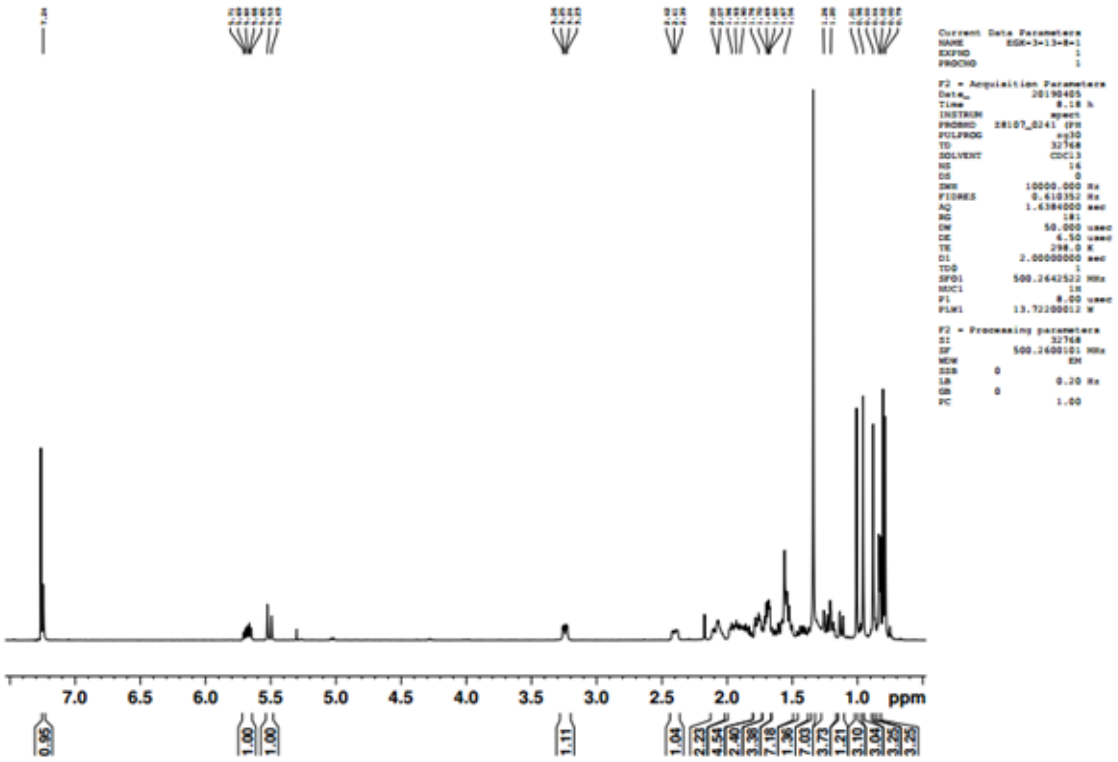


Figure 6.[1]H NMR spectrum of compound 3

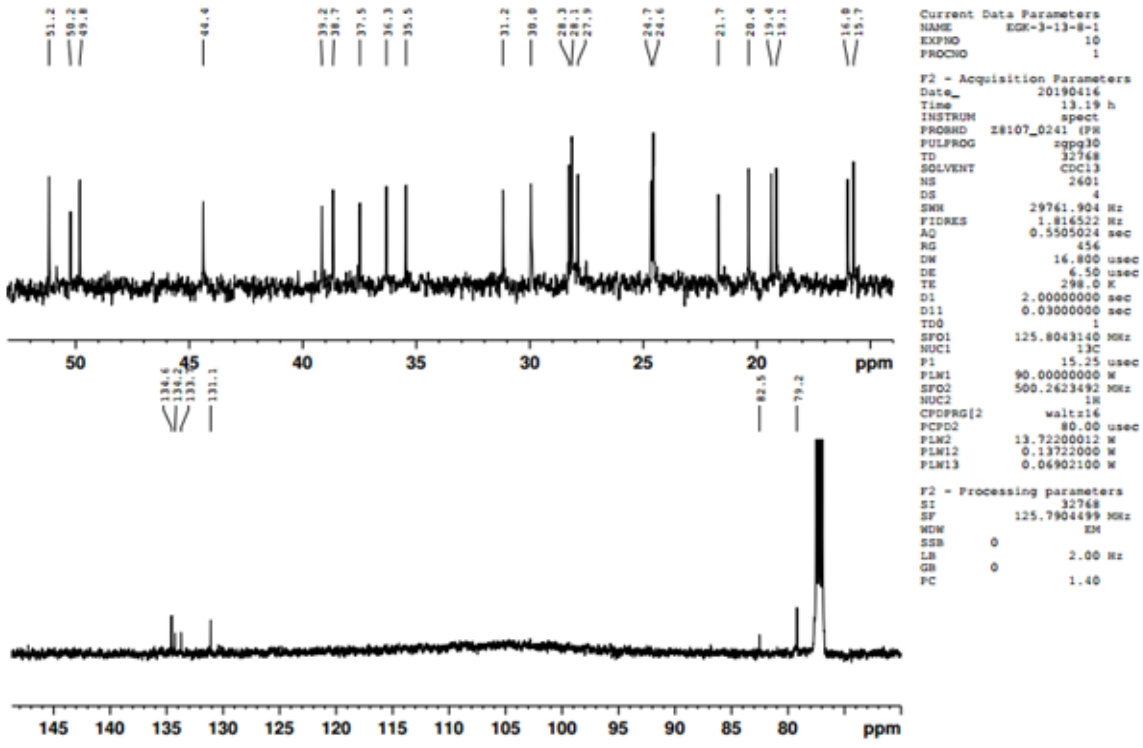
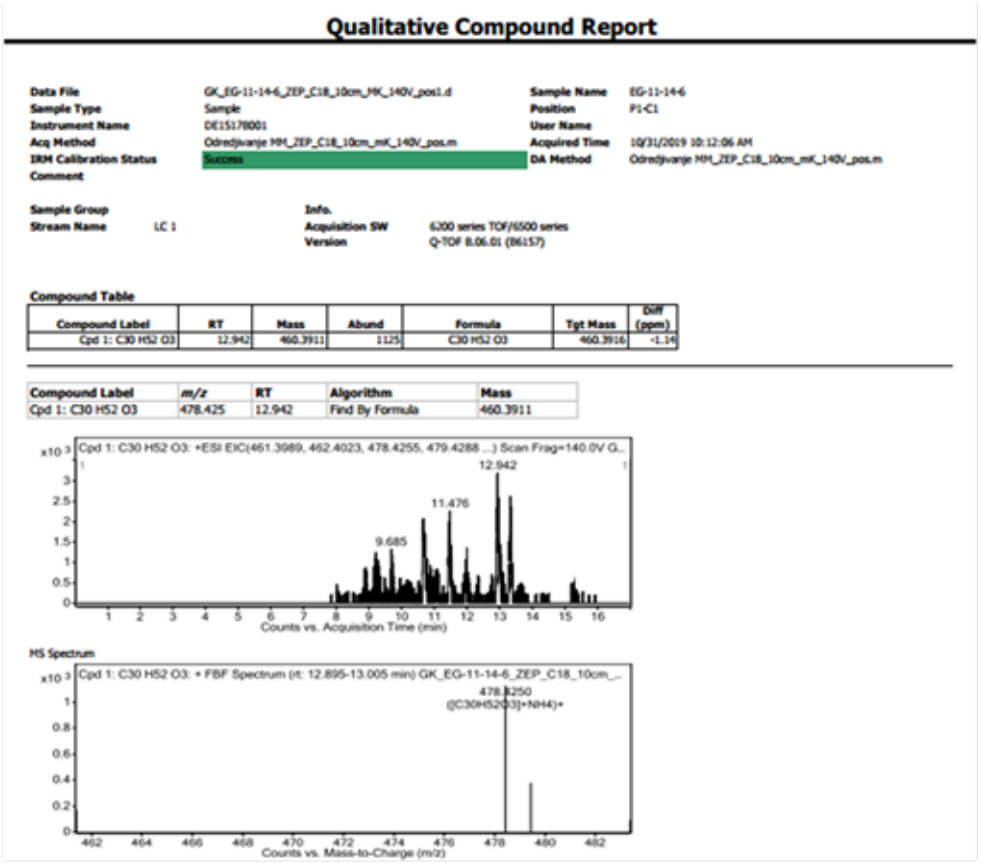


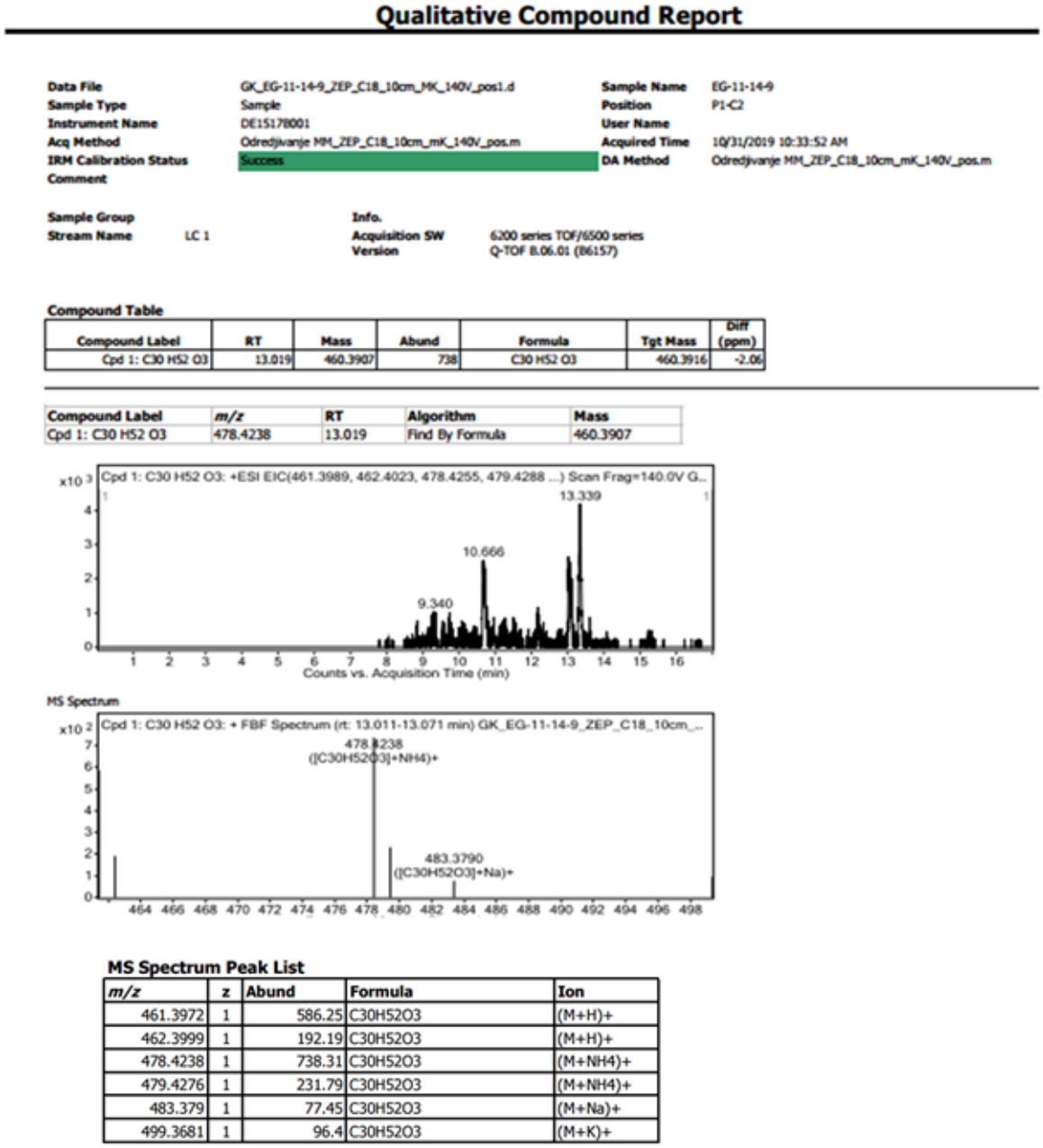
Figure 7. [13]C NMR spectrum of compound 3



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
461.398	1	171.56	C30H52O3	(M+H)+
478.425	1	1125.15	C30H52O3	(M+NH4)+
479.4285	1	377.39	C30H52O3	(M+NH4)+
483.3795	1	90.49	C30H52O3	(M+Na)+

Figure 8. HRESIMS spectrum of compound 1



Qualitative Compound Report

Data File	GK_EG-11-14-9_ZEP_C18_10cm_MK_140V_pos1.d	Sample Name	EG-11-14-9
Sample Type	Sample	Position	P1-C2
Instrument Name	DE15178001	User Name	
Acq Method	Odredjivanje MM_ZEP_C18_10cm_mK_140V_pos.m	Acquired Time	10/31/2019 10:33:52 AM
IRM Calibration Status	Success	DA Method	Odredjivanje MM_ZEP_C18_10cm_mK_140V_pos.m
Comment			

Sample Group		Info.	
Stream Name	LC 1	Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.06.01 (B6157)

Compound Table

Compound Label	RT	Mass	Abund	Formula	Tgt Mass	Diff (ppm)
Cpd 1: C30 H52 O3	13.019	460.3907	738	C30 H52 O3	460.3916	-2.06

Compound Label	m/z	RT	Algorithm	Mass
Cpd 1: C30 H52 O3	478.4238	13.019	Find By Formula	460.3907

MS Spectrum

MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
461.3972	1	586.25	C30H52O3	(M+H)+
462.3999	1	192.19	C30H52O3	(M+H)+
478.4238	1	738.31	C30H52O3	(M+NH4)+
479.4276	1	231.79	C30H52O3	(M+NH4)+
483.379	1	77.45	C30H52O3	(M+Na)+
499.3681	1	96.4	C30H52O3	(M+K)+

Figure 9. HRESIMS spectrum of compound 2

Conclusion

This manuscript describes isolation and structure elucidation of three triterpenoid compounds from milkweed *Euphorbia nicaeensis*. Even though these compounds had been isolated before, this was the first time that they were isolated from this plant species. Their structure elucidation was done using NMR analysis revealing (*3S,24S*)-tirucall-7-ene-3,24,25-triol, (*3S,24R*)-tirucall-7-ene-3,24,25-triol and inoterpene C as their structures.

Their biological activity should be further analyzed for the purpose of testing their anti-inflammatory activity since similar compounds have already shown such activity. Another reason for further analysis should be the antimicrobial activity against plant pathogens, especially for compounds 1 and 2, since they were isolated from the latex which represents the plant protection system.

Acknowledgements

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Izvod

TETRACIKLIČNI TRITERPENOIDI IZ VRSTE *Euphorbia nicaeensis* All.

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U ovom radu, tri tetraciklična triterpena: (*3S,24S*)-tirukal-7-en-3,24,25-triol (1), (*3S,24R*)-tirukal-7-en-3,24,25-triol (2) i inoterpen C (3) su izolovana iz mlečike *Euphorbia nicaeensis* All. koristeći brzu hromatografiju na suvom stubu silika gela i semipreparativnu normalno-faznu HPLC. Strukture su određene na osnovu 1D i 2D NMR spektara i poređenjem sa literaturom. Iako su ova tri jedinjenja ranije izolovana iz drugih biljnih vrsta, ovo je prvi put da su izolovana iz *E. nicaeensis*.

Ključne reči: tetraciklični triterpeni, *Euphorbia nicaeensis*, lateks, brza hromatografija na suvom stubu, NP HPLC, NMR analiza