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ORIGINAL ARTICLE

Modulation of the biosynthesis of oxyprenylated coumarins in calli from *Ferulago campestris* elicited by ferulic acid

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Abstract
Previous and recent literature acquisitions suggested that cultured calli are an efficient and meaningful model to investigate the extent and fate of prenylation of phenylpropanoid cores in plants belonging to the Rutaceae and Apiaceae families upon administration of putative biosynthetic precursors. To this concern, in the present manuscript, we investigated the effect of supplementation of ferulic acid and umbelliferone on the biosynthesis of their oxyprenylated counterparts in *Ferulago campestris* (Besser) Grecescu (Fam. Apiaceae) cultured calli. Dried plant biomass material has been extracted by an ultrasound-assisted extraction with EtOH. *O*-prenyl secondary metabolites, namely 3,3-dimethylallyloxy, geranyloxy, and farnesyloxy derivatives of both ferulic acid and umbelliferone, were identified and quantified by HPLC/DAD analyses. Supplementation with ferulic acid was the only treatment providing appreciable results. Quite surprisingly, its addition to cultured calli did not affect the formation of its oxyprenylated phytochemicals but boosted the biosynthesis of umbelliferone and its farnesyloxy-derivative umbrelliprenin. The findings reported herein enforce and underline the role of ferulic acid as an elicitor of selected classes of secondary metabolites in apiaceous species, as recently observed. In addition to these results, a novel hitherto unknown metabolite from *F. campestris*, namely 7-[[(2*E*)-7-hydroxy-3,7-dimethyl-2-octen-1-yl]oxy]-2*H*-1-benzopyran-2-one, was characterized by NMR and LC–MS analyses.

Keywords *Ferulago campestris* · Ferulic acid · *O*-prenyl coumarins · Umbelliferone · Umbelliprenin

1 Introduction

Prenylation is a metabolic reaction widely spread in the natural kingdom, in particular in terrestrial and marine plants, in fungi, protozoa, and bacteria. This biosynthetic step provides secondary metabolites of mixed origin featured by a structure in which one or more 3,3-dimethylallyl, monoterpenyl, and/or sesquiterpenyl skeletons are linked to oxygen, nitrogen, and/or carbon atoms of phenylpropanoid, polyketide, and alkaloid cores [1]. The interest towards such rare phytochemicals has been increasing during the last two decades thanks to their valuable and promising pharmacological properties as anti-diabetic, anti-lipemic [2], neuroprotective [3], cancer chemopreventive [4], and anti-inflammatory [5] agents. Furthermore, acquisition from the recent literature reveal that the addition of terpenoid moieties render such secondary metabolites more biologically efficient than the parent unprenylated samples [6]. While structural, phytochemical, and pharmacological characterization studies on this class of rare natural products have been published during the last three decades, few data are currently at disposition about the description of their biosynthetic pathways, and the factors influencing them. Such investigations have been better performed handling cultured plant calli rather than the plant itself. Biomasses obtained from calli in fact have been largely employed to these purposes in recent years. Notable example to this concern includes the characterization of phenylpropanoid biosynthesis in *Camellia sinensis* (L.) O. Kuntze (Fam. Theaceae) [7], that of picrosides in *Picrorhiza kurroa* Royle ex Benth (Fam. Plantaginaceae) [8], and of capsaicinoids in *Capsicum chinense* Jacq. (Fam.

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Solanaceae) [9] [10]. In particular, during recent years, we focused our attention in studying how the supplementation of putative phenylpropanoid precursors can influence the type and the extent of the biosynthesis of some oxyprenylated cinnamic acid and coumarin derivatives. In this context, we have shown that the supplementation of ferulic acid, umbelliferone, p-cumaric acid, and partially prenylated boropinic acid to cultured calli of dill (*Anethum graveolens* L.) was effective in leading to increase or suppress geranylation and/or elongation of oxyprenylated side chains [11, 12]. To this aim, we wish to report herein the effects evoked by the addition of ferulic acid **1** and umbelliferone **4** to cultured calli of *Ferulago campestris* (Besser) Grecescu (Fam. Apiaceae), followed by extraction of the derived biomasses. Both compounds **1** and **4** are thought to be precursors of known biologically active *O*-prenyl cinnamic acids, like boropinic acid **2**, 4′-geranyloxyferulic acid **3**, and coumarins like 7-isopentenyloxycoumarin **5**, auraptene **6**, and umbelliprenin **7**. The importance and great potential of all these five oxyprenylated secondary metabolites have been recently exhaustively reviewed [13–16]. Furthermore, studies like the one described herein and the few ones published in the last 5 years are of great importance to explore the possibility and to claim biomasses from cultured calli as a more convenient, easier to handle, and alternative route to obtain higher quantities of oxyprenylated secondary metabolites. Finally, we were also able to characterize a novel hitherto unknown metabolite from *F. campestris*, identified as 7-[[(2*E*)-7-hydroxy-3,7-dimethyl-2-octen-1-yl]oxy]-2*H*-1-benzopyran-2-one **8** by NMR and LC–MS analyses (Fig. 1).

2 Material and methods

2.1 Chemicals

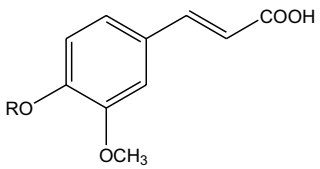
Boropinic acid **2**, 4′-geranyloxyferulic acid **3**, 7-isopentenyloxycoumarin **5**, auraptene **6**, and umbelliprenin **7** have been synthesized as previously reported [17] and their purity (> 98.7%), assessed by HPLC. Ferulic acid and umbelliferone, CH_3CN, H_2O, MeOH, and EtOH (all HPLC grade), HCOOH, all reagents employed for culturing calli, and thin layer chromatography (TLC) plates (Analtech Uniplate, SiO_2 gel GF, 20 × 20 cm, 500 µm) for preparative chromatography were purchased from Merck Sigma-Aldrich (Milan, Italy).

2.2 Plant material and extraction procedures

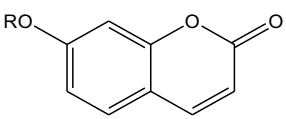
Calli of *F. campestris* have been obtained from sterile 1-month-old plant stems collected in June 2019 in Cesane Park, Urbino, Central Italy, 600 m above sea level (GPS coordinates N 43° 30′ 56″ E 12° 49′ 13″). The plant was classified by D. Fraternale. A voucher specimen (FCS01) has been stored in the Herbarium of the Botanical Garden of Urbino University "Carlo Bo." Stems were sterilized by immersion in a $HgCl_2$ 0.1% aqueous solution for 5 min., and then rinsed 5 times with sterile bi-distilled H_2O. After drying in sterile filter paper, stem explants, with a thickness of 2 mm, were placed in Petri dishes (∅ = 9 cm) containing 30 mL of MS culture medium plus 30 g/L sucrose and 4.52×10^{-7} M 2,4-dichlorophenoxyacetic acid at pH 5.8, and cultured in the dark for 28 days at 25 °C. Subsequently, small portions of cultured calli were transferred into the same Petri dishes as above containing 30 mL of the same culture medium separately added with 30 mL of 10^{-5} M solutions of ferulic acid and umbelliferone. All samples were then cultured for further 28 days adopting the same experimental conditions. Finally, calli were collected, frozen, lyophilized, and the resulting biomass extracted by an ultrasound-assisted extraction for 15 min at 40 W using EtOH as the solvent. After its removal to dryness by evaporation under vacuum, the resulting powder was re-suspended in MeOH, filtered through a 0.22-µm pore size Durapore® membrane (Merck Sigma-Aldrich, Milan, Italy), and an aliquot of 20 µL was injected into the HPLC apparatus for subsequent analyses.

2.3 *HPLC analysis and method validation: ^{1}H NMR, ^{13}C NMR, and HRMS analyses*

The same apparatus as already described was used to accomplish HPLC/DAD analyses [18]. Each chromatographic run was done in triplicate. The mobile phase consisted of a H_2O—HCOOH (99.6–0.4%) (solvent A) and CH_3CN—HCOOH (99.6–0.4%) (solvent B) mixture. The flow rate



1 R = -H
2 R = 3,3-dimethylallyl
3 R = geranyl



4 R = -H
5 R = 3,3-dimethylallyl
6 R = geranyl
7 R = farnesyl
8 R = $-OCH_2CH{=}C(CH_3)CH_2CH_2C(CH_3)_2OH$

Fig. 1 Structure of herein investigated secondary metabolites

was 1.2 mL/min. The gradient was changed over time as the following: 0.0–18.0 min, 60% A–40% B, 18.01–22.0 min from 40 to 80% B, 22.01–25.0 min from 80 to 40% B, 25.01–33.0 min 40% B, 33.01–38.0 min from 40 to 100% B, 38.01–50.0 min 100% B. The column temperature was set at 25 °C. The wavelength for the determination compounds **1–7** was set at 322 nm. Stock solutions of pure chemical standards for the calculation of calibration curves were prepared by dissolving 3 mg of each analyte into 3 mL of MeOH and storing all samples in glass-stoppered bottles at 4 °C in the dark. Standards for calibration curves and quality control (QC) samples, at concentration of 0.10, 0.50, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, and 100.0 µg/mL, were prepared by appropriate dilution of the stock solutions in MeOH mentioned before. After having optimized chromatographic conditions, the HPLC/DAD method was validated in terms of precision, accuracy, linearity, limits of detection (LODs), and limits of quantification (LOQs) according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) recommendations. These and other relevant analytical parameters are listed in Table 1.

The intra-day precision of the analytical method we set up was determined and validated by the injection of standard solutions 5 times a day applying the same optimized experimental conditions as described above. For what concerned the inter-day precision, measurements were carried out once a day on 3 consecutive days. All the precision measurements were expressed as relative standard deviations (RSDs) and ranged from 0.18 to 1.23. Precision was calculated at 3 concentration levels, namely quality control $QC_{Low} = 0.50$ µg/mL, $QC_{Medium} = 5.0$ µg/mL, and $QC_{High} = 100.0$ µg/mL, and assessed following the criteria already reported [18]. Accuracy was determined by spiking the samples obtained after the extraction process with 3 concentrations of the chemical standards (low, medium, and high spikes). LODs and LOQs were obtained by injecting serial dilutions of the corresponding standard solutions, taking the signal-to noise (S/N) ratio of 3.3 and 10 as the criteria, respectively. All analytes herein under investigation demonstrated a very good linearity providing r^2 values from 0.9980 to 0.9997. Statistical analyses were accomplished following the same route as recently reported [18].

^{1}H and ^{13}CNMR analyses have been performed following the same general procedure as already described [17]. For high-resolution mass spectrometry (HRMS) analyses, a final concentration of 50 µg/mL of the investigated compound was prepared by its dissolution in a mixture of CH_3CN/H_2O in a 70:30 ratio containing HCOOH 0.1%. This solution was then injected into the mass spectrometer apparatus through a syringe pump at a flow rate of 20 µL/min. The mass spectrometer was a Thermo Fischer Orbitrap Fusion™ Tribrid™ operating in positive mode MS scan in the m/z range of 50 to 1000 m/z by using the Orbitrap as detector type at 120,000 of mass resolution (FWHM). Precursor was isolated by the quadrupole for subsequent HCD fragmentation using a collision energy of 30%.

7-[[(2*E*)-7-hydroxy-3,7-dimethyl-2-octen-1-yl]oxy]-2*H*-1-benzopyran-2-one (8): white solid, m.p. 115–116 °C. IR (cm^{-1}) 1655. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.23 (s, 3H), 1.26 (s, 3H), 1.26–1.31 (m, 2H), 1.68 (s, 3H), 2.11–2.14 (m, 2H), 4.52–4.54 (m, 2H), 5.46–5.50 (m, 1H), 6.16 (d, 1H8, J = 7.9 Hz), 6.73–6.76 (m, 2H), 7.25–7.29 (m, 1H), 7.52 (d, 1H8, J = 7.9 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 16.3, 28.3, 28.4, 32.2, 39.5, 65.0, 67.6, 101.3, 112.6, 112.7, 121.5, 128.3, 143.0, 143.3, 155.4, 160.8, 161.3

3 Results and discussion

Cultured plant cells and tissues represent a novel and alternative method for the production in good to excellent yields of active principles and a wide range of other phytochemicals in general, not only for phytotherapeutic purposes, but also for cosmetic and nutraceutical aims [19–21]. We have recently shown how using this approach, the production, and

Table 1 Main HPLC parameters set-up in the present study

Entry	Rt (min.)	Linearity range*	Slope (a)	Intercept (b)	r^2	LOD*	LOQ*
1	2.70	0.10–100.0	119.8	43.37	0.9992	0.035	0.10
4	3.02	0.10–100.0	141.1	15.54	0.9980	0.017	0.10
2	12.37	0.10–100.0	103.9	41.54	0.9987	0.045	0.14
3	24.89	0.10–100.0	72.01	− 5.594	0.9997	0.020	0.10
5	21.89	0.10–100.0	109.8	− 39.66	0.9990	0.040	0.12
6	38.89	0.10–100.0	89.61	76.27	0.9993	0.032	0.10
7	41.16	0.10–500.0	66.85	− 28.13	0.9994	0.031	0.10

Rt retention time

*Values expressed as µg/mL

[a]For each curve the equation is $y = ax + b$, where y is the peak area, x the concentration of the analyte (µg/mL), a is the slope, b is the intercept, and r^2 the correlation coefficient. The p value was < 0.0001 for all calibration curves

biosynthesis of biologically active oxyprenylated ferulic acid and umbelliferone derivatives boropinic acid **2**, 4′-gernayloxyferulic acid **3**, 7-isopentenyloxycoumarin **5**, auraptene **6**, and umbelliprenin **7** by *Anethum graveolens* L. (dill, Fam. Apiaceae) could be enhanced and finely modulated upon supplementation to cultured calli derived from selected part of this plant by supplementation of the parent phenolic precursors [11, 12]. The encouraging results obtained in these previous studies prompted us to investigate the possibility to obtain the same *O*-prenyl phytochemicals from an alternative source, represented by cultured cells from *F. campestris* belonging to the same plant family, and possibly to get further insights into the effect of supplementation on the biosynthetic fate of the same natural products. In particular, we assessed the influence of the addition to culture media of ferulic acid **1** and umbelliferone **4** on their own prenylation. Lyophilized calli powders of *F. campestris* were finally extracted by an ultrasound assisted procedure for 15 min (performed in triplicate for each sample) with EtOH as the solvent. Qualitative and quantitative determination of secondary metabolites of interest in all extracts were accomplished by RP-HPLC/DAD analyses. The corresponding data are reported in Table 2**.**

Ferulic acid **1**, umbelliferone **4**, and umbelliprenin **7** were identified in the EtOH extract from untreated calli. The presence of umbelliprenin as the only oxyprenylated phenylpropanoid even in cultured cells extracts is in line with literature data indicating how *F. campestris* is among the main natural sources of this oxyprenylated coumarin [22]. Umbelliferone supplementation led to a slight increase of geranylation (providing auraptene **6** not detected in control samples) and farnesylation (providing umbelliprenin **7**, around 2.5-fold increase) of the same core. These set of data suggest that compound **4** can elicit also geranylation of phenolics as a metabolic biosynthetic step in *Ferulago* spp. other than farnesylation, so far indicated as "typical" of this genus by a survey of literature data [23]. Data obtained upon supplementation with ferulic acid were quite surprising. No elicitation of the corresponding *O*-prenylated metabolites have been recorded, but an increase in the biosynthesis of umbelliferone (around fivefold) and a massive one of umbelliprenin (around 211-fold) were observed. This sounds as an adaptive response by *F. campestris* leading to an enhancement in the metabolic rate of production of hydroxylated coumarins, and, of more interest, selectively of their farnesylation step. Such elicitation properties by ferulic acid have been revealed for other plants, leading to the acceleration in the biosynthesis of a wide range of antioxidant secondary metabolites including flavonoids and carotenoids [24, 25]. Also, in our previous investigations with cultured calli of *A. graveolens*, ferulic acid exhibited quite a similar trend providing an increase, although to a less extent, of geranylation of umbelliferone [12]. Sample HPLC chromatograms of extracts deriving from untreated calli and calli supplemented with ferulic acid illustrating the findings described above are reported in Fig. 2.

The substantial and main novelty of the present investigation was the structural characterization of a new chemical entity from cultured calli biomass related to a not attributable peak observed in the HPLC chromatogram with a Rt = 26.45 min. A preliminary screening was carried out by TLC analyses of the crude EtOH extract allowing to detect a strong blue-green fluorescence spot (indicating the presence of a coumarin ring) with a Rf of 0.36, not corresponding to any of the pure standards **1–7** employed in the present investigation. The purification and isolation of such an unknown compound was carried out by preparative TLC adopting a 95:5 CH_2Cl_2/MeOH as the eluting mixture. The amount of the EtOH extract processed was 20 mg. The desired spot was scraped, separated by desorption with the same mixture of solvents as above, and filtered. The white solid so obtained was then characterized by ^{1}H and ^{13}C NMR, and HRMS. Fragment ions from this latter were scanned in the ion trap (AGC target value of 100, MIT of 100 ms). The MS fragmentation pattern is illustrated in Fig. 3.

Data reported in Fig. 2 showed the most significant molecular ions at m/z 298.2733, 255.2312, 106.0861, 88.0757, and 70.0652, which suggest the formation of an auraptene derivative with an OH group in the geranyloxy chain. The most relevant peak in the mass spectra at m/z 298.2733 is linked to the molecular weight of auraptene while the base peaks at m/z 88.09 and 70.0652 are related to the presence of isopentenyl moieties with and without -OH. The presence of a hydroxyl group in the geranyloxy chain was first hypothesized to be located on C-7′. This was confirmed by data obtained from the ^{13}C NMR spectrum indicating the presence of two CH_3 on a quaternary aliphatic carbon at 67.6 ppm. Thus, the

Table 2 Quantification of compounds **1–7** in *F. campestris* cultured calli (mg/g dry weight ± SD, $n = 3$)*

	1	4	2	3	5	6	7
Ctrl	11.1 ± 0.4	6.4 ± 0.23	–	–	–	–	12.2 ± 0.9
+1	300.2 ± 1.1	270.5 ± 1.5	–	–	–	–	2580.6 ± 2.0
+4	–	30.2 ± 0.5	–	–	2.1 ± 0.1	–	40.4 ± 1.4

– not detected or below LOQ, *Ctrl* untreated controls, *+1* supplementation with ferulic acid, *+4* supplementation with umbelliferone

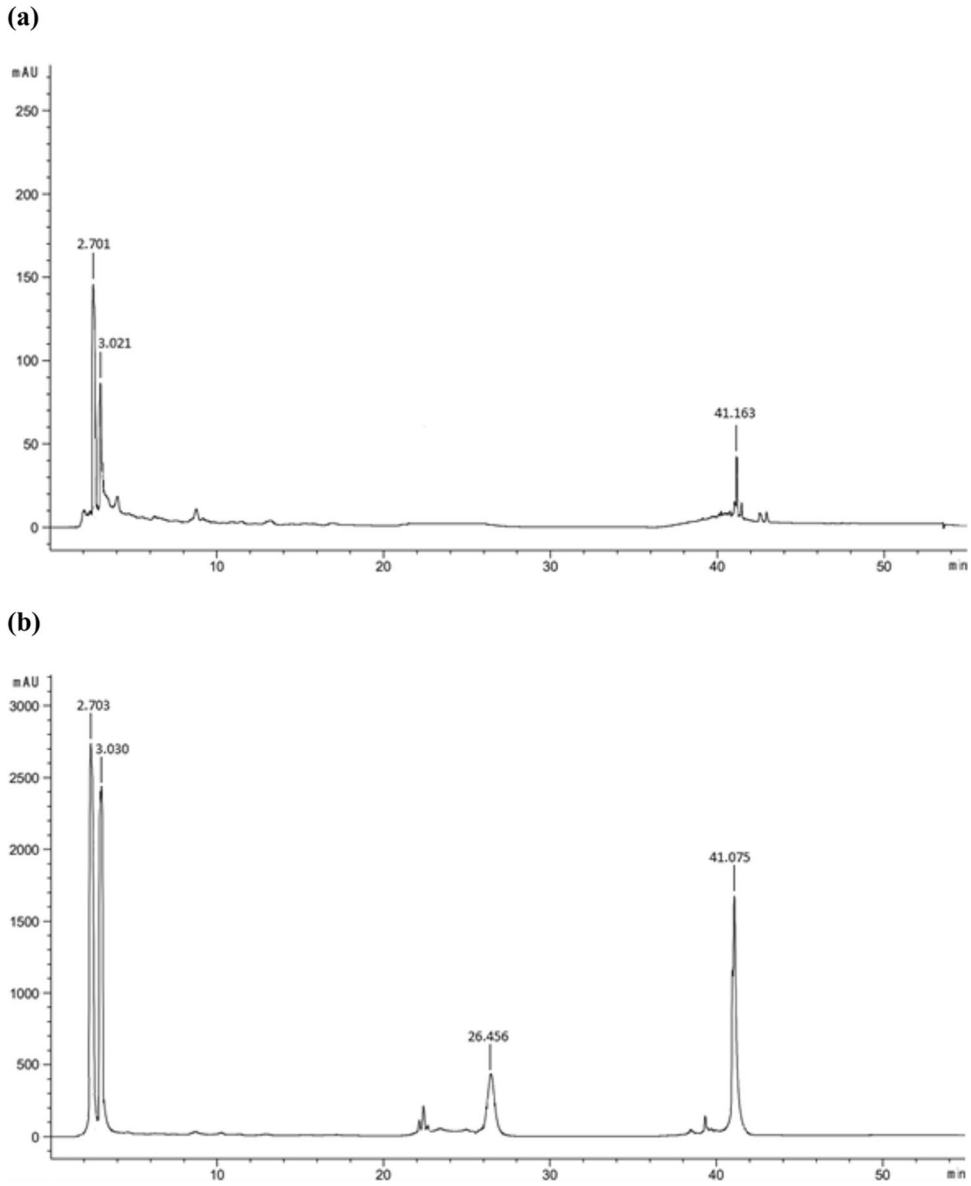


Fig. 2 Sample HPLC chromatograms of extracts from untreated calli (**a**) and calli treated with ferulic acid (**b**)

formula 7-[[(2*E*)-7-hydroxy-3,7-dimethyl-2-octen-1-yl] oxy]-2*H*-1-benzopyran-2-one was proposed for compound **8**. This oxyprenylated coumarin is reported herein for the first time in the literature and represents a unique and an additional structure of the phytochemical pool of oxyprenylated secondary metabolites so far characterized from natural sources.

4 Conclusions

As a conclusion in this manuscript, we described the role of ferulic acid as an elicitor of prenylation of phenylpropanoid cores in *F. campestris*-cultured calli. The acquisition that this compound selectively provide a huge enhancement of the biosynthesis of umbelliprenin could represent a strong rationale to get an experimental route for the production on larger scale and more interestingly from renewable and easy to handle vegetable sources of this biologically active, therapeutically promising, and valuable phytochemical. It has to be reminded in fact in this context that umbelliprenin, like in general all oxyprenylated phenylpropanoids, is quite rare in nature and the provision of even few grams of this compound requires the use and extraction of a great mass of plant material. The one indicated in the present investigation can be a valid and alternative solution to this potential drawback. Furthermore, handling biomasses from cultured calli of *F. campestris* allowed to isolate and structurally characterize a novel natural oxyprenylated secondary metabolite that was not recorded so far in any of previous phytochemical studies about *Ferula* species. Studies to optimize the extractive yields of this new compound from calli derived biomasses

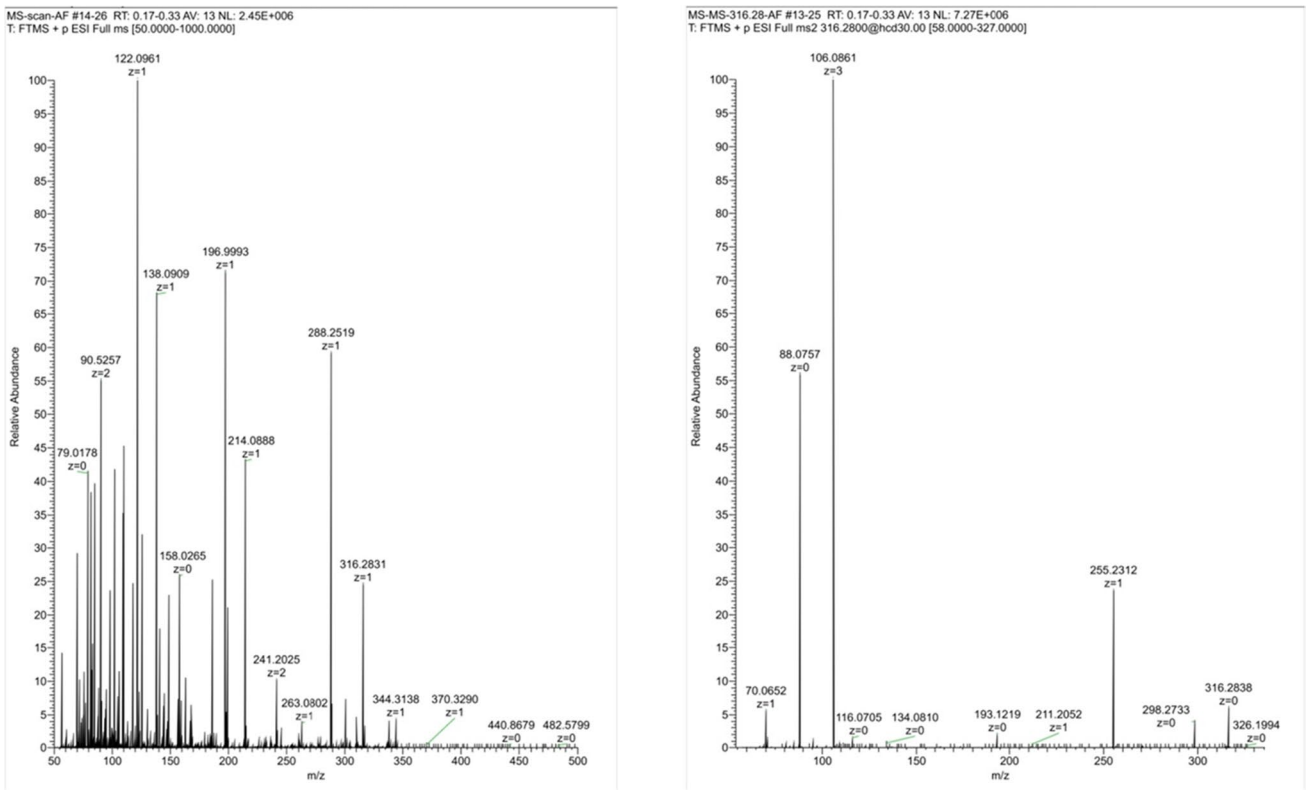


Fig. 3 Full scan acquisition in positive mode and fragmentation of 316.2831 m/z using a collision energy of 30%

also in order to get a preliminary characterization of its pharmacological properties are now in course in our laboratories.

Author contribution All authors equally contributed to this study.

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Data availability Data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval Not applicable.

Competing interests The authors declare no competing interests.

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