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Diterpenoids Profile of *E. paralias* and *E. geniculata* Using UPLC-ESI/MS Spectrometry

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Abstract

Euphorbia species contain series of macrocyclic diterpenoids which present as mono, di and sometimes triesters and were reported to possess several and diverse biological activities. The aim herein was to use the ultra-performance coupled to the positive ion mode electron spray ionization mass spectroscopy (UPLC-ESI-MS) as an analytical tool for rapid screening and tentative identification of diterpenoids in the methylene chloride fractions of Euphorbia paralias and Euphorbia geniculata. The identification of compounds was based on ESI-MS and ESI-MS/MS fragmentation pattern, in addition to comparison with reported literature. Five classes were determined including tigliane, ingenane, lathyrane, daphnane and jatrophane diterpenoids esters. Each class of the detected diterpenoids obtained its characteristic ESI-MS/MS fragmentation which facilitates its identification. As a result, a total of fifty-two diterpenoids were identified and characterized in Euphorbia paralias and Euphorbia geniculata. All of these compounds are first report in the two plants by this method. Peak 35 was tentatively identified as ingenol-20-hydroxyheptanoate which expected to be a new compound. The results demonstrated that the used method could be a rapid, and an effective analytical tool for screening and characterization of diterpenoids in the complex systems of plant extracts.

Keywords: Euphorbia, Terpenoids, UPLC-ESI-MS, ingenanes, and tiglianes.

1. Introduction

The genus Euphorbia is one of the largest genera with more than 2000 species worldwide [1]. Euphorbia species are characterized by the presence of a vast array of macrocyclic diterpenoids with various types of carbon skeletons. Several hundred diterpene compounds were isolated from the Euphorbia species with more than twenty different skeletons [2,3] as jatrophane (5/12-bicyclic ring system), ingenane (5/7/7/3-tetracyclic ring system), tigliane (5/7/6/3 tetracyclic ring structure), daphnane (5/7/6-tricyclic system), segetane (5/9/5 tricyclic ring system) and skeleton), myrsinane (5/7/6-fused tricyclic ring lathyrane (5/11/3-tricyclic ring system) [2]. These macrocyclic diterpenoids are valuable due to their diverse biological and therapeutic activities as cytotoxic [4], antitumor [5,6], multi-drug-resistance-reversing [7], immuno-modulatory [8], antimicrobial [9], anti-inflammatory [10], pesticidal, pro-inflammatory [3], molluscicidal [11], vascular-relaxing, and neuroprotective activities [10,12]. So the authors aimed in this work to study the UPLC-ESI-MS/MS analysis of nonpolar fractions of *E. paralias* and *E. geniculata* to detect their diterpene constituents.

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Material and methods:

Plant material:

The aerial parts of *Euphorbia paralias* L. (Euphorbiaceae) used in this work was collected in the flowering stage on May 2015 from the North beach of Alexanderia, Egypt, while *Euphorbia geniculata* Ortega was collected in August 2015 from the roadsides and beside farms in the vicinity of Benha, Qalubya Governorate, Egypt. The identification was kindly verified by Dr. Ahmed Abd El-Razik respective lecturer of Plant Taxonomy, Department of Botany, Faculty of Science, Banha University, Banha, Qalubya Governorate, Egypt. The voucher specimens (no. S303 (for *E. paralias*) and S304 (for *E. geniculata*)) were deposited in National Research Center, El Doki, Egypt. The aerial parts of *E. paralias* L. and *E. geniculata* Ortega were air-dried and ground into coarse particles till use.

Extraction of plant material:

The air-dried powdered aerial parts of *E. paralias* and *E. geniculata* (200 gm of each) were extracted by cold maceration with 70 % methanol (1 L for each) till complete exhaustion. The combined methanolic extract of each was evaporated under reduced pressure at 45 ^{o}C to give 32 and 30 gm respectively, of greenish brown viscous residue. Each residue was suspended in MeOH-H_2O mixture (150 ml, 1:9 v/v) and extracted with methylene chloride (5 x 100 ml). The combined methylene chloride fractions of *E. paralias* and *E. geniculata* were separately washed with distilled water, dried over anhydrous sodium sulphate, then distilled off under vacuum at 45 ^{o}C to afford 1.5 and 1.2 g respectively.

UPLC- ESI- MS/MS analysis:

The methylene chloride fraction samples of *E. paralias* and *E. geniculata* were prepared as solutions of 100 µg/mL using HPLC grade methanol, filtered using a membrane disc filter (0.2 µm) then subjected to LC-ESI-MS analysis. The injection volume was 10 µL. The separation of samples was performed on UPLC instrument with ESI-MS detector equipped with reverse phase C-18 column (ACQUITY UPLC – BEH 2.1 × 50 mm, 1.7 µm). Mobile phase elution was made with the flow rate of 0.2 ml/min using gradient mobile phase comprising two eluents: eluent A is H_2O acidified with 0.1 % formic acid and eluent B is MeOH acidified with 0.1 % formic acid. Elution was performed using the following gradient: 20 % B, 0-1 min; 20-90 % B, 1-18 min; 20 % B, 18-20 min. Mass spectra were detected in the ESI-MS positive ion mode between m/z 50-900 at 30 ev capillary cone and capillary voltage 3 kV. The peaks and spectra were processed using the Maslynx 4.1 software and tentatively identified by comparing its retention time (Rt) and mass spectrum with reported data. For fragmentation collision energy 40 eV was used.

Results:

Electrospray ionization mass spectroscopy is an important, sensitive and reliable technique used for the analysis and rapid tentative identification of various classes of compounds. This helped in studying the fragmentation pathways of five classes of diterpenoids, ingenane, daphnane, tigliane, lathyrane and jatrophane, in methylene chloride fractions of two Euphorbia species. As a result, a total of fiftytwo diterpenoids (Fig. 1-13, Table 1) in *E. paralias* and *E. geniculata* were screened. The tentative identification of these compounds was based on matching the molecular ion peaks detected in positive ESI-MS and MS^2 fragmentation pattern with the previous literature. The major compounds in *E. paralias* were phorobol-12-tetradecanoate (4.93 %), 20-Oxo-phorbol-12, 13-dibutyrate (4.67%) and 20-Oxo-13-deoxy phorobol-12-propionate (4.18%), while the major compounds in *E. geniculata* were 20-Oxo-phorbol-12, 13-dibutyrate, 20-Oxo-13-deoxy phorobol-12-propionate and 13-*O*-isobutyryl-12-deoxyphorbol-20-acetate in ratio of 10.4%, 8.87% and 7.74 % respectively.

Identification of diterpenoids in Methylene Chloride Fractions of E. paralias and E. geniculate

Depending on the class of core structure and the substitution pattern, each class of diterpenoids gave either the molecular adduct ion $[M^+$+Na] and sometimes $[M^+$+H]/ $[M^+$+K]. In the MS/MS of ingenanes (ingenol esters), the molecular adduct ion gave characteristic product ions corresponding to the loss of angelic, acetic, benzoic, or hydroxyheptanoic acid, followed by the loss of water to yield the prominent characteristic fragment ion at *m/z* 295 (ingenol residual structure), the fragment at *m/z* 267 was sometimes observed which showed the loss of C=O. The tigliane class (phorobol esters) produced fragment ions showing the subsequent losses of the acyl moieties as acetic, benzoic, decanoic acids, N-methyl anthranilic moiety etc., in addition to the loss of three molecules of water leaving the characteristic fragment ion at *m/z* 293 (phorobol residual structure). While lathyrane diterpenes showed $[M^+$+H], $[M^+$+Na] and $[M^+$+K] in ESI-MS. In ESI-MS/MS spectrum of lathyrane diterpenes the loss of acyl moieties with the subsequent loss of water molecules were observed producing the diagnostic fragment ion at *m/z* 281 (for lathyrol skeleton). Furthermore, the jatrophane type showed the MS^2 fragment ions at *m/z* 327, 309, 299 and 281.

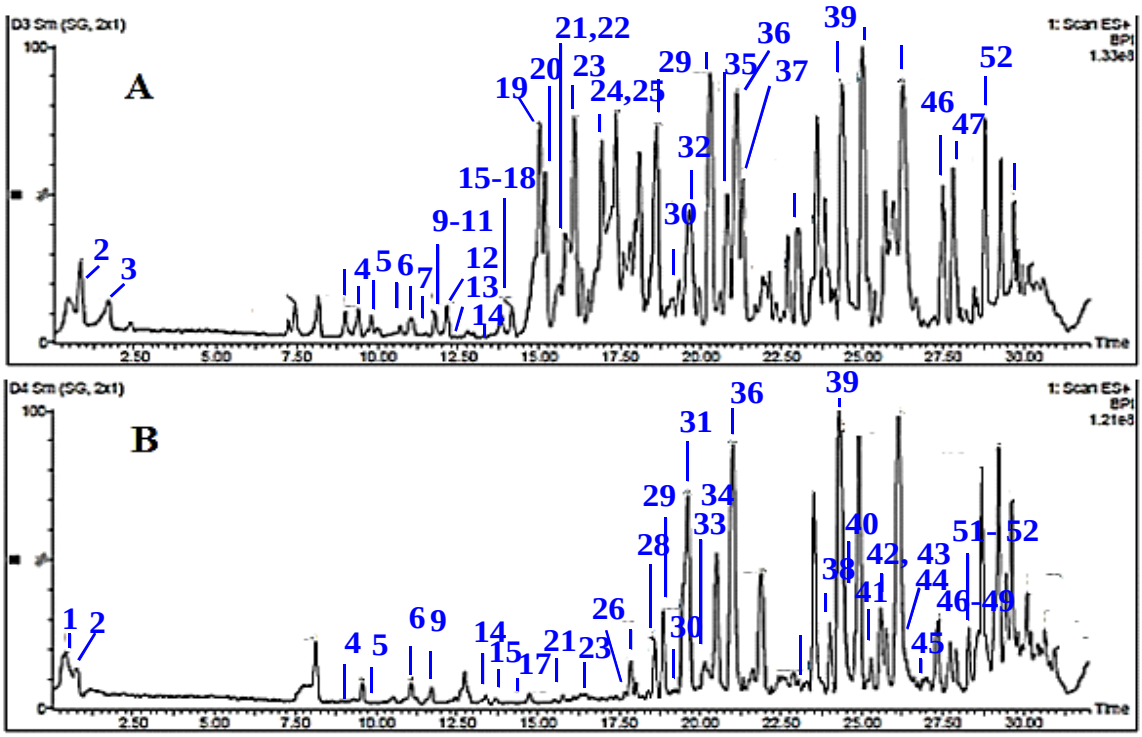


Figure 1: LC-ESI-MS profiles of methylene chloride fractions of *E. paralias* (A) and *Egeniculata* (B) (Positive mode).

Table 1: The diterpenoids identified in Methylene Chloride Fractions of *E. paralias* and *Egeniculata*:

Peak No	Rt (min)	[M⁺+Na] (M⁺)	Daughter fragments (*m/z*) of MS²	E.p % area	E.g % area	Proposed compound
Ingenane diterpenoids (Ingenol esters):						
1	0.74	453 (430)	353, 295	-	+	Ingenol-3- mebutate (Ingenol-3- angelate)
23	15.9	579 (556)	457, 335, 317, 313, 307, 295, 285	-	+	Ingenol-3,20-dibenzoate
27	17.32	579(556)	457, 335, 317, 313, 307, 295, 285	-	+	Ingenol-3,20-dibenzoate isomer
28	18.60	579(556)	457, 335, 317, 313, 307, 295, 285	-	1.17	Ingenol-3,20-dibenzoate isomer
29	18.86	579(556)	457, 335, 317, 313, 307, 295, 285	-	1.74	Ingenol-3,20-dibenzoate isomer
30	19.45	579 (556)	457, 335, 317, 313, 307, 295, 285	-	+	Ingenol-3,20-dibenzoate isomer
33	20.15	437 [M⁺+Na], 453 [M⁺+K](414)	353, 313, 295, 267, 255	-	1.19	3-Deoxy-3-oxoingenol 20-butenoate
34	20.34	579[M⁺+Na]	457, 335, 317, 313, 307, 295, 285	-	+	Ingenol-3,20-dibenzoate isomer
35	20.82	515 [M⁺+K], 499[M⁺+Na], 477 [M⁺+H] (476)	313, 295, 267	1.63	-	Ingenol-20-hydroxyheptanoate
Tigliane diterpenoids (Phorobol and phorobol esters)						
2	0.82	541(518)	369, 351, 333, 329, 315, 311, 293	1.19	+	Phorbol-12-decanoate
3	1.69	387(364)	387, 369, 351, 333, 315, 311, 293	0.77	-	Phorbol
4	8.89	429 (406)	369, 351, 333, 329, 315, 311, 293	0.40	+	Phorbol-12-acetate
5	9.82	429 (406)	369, 351, 333, 329, 315, 311, 293	0.15	+	Phorbol-13-acetate
6	11.05	471 (448)	411, 351, 333, 329, 315, 311, 293	0.36	0.32	Phorbol-12,13-diacetate
7	11.50	471 (448)	411, 351, 333, 329, 315, 311, 293	-	+	Phorbol-12, 13-diacetate isomer
9	11.78	562 (539)	411, 502, 351, 333, 329, 315, 311, 293	0.30	0.44	12-*O*-(*N*-methylanthranilate) phorbol-13-acetate

10	**11.80**	**562 (539)**	**411, 502, 351, 333, 329, 315, 311, 293**	**+**	**+**	**12-*O*-(*N*-methylanthranilate) phorbol-13-acetate isomer**
11	**11.85**	**562 (539)**	**411, 502, 351, 333, 329, 315, 311, 293**	**+**	**+**	**12-*O*-(*N*-methylanthranilate) phorbol-13-acetate isomer**
13	**12.30**	**583 (560)**	**467, 351, 333, 329, 315, 311, 293**	**0.45**	**-**	**Phorbol-12,13-dihexanoate**
14	**12.82**	**541 (518)**	**387, 369, 351, 333, 329, 315, 311, 293**	**+**	**+**	**Phorbol-12-decanoate isomer**
15	**13.03**	**531 (508)**	**489, 471, 371, 335, 317, 313, 295**	**+**	**+**	**13-*O*-phenylacetyl-12-deoxyphorbol-20-acetate**
16	**13.20**	**541 (518)**	**387, 369, 351, 333, 329, 315, 311, 293**	**+**	**-**	**Phorbol-12-decanoate isomer**
17	**14.10**	**429 (406)**	**369, 351, 333, 329, 315, 311, 293**	**0.38**	**+**	**Phorbol-12-acetate isomer**
19	**15.02**	**597 (574)**	**369, 351, 333, 329, 315, 311, 293**	**4.93**	**-**	**Phorobol-12-tetradecanoate**
20	**15.18**	**597 (574)**	**369, 351, 333, 329, 315, 311, 293**	**1.97**	**-**	**Phorbol-13-tetradecanoate**
21	**15.63**	**695 (672)**	**523, 351, 333, 329, 315, 311, 293**	**0.95**	**+**	**Phorbol-12,13-didecanoate**
22	**15.82**	**429 (406)**	**369, 351, 333, 329, 315, 311, 293**	**2.18**	**-**	**Phorbol-13-acetate isomer**
24	**16.08**	**695 (672)**	**523, 351, 333, 329, 315, 311, 293**	**3.18**	**-**	**Phorbol-12,13-didecanoate isomer**
25	**16.78**	**597 (574)**	**369, 351, 333, 329, 315, 311, 293**	**1.97**	**-**	**Phorbol-13-tetradecanoate isomer**
26	**16.93**	**597 (574)**	**369, 351, 333, 329, 315, 311, 293**	**2.74**	**-**	**Phorbol-13-tetradecanoate isomer**
31	**19.61**	**483 (460)**	**335, 317, 313, 307, 295**	**-**	**7.74**	**13-*O*-isobutyryl-12-deoxyphorbol-20-acetate**
36	**21.01**	**425 (402)**	**347, 331, 291 (100%)**	**4.18**	**8.87**	**20-Oxo-13- deoxy phorobol-12-propionate**
37	**21.31**	**535 (512)**	**495, 395, 313, 295, 267**	**1.79**	**-**	**12-Deoxyphorobol-13, 20 diangelate**
38	**24.20**	**639 (616)**	**579, 411, 351, 333, 329, 315, 311, 293**	**-**	**+**	**12-*O*-Tetradecanoylphorbol-13-acetate (TPA)**
39	**24.28**	**525 (502)**	**507, 437, 349, 331, 309, 291**	**4.67**	**10.4**	**20-Oxo-phorbol-12,13-dibutyrate**
40	**24.78**	**525 (502)**	**507, 437, 349, 331, 309, 291**	**-**	**+**	**20-Oxo-phorbol-12,13-dibutyrate isomer**
41	**24.98**	**525 (502)**	**507, 437, 349, 331, 309, 291**	**-**	**+**	**20-Oxo-phorbol-12,13-dibutyrate isomer**
42	**25.0**	**639 (616)**	**411, 351, 333, 329, 315, 311, 293**	**-**	**+**	**12-*O*-tetradecanoylphorbol-13-acetate (TPA) isomer**
43	**25.61**	**531 (508)**	**489, 471, 371, 335, 317, 313, 295**	**-**	**1.97**	**13-*O*-phenylacetyl-12-deoxyphorbol-20-acetate**
44	**26.34**	**531 (508)**	**487, 471, 371, 335, 317, 313, 295**	**-**	**1.0**	**13-*O*-phenylacetyl-12-deoxyphorbol-20-acetate isomer**
45	**27.57**	**471 (448)**	**411, 351, 333, 329, 315, 311, 293**	**-**	**+**	**Phorbol-12, 13-diacetate isomer**
46	**27.67**	**541 (518)**	**387, 369, 351, 333, 329, 315, 311, 293**	**2.11**	**0.86**	**Phorbol-12-decanoate isomer**
47	**27.69**	**471 (448)**	**411, 351, 333, 329, 315, 311, 293**	**-**	**+**	**Phorbol-12,13-diacetate isomer**
48	**27.73**	**531 (508)**	**489, 471, 371, 335, 317, 313, 295**	**-**	**+**	**13-*O*-phenylacetyl-12-deoxyphorbol-20-acetate isomer**
49	**28.11**	**471 (448)**	**411, 351, 333, 329, 315, 311, 293**	**-**	**+**	**Phorbol-12, 13-diacetate isomer**
51	**28.58**	**695 (672)**	**387, 523, 351, 333, 329, 315, 311, 293**	**-**	**1.02**	**Phorbol-12,13-didecanoate isomer**
52	**28.70**	**541 (518)**	**369, 351, 333, 329, 315, 311, 293**	**2.18**	**4.47**	**Phorbol-12-decanoate isomer**
Lathyrane diterpenoids (Lathyrol and hydroxylathyrol esters)						
8	**11.69**	**539 [M^++H],561 [M^++Na], 577[M^++K](538)**	**461, 377, 317, 299, 281, 259**	**+**	**-**	**5-Pentanoyl-3-benzoyl-hydroxylathyrol**
12	**12.14**	**539 [M^++H],561.3[M^++ Na], 577[M^++K] (538)**	**461, 377, 317, 299, 281, 259**	**0.35**	**-**	**5-Pentanoyl-3-benzoyl-hydroxylathyrol isomer**

32	**19.94**	**561[M^++K], 545 [M^++Na], 523 [M^++H] (522)**	**463, 403, 375, 341, 299, 281 (100%)**	**0.37**	**-**	**Euphorbia factor L 3**
			Jatrophane diterpene ester			
18	**14.16**	**647 [M^++K], 631 [M^++Na] (608)**	**591, 549, 531, 489 471, 429 411, ,387, 369, 341, 327, 309 , 299, 281**	**+**	**-**	**3,5,7,8,15, pentacetyl-2-hydroxy-9,14 dioxo-jatropha-6(16), -11(12)-diene**
Daphnane diterpene ester						
50	**28.30**	**651[M^++Na] (628)**	**507, 441, 371, 355, 333**	**-**	**1.1**	**Resiniferatoxin**

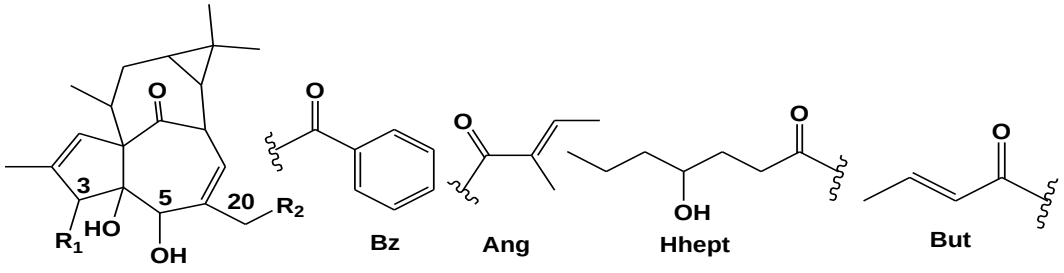
+ = present - = absent E.p= *Euphorbia paralias* E.g = *Euporbia geniclata*

Ingenol esters:

Nine ingenol esters were tentatively identified in the methylene chloride fractions of *E. paralias* and *E. geniculata***.** Eight compounds including ingenol-3-mebutate (ingenol-3-angelate), ingenol-3, 20-dibenzoate with its five isomers, in addition to 3-deoxy-3-oxoingenol 20-butenoate were identified in *Egeniculata* and one in *E. paralias* (ingenol-20-hydroxyheptanoate)**.**

Peak 1 (Rt 0.74 min.) generated [M^++ Na] at *m/z* 453. It gave diagnostic fragment ions at *m/z* 353 [M^++Na-100 amu (angelic acid)] and 295 [M^++H-100 amu (angelic acid)-36 amu] corresponding to the loss of angelic acid ion and two molecules of water (*m/z* 36). Thus, it was characterized tentatively as ingenol-3-mebutate **[13].** While peaks 23, 27-30 and 34 eluted at Rt.15.90, 17.32, 18.60, 18.86, 19.45 and 20.34 min. were observed to possess the same ESI-MS^1 at *m/z* 579 [M^++Na]. The dominant ESI-MS^2 fragment ions at *m/z* 457 [M^++Na- $C_7H_5O_2$], 335 [M^++Na-2 $C_7H_5O_2$], and 317 [M^++Na-2 $C_7H_5O_2$-H_2O], showing the subsequent losses of one and two benzoic acid moieties then one molecule of H_2O. the deprotonated base peak fragment ion at *m/z* 295 [M^+ +H-2$C_7H_5O_2$-H_2O] was corresponding to the loss of two molecules of benzoic acid and one molecule of water and corresponding to the residual ingenol skeleton. Consequently, peak **23** and its isomer were tentatively characterized as ingenol-3, 20-dibenzoate **[13].**

In addition**,** peak **35** (Rt, 20.82 min) displayed molecular ion peaks at *m/z* 515 [M^++K], 499 [M^++Na], 477 [M^++H]. Its further fragmentation resulted in [M^++H- $C_7H_{13}O_3$-H_2O] at *m/z* 313 for the loss of hydroxyheptanoic acid and one molecule of water. The prominent fragment ion at *m/z* 295 [M^++H-$C_7H_{13}O_2$-2H_2O] showing the loss of hydroxyheptanoic acid and two molecules of water and is characteristic for ingenol skeleton **[4,13**]. Furthermore, the remaining fragment at *m/z* 267 showed the loss of C=O group. By comparing the fragmentation pattern with the previous literature, the nucleus of peak **35** is ingenol while the substitution is hydroxyheptanoate which did not previously reported. Hence, peak **35** was tentatively identified as ingenol-20-hydroxyheptanoate which expected to be a new compound. Similarly, peak **33** eluted at Rt 20.15 min generated its ESI-MS at *m/z* 437 [M^++Na], and MS^2 fragment ion at *m/z* 353 [M^++Na-$C_4H_5O_2$] which was produced by the loss of butenoic acid moiety, while the product ion at *m/z* 313 [M+H-$C_4H_5O_2$-H_2O] showed the loss of one molecule of water, furthermore the fragment ion at *m/z* 295 [M^++H-$C_4H_5O_2$ (86 amu) -2H_2O (36 amu)] was resulted from the loss of two molecules of H_2O leaving the residual ingenol skeleton [**4,13**]. The next fragment ion at *m/z* 267 [M^++H-$C_4H_5O_2$-2H_2O-CO] which showed the loss of carbonyl group was also observed. So peak **33** was identified as 3-deoxy-3-oxoingenol 20-butenoate **[4,13**].



Compound No.	R_1	R_2	Compound No.	R_1	R_2
1	**O-Ang**	**OH**	**23, 27-30, 34**	**O-Bz**	**O-Bz**
33	**O**	**O-But**	**35**	**OH**	**O-Hhept**

Figure 2: The chemical structures of ingenol esters identified in methylene chloride fractions of *E. paralias* and *E. geniculate*

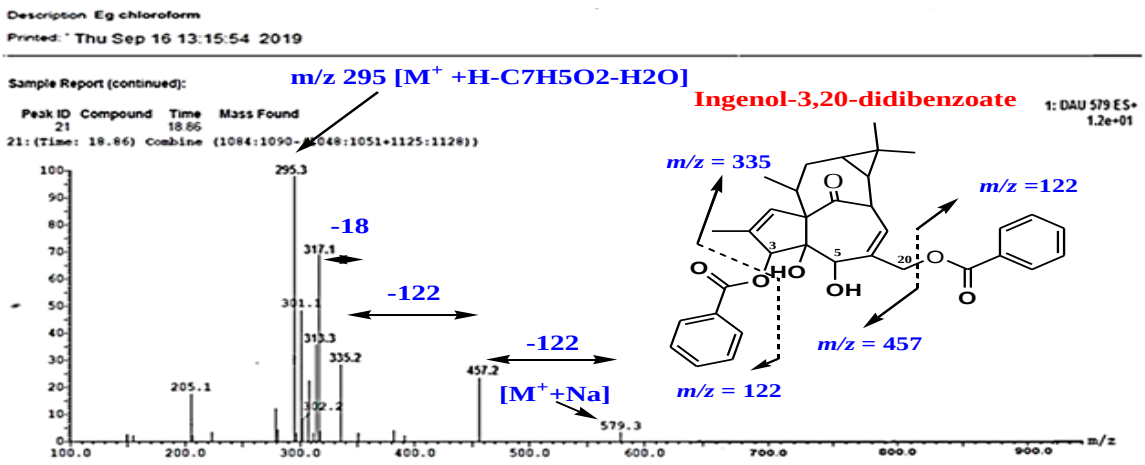
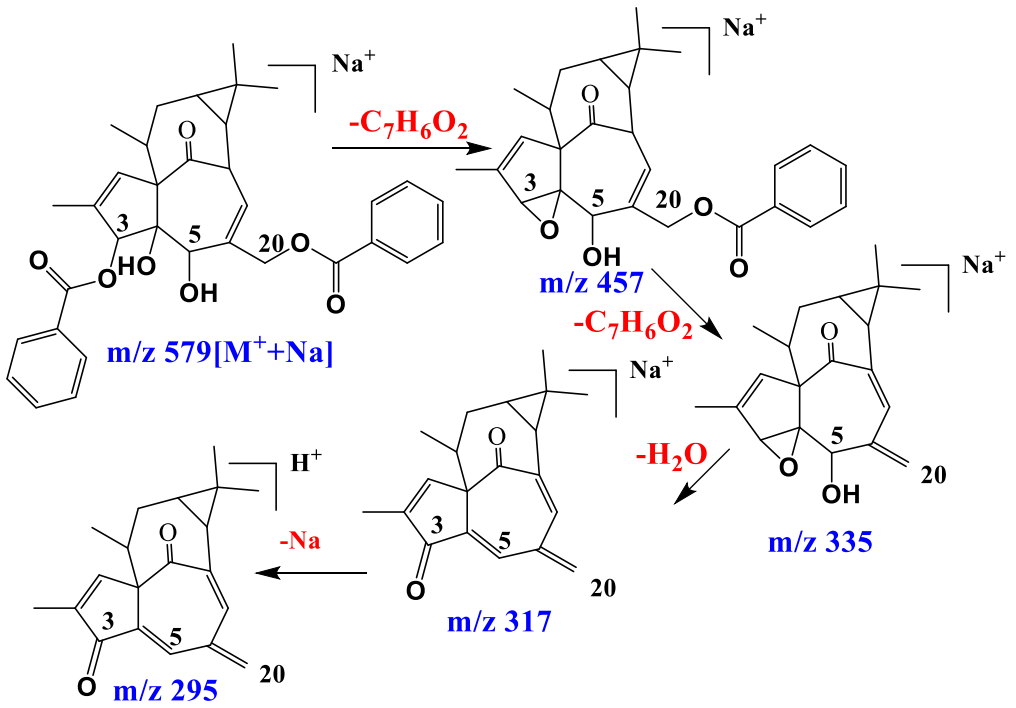


Figure 3: Positive ESI-MS/MS spectrum of ingenol-3, 20- dibenzoate (peak 23, *m/z* 579[M⁺+Na])



Scheme 1: Characteristic ESI-MS/MS fragmentation pattern of ingenol-3, 20-dibenzoate in positive ion mode

Phorbol esters:

Phorbol and Phorbol monoesters

Fourteen (one phorbol, and thirteen phorbol monoester) diterpenoids were determined in both Euphorbia species including phorbol-12-tetradecanoate, phoropol-12- acetate, phorpol-13-acetate and phorpol-12-decanoate and their isomers. Peak 3, (Rt, 1.6 min), its ESI-MS spectrum showed a molecular ion peak at *m/z* 387 [M⁺+Na] and MS² fragment ions at *m/z* 369 [M⁺+Na-H_2O], 351 [M⁺+Na-$2H_2O$] and 333 [M⁺+Na-$3H_2O$] were yielded due to losses of one, two and three molecules of water respectively. Base peak fragment at *m/z* 293 [M⁺+H -$4H_2O$] was observed. From this behavior in ESI-MS/MS and through comparison with literature, peak 3 was identified as phorbol [13]. Peaks 19, 20, 25 and 26 eluted at Rt, 15.02, 15.18, 16.78, 16.93 min exhibited the same MS^1 and MS^2 data. They gave molecular ion peak at *m/z* 597 [M⁺+Na]. Series of MS^2 fragment ions showing the loss of tetradecanoic acid and several molecules of water at *m/z* 369 [M⁺+Na-tetradecanoic acid], 351 [M⁺+Na-tetradecanoic acid-H_2O], 333 [M⁺+Na-tetradecanoic acid-$2H_2O$], 315 [M⁺+Na-tetradecanoic acid- $3H_2O$] and 293 [M⁺-H-tetradecanoic acid-3 H_2O] were observed. Accordingly peaks 19 was tentatively identified as phorbol-12-tetradecanoate while peak 20 as its isomers (peaks 25 and 26) were tentatively identified phorbol-13-tetradecanoate [13].

Peaks 2, 14, 16, 46 and 52 (Rt, 0.82, 12.82, 13.20, 27.67, 28.70) were tentatively identified as phoropol-12-decanoate and its four isomers [**11]**. Where the ESI-MS spectra displayed the same molecular ion peak at *m/z* 541 [M^++Na] and MS^2 spectra produced a fragment ion showing the loss of decanoate moiety at *m/z* 387 followed by several fragment ions at *m/z* 369 [M^++Na-decanoate-H_2O], 351 [M^++Na- decanoate -$2H_2O$] and 333 [M^++Na- decanoate -$3H_2O$] which showed repeated loss of 18 amu for loss of several molecules of water.

Peaks 4, 5, 17 and 22, (Rt, 8.89, 9.82, 14.1, 15.82 min) possessed the same molecular ion peak at *m/z* 429 [M^++Na] and displayed MS^2 fragment ions at *m/z* 387 [M^++Na-$COCH_3$], 369 [M^++Na-CH_3CO+H_2O], 351 [M^++Na-CH_3CO-$2H_2O$], 333 [M^++Na-CH_3CO-$3H_2O$] and 315 [M^++Na- CH_3CO-$4H_2O$]. In addition, a base peak fragment at *m/z* 293 [M^+ +H-CH_3COOH-$4H_2O$] was observed. Thus peak 4 and its isomer (peak 17) were tentatively identified as phoropol-12-acetate, while peak 5 and its isomer (peak 22) were tentatively identified as phoropol-13-acetate [**13**]. Figure 5 showed the fragmentation pattern of phorobol-12-acetate (Fig. 5, Scheme 2).

Phorbol diesters:

Fifteen phorbol diesters were identified in both euphorbia species (13 in *E. paralias* and 15 in *E. geniculata*) as follow:

Peaks 6, 7, 45, 47 and 49 (Rt, 11.05, 11.50, 27.57, 27.69 and 28.11 min) produced the same molecular ion peak at ***m/z*** 471 [M^++Na] and all of their molecular ion peaks showed series of MS^2 fragment ions at *m/z* 411 [M^++Na-CH_3COOH], 351 [M^++Na-$2CH_3COOH$], 333 [M^++Na-$2CH_3COOH$ -H_2O], 315 [M^++Na-$2CH_3COOH$ -$2H_2O$], 293 [M^++H-$2CH_3COOH$ -$2H_2O$], which conferred the identification of these compounds as phorbol-12,13-diacetate and its four isomers (Fig. 4) **[13].** In addition, peaks 9, 10 and 11 (Rt. 11.78, 11.80, 11.85 min.) showed the same [M^++Na] ion at *m/z* 562. These peaks generated [M^++Na-CH_3COOH] at *m/z* 502 by losing acetic acid, beside the fragment ion at *m/z* 411 showing the loss of $C_8H_8NO_2$ (*N*-methylanthranilate). Other MS/MS fragments at *m/z* 333 corresponded to subsequent loss of acetic acid moiety plus one molecule of water after loss of *N*-methylanthranilate. Moreover, the fragment ion at *m/z* 293 for [M^++H -$2H_2O$+CH_3COOH+$C_8H_8NO_2$] was produced. This fragmentation of peak **9 (Fig. 6)** was in a good agreement with that for 12-*O*-(*N*-methylanthranilate) phorbol-13-acetate (Fig. 6, scheme 3) which is known as sapintoxin D **[13**] and its isomers. While peaks 21, 24 and 51 eluted at Rt, 15.63, 16.08, 28.58 min were identified as phorbol-12, 13-didecanoate and its isomers from MS^1 and MS^2 spectral data (Table 1). Prominent MS^1 pseudo molecular ion peak at *m/z* 695 was formed. The MS^2 product ions at *m/z* 523 [M^++Na-$C_{10}H_{19}O_2$] and 351[M^++Na -$2C_{10}H_{19}O_2$] corresponding to neutral losses of one and two molecules of decanoic acid. In addition to fragment ions at *m/z* 333 [M^++Na-$2C_{10}H_{19}O_2$ -H_2O] and 315 [M^++Na -$2C_{10}H_{19}O_2$-$2H_2O$] which showed the subsequent losses of one and two molecules of water. The base peak fragment at *m/z* 293 [M+H-$2C_{10}H_{19}O_2$-$2H_2O$] was also observed. The mass data of these compounds are in a good agreement with those of phorbol-12, 13-didecanoate **[13].** Peak 13 (Rt, 12.30) gave a pseudo-molecular ion peak at *m/z* 583 [M^++Na] and a fragment ion at *m/z* 467 [M^++Na-$C_6H_{11}O_2$] which involve loss of one hexanoic acid, further fragmentation gave *m/z* 351 [M^++Na- $2C_6H_{11}O_2$] for further loss of one hexanoic acid molecule. Moreover, series of fragments were produced at *m/z* 333 [M^++Na-2 hexanoic acid-H_2O], 315 [M^++Na-2 hexanoic acid-$2H_2O$] and 293 [M^++H -2 hexanoic acid-$2H_2O$]. Consequently, compound 13 was identified as phorbol-12,13-dihexanoate [13].

Similarly, peaks 38 and 42 (Rt, 24.20, 25.0 min) exhibited the same molecular ion peak at *m/z* 639 [M^++Na]. MS^2 yielded fragment ion peaks at *m/z* 579 [M^++H-$2H_2O$] and 411[M^++Na-$C_{14}H_{27}O_2$] which showed the loss of two molecules of water and one molecule of tetradecanoic acid respectively. Further fragmentation gave [M^++Na-$C_{14}H_{27}O_2$-CH_3COOH] at m/z 351 showing the loss of one molecule of acetic acid and tetradecanoic acid. The base peak fragment at *m/z* 293 [M++H-acetic acid and tetradecanoic acid-$2H_2O$] was observed. Therefore, peak 38 was tentatively identified as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and its isomer [13].

Deoxyphorobol esters:

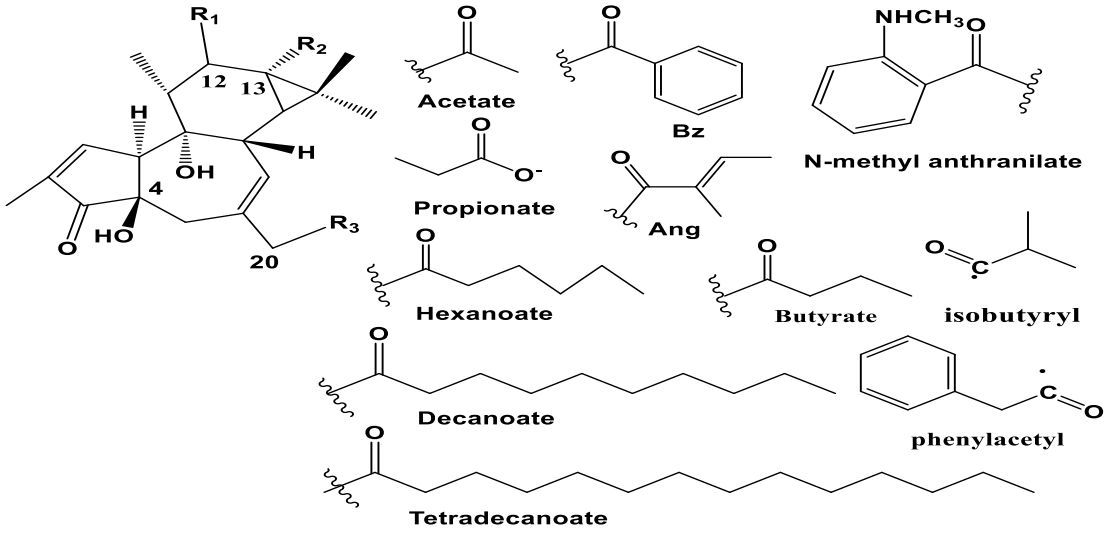
Six deoxyphorobol esters were characterized in both Euphorbia species (three in *E. paralias* and five in *E. geniculata*). Peaks 15, 43, 44 and 48 with time retention 13.03, 25.61, 26.34 and 27.73 min, respectively, gave the same molecular ion peaks at *m/z* 531 [M^++Na]. MS^2 fragment ions produced at *m/z* 489 [M^++Na-CH_3CO], 471 [M^++Na -CH_3COOH], 335 [M^++Na -CH_3COOH-$C_8H_7O_2$] showed the loss of acetyl, acetic acid and acetic acid with phenylacetyl moieties respectively. The fragment at *m/z* 295 [M^++H-CH_3COOH-$C_8H_7O_2$-H_2O] was observed. Accordingly peak 15 was tentatively characterized as 13-*O*-phenylacetyl-12-deoxyphorbol-20-acetate and its isomers **[13].** While peak 31 (Rt, 19.61 min.) exhibited a molecular ion peak at *m/z* 483 [M^++Na] and MS^2 fragment at *m/z* 335 which showed the loss of acetic and isobutyric acid moieties. It gave a fragment ion at *m/z* 317 owing to the subsequent loss of one molecule of water. The base peak fragment ion at *m/z* 295 [M+H- acetic acid- isobutyric acid-H_2O] was observed. These data confirmed the existence of butyryl and acetyl moieties. Therefore, peak 31 was tentatively identified as 13-*O*-isobutyryl-12-deoxyphorbol-20-

acetate **[13].** Similarly, peak 37**,** (Rt, 21.31 min), its ESI-MS spectrum showed *m/z* 535 [M^++Na], 551 [M^++K] and 513 [M^++H]. The dominant characteristic fragment ions at *m/z* 495 [M^++H-H_2O] showing the loss of one molecule of water, *m/z* 395 [M^++H-H_2O-$C_4H_7O_2$] exhibited the loss of angelic acid and water *m/z* 295 [M^++H-H_2O-2C_4H_7COOH] showing loss of two angelic acid moieties and one molecule of water, the loss of C=O moiety was observed from fragment at *m/z* 267 [M^++H-H_2O-2C_4H_7COOH-CO]. Thus peak 37 was tentatively identified as 12-deoxyphorobol-13, 20 diangelate (Fig. 7) **[13].**

20-oxo-phorbol esters

One 20-oxo-phorbol ester was tentatively identified in *E. paralias* and three in *E. genicuata* as follow:

Peak 36 (Rt, 20.01 min) showed a molecular ion peak at *m/z* 425 [M^++Na]. The ESI-MS/MS product ion at *m/z* 331 [M++H-$C_3H_5O_2$] is produced by cleavage of $C_3H_5O_2$ of propionic acid moiety. The base peak fragment at *m/z* 291 showed the subsequent loss of 40 amu [Na+H_2O] from 331 which showed the loss of one molecule of water. This fragment is characteristic for 20-oxophorobol skeleton **[13].** These data led to tentative identification of peak 36 as 20-oxo-13-deoxy phorobol-12-propionate. By the same manner peaks 39, 40 and 41 (Rt, 24.28, 24.78, 24.98 min.) which gave their molecular ion peak at *m/z* 525 [M^++Na] were tentatively identified as 20-oxophorbol-12, 13-dibutyrate isomers (Fig. 8, Scheme 5) **[13].** The MS^2 fragment ions at *m/z* 507 [M^++Na-H_2O] showed the loss of one molecule of water, In addition to *m/z* 437 [M^++Na-$C_4H_8O_2$] showed the loss of butyric acid, 331 [M^++Na-2$C_4H_8O_2$-H_2O] exhibit the loss of two molecule of butyric acid and one molecule of water.



Compound No.	R_1	R_2	R_3
19, 20, 25 , 26	**O-Tetradecanoate**	**OH**	**OH**
2, 14, 16, 46	**O- decanoate**	**OH**	**OH**
3	**OH**	**OH**	**OH**
4, 17	**O-acetate**	**OH**	**OH**
5, 22	**OH**	**O-acetate**	**OH**
6, 7, 45, 47 , 49	**O-acetate**	**O-acetate**	**OH**
9-11	**N-methyl anthranilate**	**O-acetate**	**OH**
21, 24, 51	**O- decanoate**	**O- decanoate**	**OH**
13	**O-Hexanoate**	**O-Hexanoate**	**OH**
38 , 42	**O-Tetradecanoate**	**O-acetate**	**OH**
15, 43, 44 , 48	**H**	**O-Phenylacetyl**	**O-Acetate**
31	**H**	**O-Isobutyryl**	**O-Acetate**
36	**O-Propionyl**	**H**	**O**
37	**H**	**O-Angelate**	**O-Angelate**
39, 40 , 41	**O- Butyrate**	**O- Butyrate**	**O**

Figure 4: The chemical structures of phorbol esters identified in methylene chloride fractions of *E. paralias* and *Egeniculata*

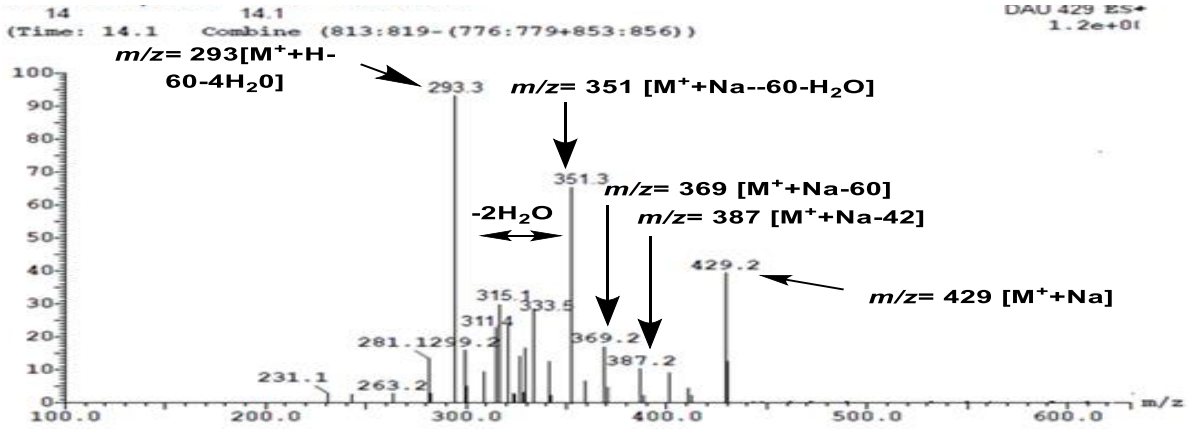
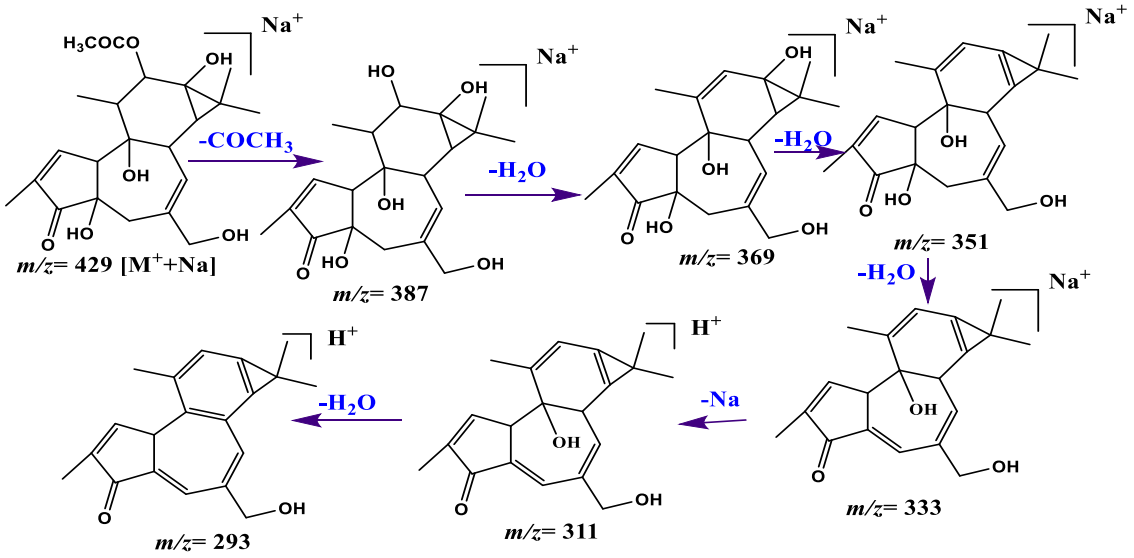


Figure 5: Positive ESI-MS/MS spectrum of phorpol-12-acetate (peak 4 *m/z* 429[M$^+$+Na])



Scheme 2: Characteristic ESI-MS/MS fragmentation pattern of phorpol-12-acetate in positive ion mode

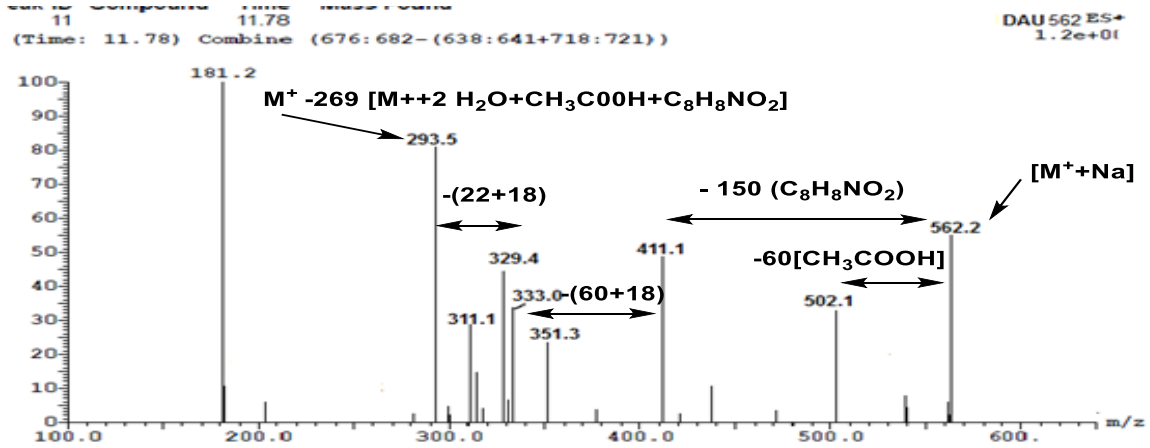
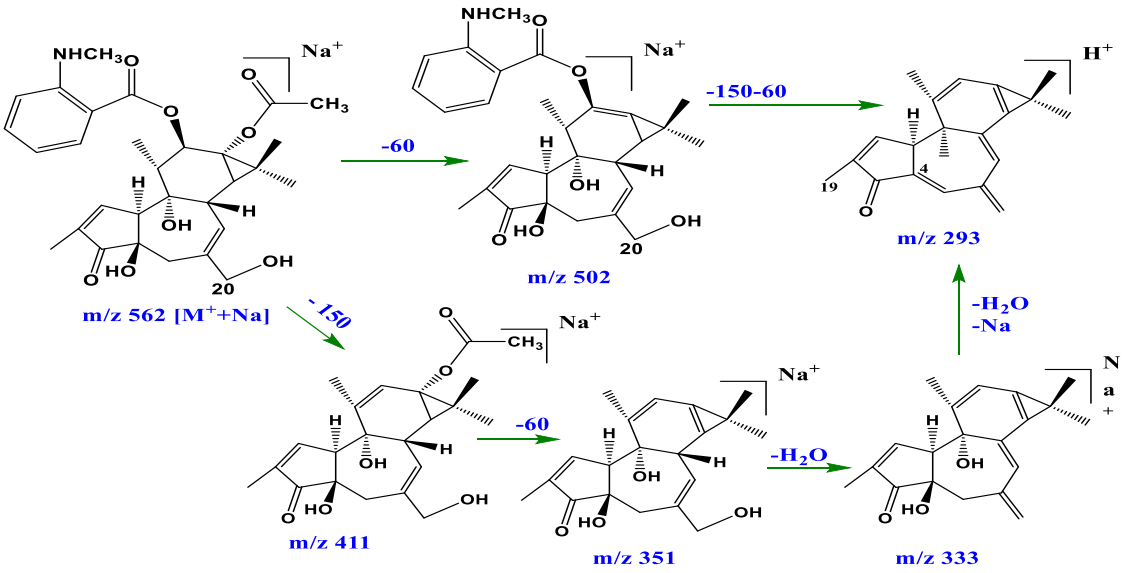


Figure 6: Positive ESI-MS/MS spectrum of 12-*O*-(*N*-methylanthranilate) phorbol-13-acetate (peak 9 *m/z* 562[M$^+$+Na])



Scheme 3: Characteristic ESI-MS/MS fragmentation pattern of 12-*O*-(*N*-methylanthranilate) phorbol-13-acetate in positive ion mode

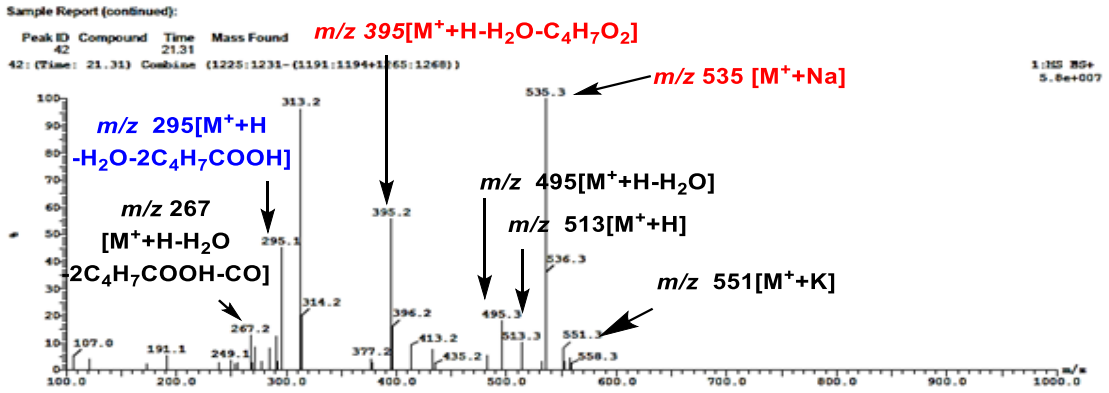
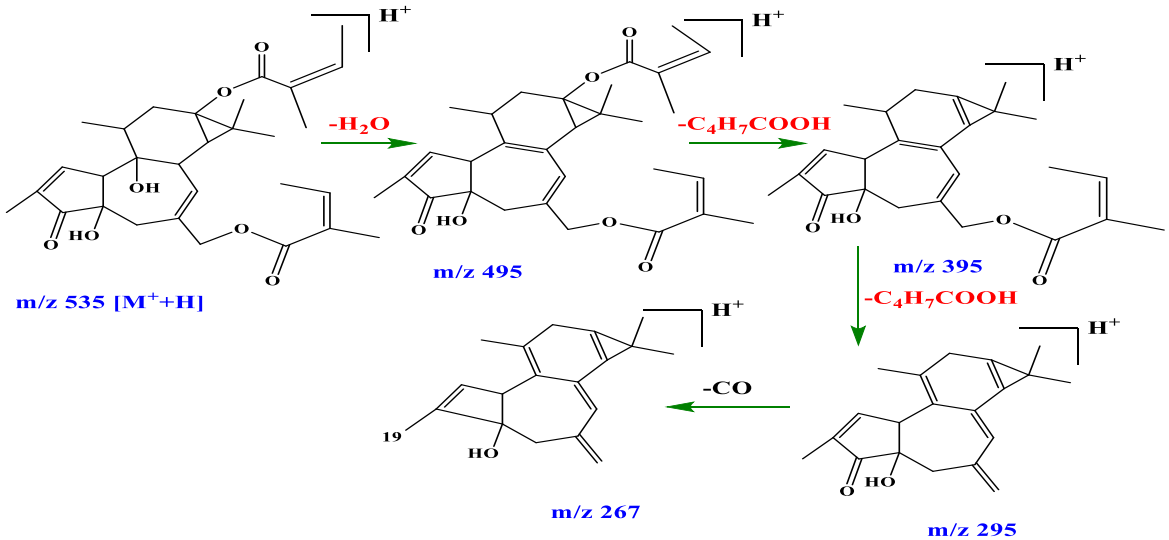


Figure 7: Positive ESI-MS/MS spectrum of 12-deoxyphorobol-13, 20 diangelate (peak 37 *m/z* 535 [M+Na])



Scheme 4: Characteristic ESI-MS/MS fragmentation pattern of 12-deoxyphorobol-13, 20-diangelate in positive ion mode

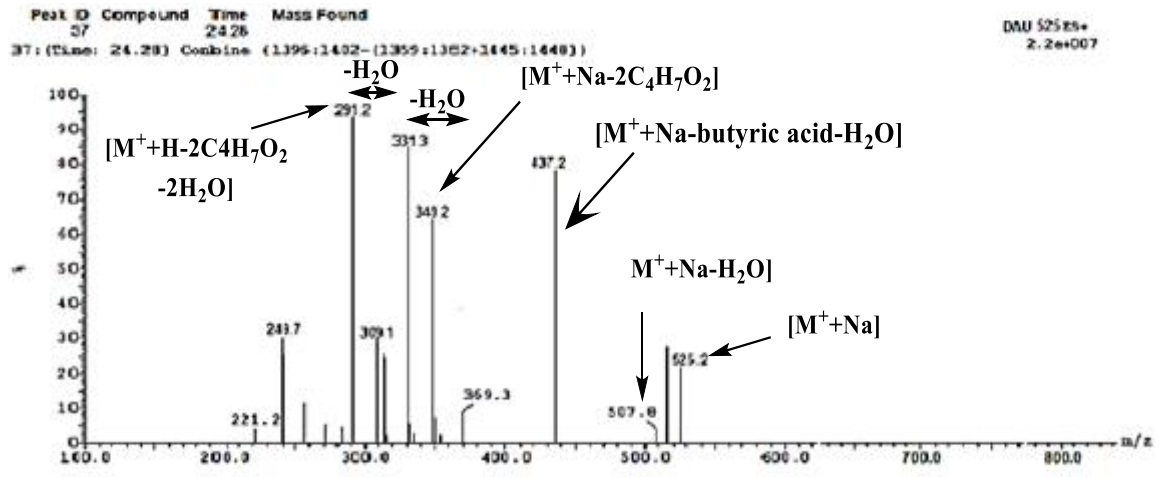
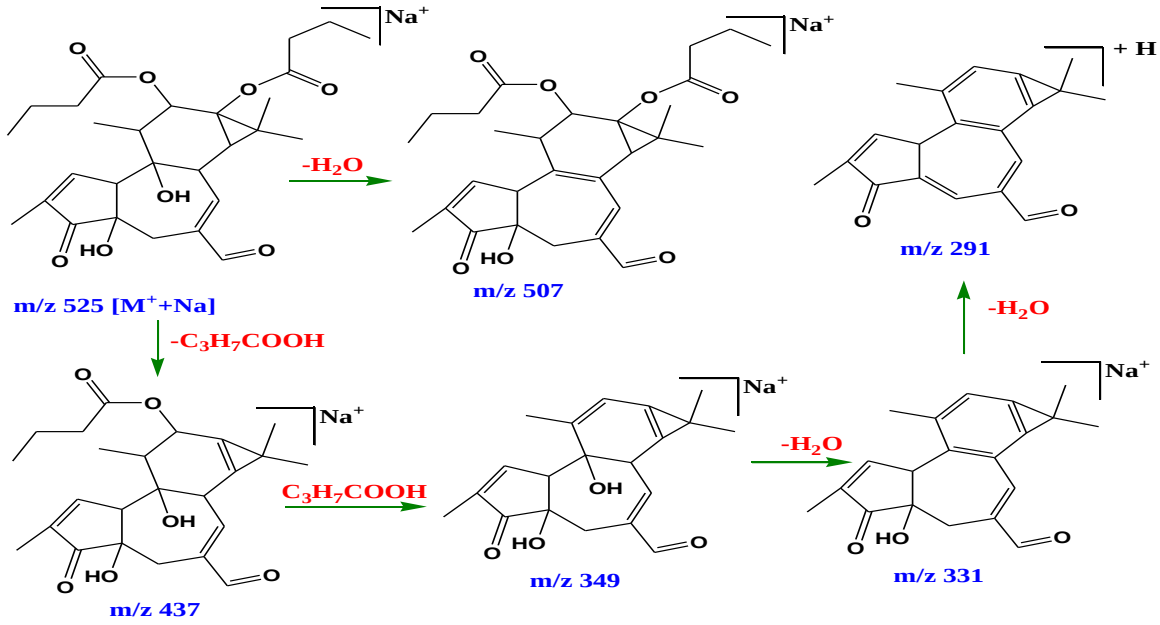


Figure 8: Positive ESI-MS/MS spectrum of 20-oxo-phorbol-12, 13-dibutyrate (peak 39 *m/z* 525[M⁺+Na])

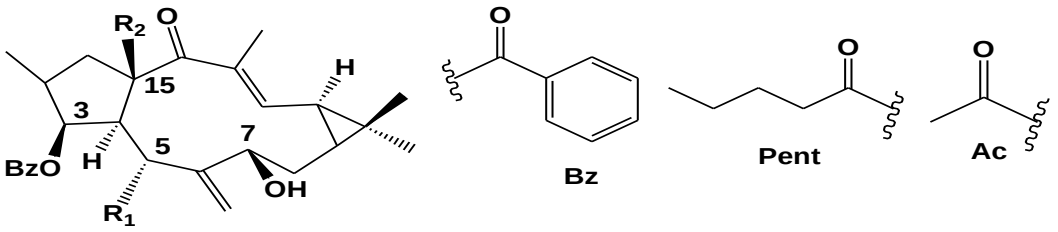


Scheme 5: Characteristic ESI-MS/MS fragmentation pattern of 20-oxo-phorbol-12, 13-dibutyrate in positive ion mode

Lathyrol and hydroxylathyrol esters

Only one lathyrol **(**Euphorbia Factor L_3) and two hydroxylathyrol (5-pentanoyl-3-benzoyl-hydroxylathyrol and its isomer) were determined in *E. paralias* while it is completely absent in *E. geniculata*.

Peaks 8 and 12 (Rt, 11.69 and 12.14 min) have the same molecular ion peaks at 567 [M⁺+K], 561 [M⁺+Na], *m/z* 539 [M⁺+H]. The MS^2 diagnostic fragment ion appear at *m/z* 461 for [M⁺+Na-$C_5H_9O_2$] which showed the loss of pentanoic acid and the fragment ion at *m/z* 317 [M⁺+H- $C_7H_5O_2$-C7H5O2] showed the loss of pentanoic and benzoic acid moieties as shown in (Fig 9). The fragment ions at *m/z* 299 and 281 showed the loss of two molecules of water which are diagnostic fragments corresponding to the residual lathyrane skeleton **[14].** From these results and through comparison with previous reports it was concluded that peaks 8 and 12 were tentatively identified as 5-pentanoyl-3-benzoyl-hydroxylathyrol and its isomer **[12].** Similarly, peak **32** (Rt, 19.94 min) was identified as Euphorbia Factor L_3 which has a molecular ion peak at *m/z* 561 [M⁺+K], 545 [M⁺+Na], 523 [M⁺+H] (Fig. 10, Scheme 6). It displayed MS^2 fragment ions at *m/z* 463 [M⁺+H-CH_3COOH], 403[M⁺+H-2CH_3COOH], 375 [403-CO], 341, 281(100%) [M⁺+H-2CH_3COOH-C_6H_5COOH] due to the loss of two molecules of acetic acid and one benzoic acid moieties. These data are in a good agreement with the reported data **[12].**



Compound No.	R_1	R_2
8, 12	O-Pent	OH
32	O-Ac	O-Ac

Figure 9: The chemical structures of lathyrane diterpene esters identified in methylene chloride fractions of *E. paralias* and *E. geniculata*

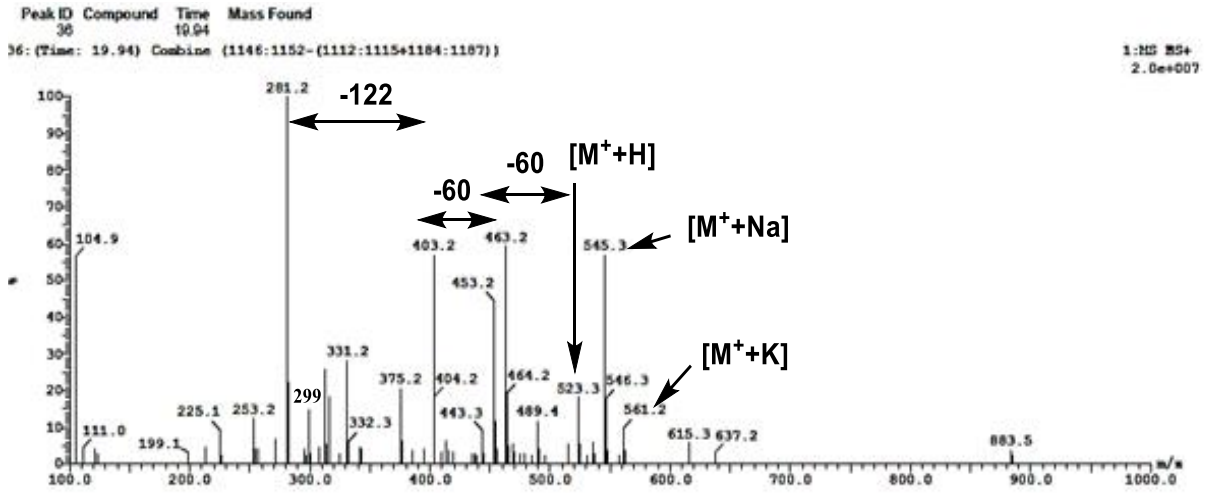
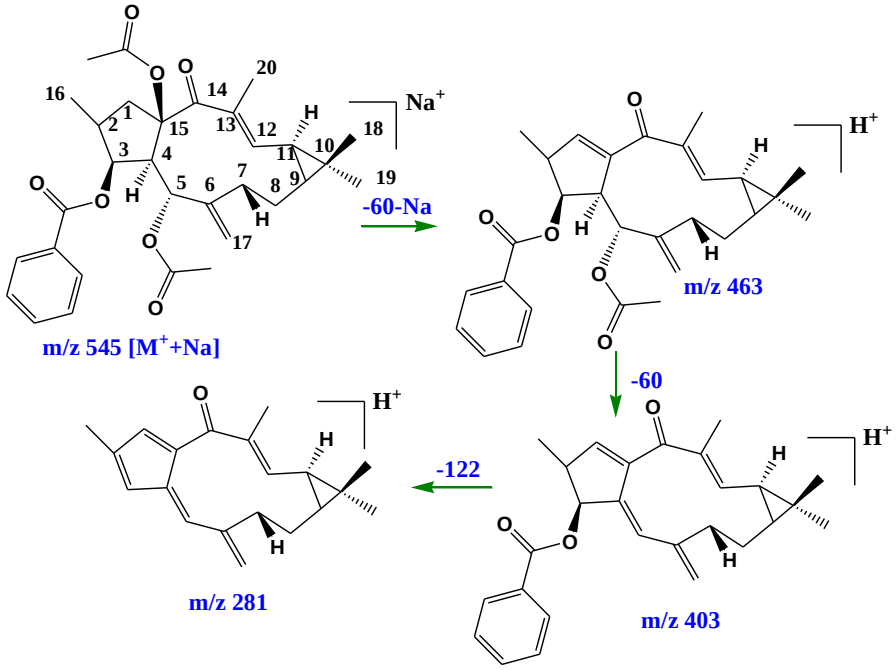


Figure 10: Positive ESI-MS/MS spectrum of Euphorbia factor L_3 (peak 32 *m/z* 545 [M^++Na])



Scheme 6: Characteristic ESI-MS/MS fragmentation pattern of Euphorbia Factor L_3 in positive ion mode

Jatrophane diterpene:

Only one Jatrophane diterpene was determined in *E. paralias*. Compound 18 (R_t of 14.16 min) (Table 1) was identified as 3,5,7,8,15, pentacetyl-2-hydroxy-9,14-dioxo-jatropha-6(16)-11(12)-diene with molecular ion peak at m/z 647 [M^++K], 631 [M^++Na] and MS^2 fragmention displayed several fragment ions for loss of several acetic acid moieties at *m/z* 591 [M^++H-H_2O], 549 [M^++H-H_2O-$COCH_3$], 531 [M^++H-2H_2O-CH_3COOH], 489 [549-60(CH_3COOH)] 471[M^++H-H_2O-2CH_3COOH], 429 [489-60 (CH_3COOH)] and 411[M^++H-H_2O-3CH_3COOH]. All these fragment ions showed the probability of loss of several acetyl groups as acetic acid (60 amu) or as acetyl group (42 amu). Furthermore, fragmentation showed fragment ions at *m/z* 387. 369, 341, 327, 309, 299 and 281 corresponding to the residual jatrophane nucleus which are in a good agreement with the previously reported data of 3, 5, 7, 8, 15-pentaycetyl-2-hydrox-9,14 dioxo-jatropha-6(16)-11(12)-diene (Fig. 11 & 12 (A)) **[13].**

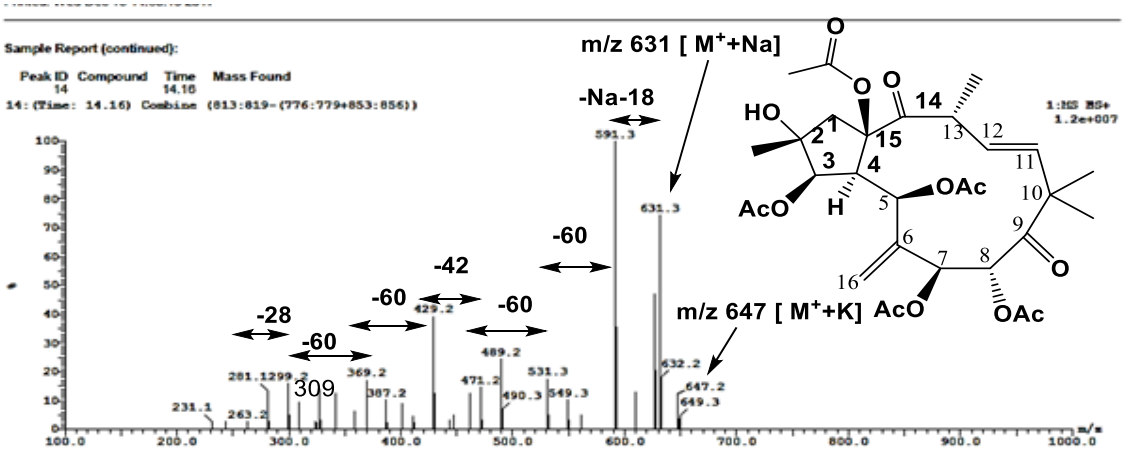


Figure 11: Positive ESI-MS/MS spectrum of 3, 5, 7, 8, 15- pentacetyl-2-hydroxy- 9, 14 dioxo-jatropha-6(16)-11(12)-diene (peak 18 *m/z* 631[M^++Na])

Daphnanes diterpenes:

Resiniferatoxin was the only daphnanes diterpene identified in *E. geniculata*. This compound is reported to have a prolonged effect on relief of spasm and pain and more potent than capsaicin with reduced side effects at nontoxic dose and may therefore be a better therapeutic option **[15].** Compound 50 (Rt. 28.30 min) gave [M^++ Na] at *m/z* 651 in the MS/MS spectrum (Fig 12 (B)) yields the diagnostic transition corresponding to the loss of $C_7H_7O_2$ moiety at *m/z* 507 [M^++H-$C_7H_7O_2$], in addition to fragment ion at *m/z* 371 [M^++H-$C_9H_9O_4$-C_6H_5] and *m/z* 355 [M^++Na-$C_9H_9O_4$-C_7H_7] which showed loss of $C_9H_9O_4$ for 4-hydroxy-3-methoxyphenyl acetic acid and C_7H_7 for benzyl moiety, respectively. Also MS^2 fragment at *m/z* 333 [M^++H-$C_9H_9O_4$-C_7H_7], comparing these results with the reported literature **[13]** peak 50 was tentatively identified as resiniferatoxin (Fig. 13).

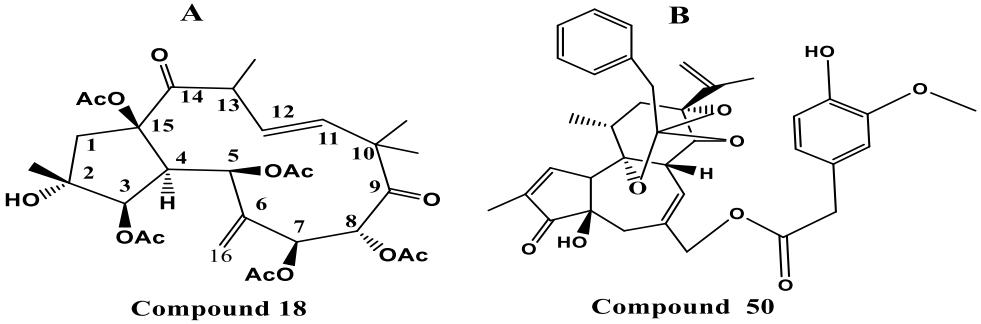


Figure 12: The chemical structures of jatrophane (A) and daphnane (B) diterpenes identified in methylene chloride fractions of *E. paralias* and *Egeniculata*

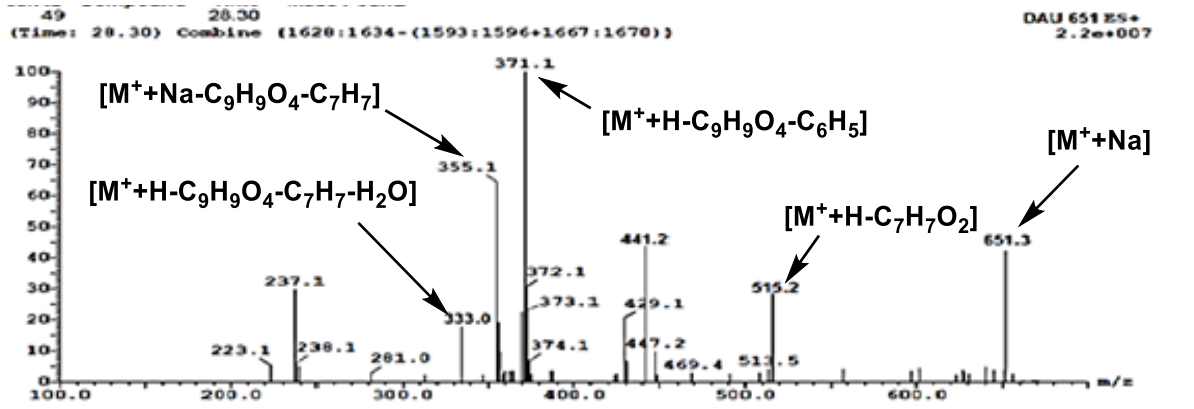


Figure 13: Positive ESI-MS/MS spectrum of resiniferatoxin (peak 50, *m/z* 651 [M$^+$+Na])

Discussion:

According to the characteristic fragmentation pathways of the detected diterpenoids in comparison with literature, totally 52 compounds were preliminary identified for the first time in the methylene chloride fractions of *E. paralias* (30 compounds) and *Egeniculata* (36 compounds). It is noteworthy to say that lathyrane diterpenes and jatrophane diterpene were determined only in *E. paralias* and were absent in *Egeniculata* while daphnane diterpene was determined only in *Egeniculata.* Moreover, the tigliane and ingenane diterpenoids were determined in both Euphorbia species. The compounds determined in both plants were reported to have powerful anti-chikungunya virus (anti-CHIKV) activity **[2].** In particular compounds 2, 6, 9, 14, 16, 21, 23, 24, 26, 31, 38 and 42 proved to be the most promising anti-CHIKV agents till now. Chikungunya virus is spread to people by bite of infected mosquito this cause fever, joint pain and swelling, headache, muscle pain, rash and there is no vaccine to prevent this viral infection. Thus, *E. paralias* and *E. geniculata* offer interesting opportunity as potential therapeutic agents for anti-chikungunya virus.

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