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A fast and efficient LC–MS/MS method for detection, identification and quantitative analysis of bioactive sesterterpenes in *Salvia dominica* crude extracts

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ABSTRACT

Sesterterpenes are a small group of terpenoids showing a number of interesting pharmacological properties, including cytotoxicity, anti-inflammatory, anti-microbial and anti-angiogenic activities and platelet aggregation inhibition. Recently, some sesterterpene lactones isolated from *Salvia dominica* have been shown to modulate enzymatic activity of tubulin tyrosine ligase (TTL), a promising target for new anti-cancer therapeutic strategies. However, to allow a direct use of *S. dominica* extracts as a source of TTL inhibitors, analytical method aimed to their fast qualitative and quantitative characterization is required. Despite the structural features and diverse biological activities of sesterterpenoids, actually no analytical method for their quantization into complex mixtures has been published. Here we describe an LC–MS/MS method aimed to qualitative and quantitative analysis of sesterterpenes lactones in the crude extracts obtained from different parts of *S. dominica*. This approach allowed us to characterize all the sesterterpenes by a single step analysis and also to identify two unknown compounds. Moreover, a quantitative comparison of the composition in sesterterpenes of extracts obtained from *S. dominica* leaves, roots and leaf galls was performed, leading to the definition of both leaves and leaf galls as suitable sources of TTL inhibitors.

1. Introduction

Sesterterpenes are a small group of terpenoids, obtained from widespread sources having been isolated from terrestrial, lichens, higher plants, insects and various marine organisms. Sesterterpenes show a number of interesting pharmacological properties, including cytotoxicity, anti-microbial and anti-angiogenic activities, and platelet aggregation inhibition; however the anti-inflammatory activity is outstanding and dominating feature in this compounds class [1,2]. The structural features and diverse biological activities of sesterterpenoids have made them attractive targets for both biomedical and synthetic purpose [3].

A small group of these compounds have been isolated from plant belonging to *Salvia* genus, many of which are medicinal plants included in some pharmacopoeias, and that are also used for alimentary and cosmetic purposes [4–9]. Previous studies performed by our research group on secondary metabolites of *S. dominica* led to the isolation of 22 new sesterterpene lactones (Fig. 1) [10]. Chemical–biological studies carried out on these compounds showed that some of them interact with tubulin tyrosine ligase (TTL), an enzyme involved in the tyrosination cycle of the C-terminal of tubulin, inhibiting its activity into the cells [11,12]. TTL has been demonstrated to play a key role in many physiological processes and to be essential for neuronal organization [13]. Moreover, misregulation of tyrosination/detyrosination cycle of tubulin, frequently observed during cancer progression, is associated with increased tumour aggressiveness [14–16]. Thus, TTL could be a target for developing novel therapeutic strategies against cancer, since modulators of TTL, by restoring normal Tyr-tubulin, could impair tumour progression [13,16]. Moreover, our studies demonstrated that inhibitory activity of those compounds towards TTL is strongly affected by their own structural characteristic [10]. Therefore, to allow a direct use of *Salvia dominica* extracts as a source of TTL inhibitors, analytical method aimed to their fast qualitative and quantitative characterization is required. In this light, we carried out the present work in an effort to obtain sesterterpenes with different structural features from various *S. dominica* organs.

The *Salvia* ssp. sestertepene lactones are characterized by an α–β unsaturated butenolide moiety, by a bicyclic portion carrying a variable number of hydroxyl groups, and by a side chain connecting them. The various compounds mainly differ in the substituents at C-4, C-6, C-8 of bicyclic part, and in the oxidation level of the

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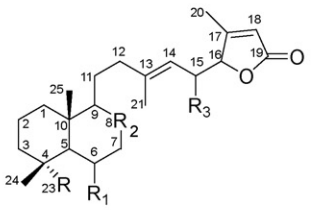
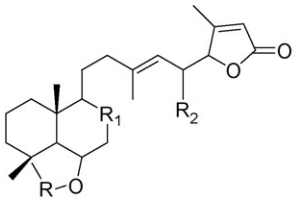
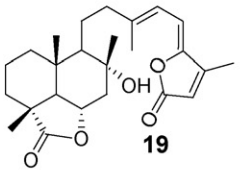
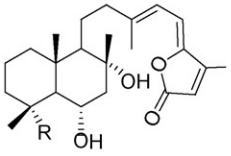


	R	R_1	R_2	R_3
1	$-CH_2OH$	-OH	OH	-OH
2	$-CH_2OH$	-OH	OH	-H
3	$-CH_3$	-OH	OH	-OH
4	-COOH	-OH	OH	-OH
5	-COOH	-OH	OH	-H
6	-CHO	-OH	OH	-OH
7	-CHO	-OH	OH	-H
13	$-CH_2OH$	-OH	=	-OH
14	-CHO	-OH	=	-H

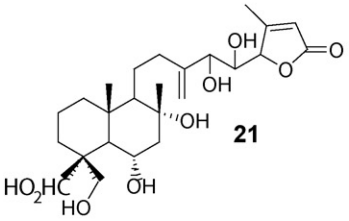


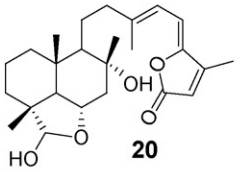
	R	R_1	R_2
8	-CHOH	OH	-OH
9	-CHOEt	OH	-OH
10	-CHOEt	OH	-H
11	$-CH_2$	OH	-OH
12	-CO	OH	-OH
15	-CHOH	=	-OH





	R
16	$-CH_2OH$
17	-COOH
18	-CHO





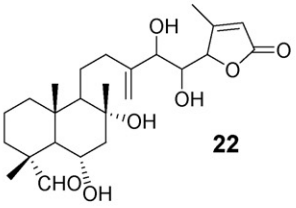


Fig. 1. Structures of compounds **1**–**22**.

side chain. Many of these compounds are isomers and show similar chromatographic behaviours; therefore, the development of a fast and efficient analytical approach to identify and quantify all compounds could be quite intricate. Indeed, classical approaches used to analyse complex mixtures of similar compounds consists of multiple steps approaches involving solvent–solvent partitioning, chromatographic separations and spectroscopic techniques [17–19]. Besides, despite the structural features and diverse biological activities of sesterterpenoids, actually no analytical method for their quantization into complex mixtures has been published. We therefore optimized an LC–MS/MS method based on the use of a highly informative post-run data analysis. This approach allowed us to characterize all the previously described components by a single step analysis of *S. dominica* leaves crude extract; moreover, two unknown sesterterpenes lactones were detected, and their structures were attempted on the basis of the MS and MS/MS data. Besides, this analytical method was also used to compare the quali-quantitative composition in sesterterpenes of *S. dominica* extracts from leaves, roots and leaf galls, abnormal growth of plant tissue caused by wound, infections or insects. Therefore, the best plant organ to be used as a source of TTL inhibitors was defined.

2. Materials and methods

2.1. Plant material

The leaves, the leaf galls and the roots of *S. dominica* L. were collected in April 2005 at As-Subayhhi, in Al-Balqa Provine, Jordan, and

identified by Prof. Ammar Bader. A voucher specimen number has been deposited in the Herbarium of Laboratory of Pharmacognosy and Phytochemistry at Al-Zaytoonah Private University of Jordan.

2.2. General experimental procedures

High resolution mass spectra were acquired on a Q-Tof Premier instrument (Waters, Milford, MA, USA), equipped with an electrospray ion source; to achieve high accuracy mass measurements, both external and internal calibrations of the spectrometer were performed using amentoflavone (molecular mass 538.0900) as standards. A system including a Surveyor Autosampler, Surveyor LC pump LCQ Advantage ion-trap mass spectrometer (ThermoFinningan, San Jose, CA, USA) was used for ESIMS and LC/MS^n analyses.

2.3. Chemicals

Acetone applied for the extraction was of analytical reagent quality (minimum 99.5%), purchased from Carlo Erba, Milano, Italy. HPLC grade water was prepared with a Millipore (Billerica, MA, USA) Direct Q5 equipment. Solvents employed for the sample preparation and for LC–MS analysis were of HPLC super gradient grade quality (Romil Ltd., Cambridge, UK). Solvents prepared for LC–MS analysis were additionally filtered through 0.45 μm (Millipore) membrane. Pure compounds used to optimize MS and MS/MS conditions were purified as reported elsewhere [7]; their purity was verified by HPLC/UV/MS analyses and it resulted ≥95% for all the used compounds. Amentoflavone used as HPLC standard to perform LC–MS/MS quantitative analyses was purchased from Sigma–Aldrich, Milano, Italy.

2.4. Sample preparation

A representative amount (1 g) of leaves, roots, and leaf galls were dried, powdered, and extracted at room temperature. Each part dried was defatted with *n*-hexane and successively extracted for 48 h with acetone by exhaustive maceration (4× 20 ml). The yield of acetone extracts of each organ was 55 mg from leaves, 19 mg from roots, and 62 mg from leaf galls. Solutions (3 mg/ml) of each extracts were prepared and 10 μl of each solution were injected for analysis. Triplicate injections were made for each sample.

2.5. LC–MS/MS analyses

LC–MS/MS analyses were performed on a Surveyor HPLC system (Thermo Electron Corporation) coupled with an LCQ Advantage electrospray mass spectrometer. Chromatographic separation was conducted injecting 10 μl (30 μg) of each extract on a C18 Synergy Fusion (15 cm × 2.1 mm, flow rate 0.15 ml/min) column (Phenomenex), using water–formic acid–trifluoracetic acid (97.9:2:0.1, v/v/v) (A) and methanol (B) as mobile phases. Initial ratio of eluents was A–B 60:40 (v/v). In the first 10 min the ratio changed to 35:65 (v/v). This was followed by a linear gradient up to 0:100 (v/v) in 24 min. Mass analyses were performed in positive mode, using a dependent MS/MS function; the following instrumental parameters were used: nitrogen gas temperature 300 °C, drying gas flow rate 9 l/min. Capillary voltage was 4000 V, while fragmentation voltage was 40 V. The full mass scan ranged between *m*/*z* 350 and 500.

2.6. Quantitative analysis

Quantitative LC–MS/MS analyses were performed using amentoflavone ($[M+Na]^+$ ion at *m*/*z* 561.5) as internal standard (IS). 50 ng of IS were added to each sample before of the injection. To perform a quantitative analysis of different compounds into each extract, selected ion chromatograms of the individual species were evaluated. For each analysis, the observed peaks underwent automatic integration and the ratios of the peak areas of investigated compounds to that of the IS were calculated. All experiments were performed in triplicate and the results were expressed as mean ± SEM.

2.7. Validation of the quantitative analysis

2.7.1. Calibration curves

A stock solution containing identical amounts of 10 *S. dominica* sesterterpenes (compounds **1**, **2**, **7**, **13**, **14**, **16**, **18**, **19**, **21**, **22**) was prepared by dissolving the reference compounds in methanol and then diluting it with methanol to appropriate concentrations for achieve calibration curves. Compounds were selected on the basis of their relative abundance into the extracts; attention was paid to include products showing different chromatographic behaviours. Solutions at different concentrations of the 10 analytes were injected in triplicate. Calibration curves were peak area/internal standard area versus injected amount for each analyte.

2.7.2. Limits of detection and quantification

Stock solution containing 10 reference compounds was diluted to a series of appropriate concentrations with methanol and an aliquot of the diluted solutions underwent LC–MS/MS analysis; the limit of detection (LOD) and limit of quantification (LOQ) for each analyte were calculated with corresponding standard solution on the basis of signal-to-noise ratio (S/N) of 3 and 10, respectively.

3. Results and discussion

To optimize a mass spectrometry-based method, analyses of pure compounds were initially performed by ESIMS/MS. In particular, full characterization of fragmentation pattern was performed for compounds **1**, **5**, **6**, **8**, **16** and **21** (Fig. 2): these molecules were selected on the basis of their chemical features, in order to evaluate the effects on fragmentation process of the different substituents at C-4 and of the presence of hydroxyl groups or double bonds into the side chain.

In the MS/MS spectrum of compound **1** (parent ion at *m*/*z* 459 $[M+Na]^+$) ions at *m*/*z* 429, produced by the loss of a formaldehyde molecule, and at *m*/*z* 361, due to the elimination of one 2-furanone, 4-methyl unit, were observed. Besides, a signal at *m*/*z* 217 was detected, corresponding to the whole side chain (C-11–C-21) added of a sodium ion. Ions at *m*/*z* 441, *m*/*z* 423 and *m*/*z* 405 were generated by the elimination of one, two or three water molecules from the parent ion, respectively; the elimination of one or two water molecules from the ion at *m*/*z* 429 produced signals at *m*/*z* 411 and *m*/*z* 393. Collision induced fragmentation of compound **5** (parent ion at *m*/*z* 457 $[M+Na]^+$) produced three diagnostic ions at *m*/*z* 413 (loss of a CO_2 molecule), *m*/*z* 347 (elimination of a 2-furanone, 4-methyl-5-methylen unit) and *m*/*z* 201 (the sodium adducted side chain). Also for this compound, signals due to the elimination of some water molecules were detected (i.e. ions at *m*/*z* 439, *m*/*z* 421 and *m*/*z* 395). MS/MS spectra of the isomeric compounds **6** and **8** were very similar, both showing peaks at *m*/*z* 359 and *m*/*z* 217, but differing in the relative abundance of these ions and in the number of water molecules which can be lost (three in the case of compound **6** and two for compound **8**). Interestingly, neither of these two compounds underwent elimination of functional group at C-4.

Finally, the analysis of CID spectra of compounds **16** and **21** allowed evaluating the effects of structural differences in the side chain on the fragmentation process; indeed, tandem mass spectrum of **16** (parent ion at *m*/*z* 457 $[M+Na]^+$) showed that the

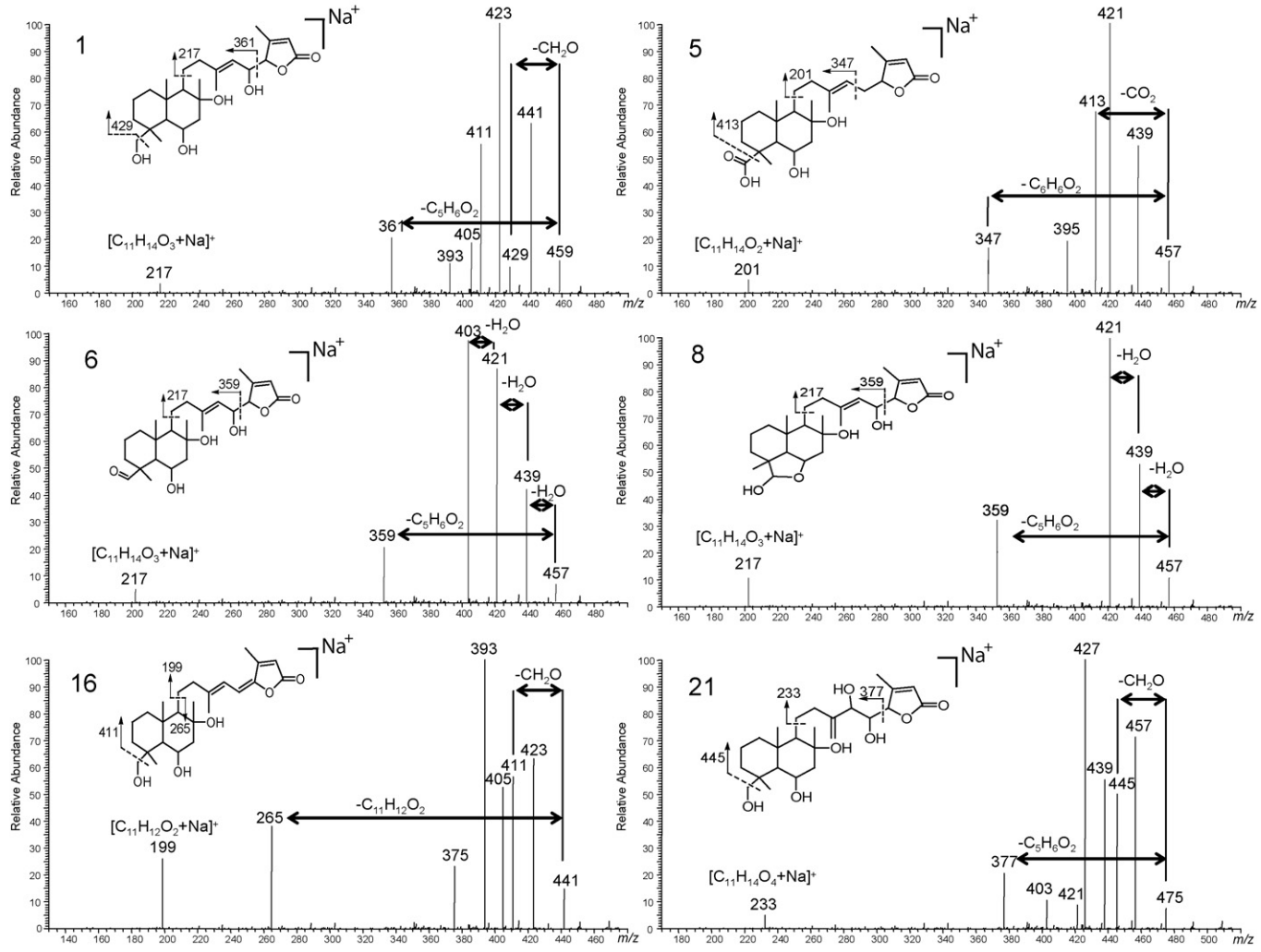


Fig. 2. MS/MS spectra of pure compounds **1**, **5**, **6**, **8**, **16** and **21**. The most informative ions have been described in terms of neutral loss producing them, or indicating their formula.

presence of an highly conjugated double bonds system let a fragmentation event to occur at the C-9–C-11 bond, producing the complementary ions at *m*/*z* 265 (loss of the whole side chain) and 199 (sodium adducted side chain). On the other hand, the presence of two hydroxyl group into the side chain of compound **21** can be deduced by the presence of the product ion at *m*/*z* 233, whereas the observed the elimination of one 2-furanone, 4-methyl unit producing the fragment ion at *m*/*z* 377 was similar to that described in the case of compounds carrying a single hydroxyl group. Both the MS/MS spectra of **16** and **21** showed a $[M\text{-}30+Na]^+$ ion (at *m*/*z* 411 in the case of **16** and at *m*/*z* 445 for **21**) due to the elimination of a formaldehyde unit.

These data, taken together, let to hypothesize some fragmentation rules of *S. dominica* sesterterpenes, allowing to identify the different compounds on the basis of mass spectrometry data. In particular: concerning the substituents at C-4, the presence of a $-CH_2OH$ can be demonstrated by a neutral loss of a formaldehyde molecule (−30 amu) and the presence of a carboxyl group by a loss of carbonic anhydride unit (−44 amu); no elimination was observed when an aldehyde or methyl function was present, or when there was a ring involving the –OH group at C-6 and an aldehyde or a carboxyl group at C-23.

Also the structural features of the side chain can be deduced by MS/MS experiments: in particular, the presence of neutral loss ions at −98 amu reveals the presence of an hydroxyl group at C-15, a neutral loss ion at −110 amu indicates that a single bond occurs between C-15 and C-16, whereas a neutral loss ion at −176 amu occurs when conjugated double bonds are present between C-13 and C-14 and between C-15 and C-16. Moreover, the oxidation level of the region C-11–C-21 can also be estimated on the basis of the *m*/*z* value of the product ion consisting of the sodium cationized side chain: ions at *m*/*z* 199 and *m*/*z* 201 are representative of the presence of no hydroxyl group and of two or of one double bonds, respectively; product ions at *m*/*z* 217 and *m*/*z* 233 are produced by the fragmentation of molecules carrying one or two –OH groups. Finally, the presence of an hydroxyl group on C-6, C-8, C-14 and C-15 can be deduced by MS/MS data, since a water molecule elimination was observed for each –OH group linked to one of these carbon atom.

These observation were therefore used to perform a post-analysis elaboration of the dependent scan LC–MS/MS analysis conducted on the crude acetone extract of the leaves of *S. dominica* (Fig. 3a): 8 new chromatograms were obtained selecting 5 specific neutral loss transitions (i.e. −176, −110, −98, −44 and −30 amu) and 3 product ions (i.e. *m*/*z* 201, 217, 233) (Fig. 3). This approach led to the detection of 25 peaks, all ascribable to sesterterpenes, with the only exception of that at 28 min. Fifteen compounds were detected undergoing a −98 amu neutral loss (Fig. 3b) and then possibly carrying an hydroxyl group on C-15. The fragmentation of 12 of these molecules also generated a product ion at *m*/*z* 217

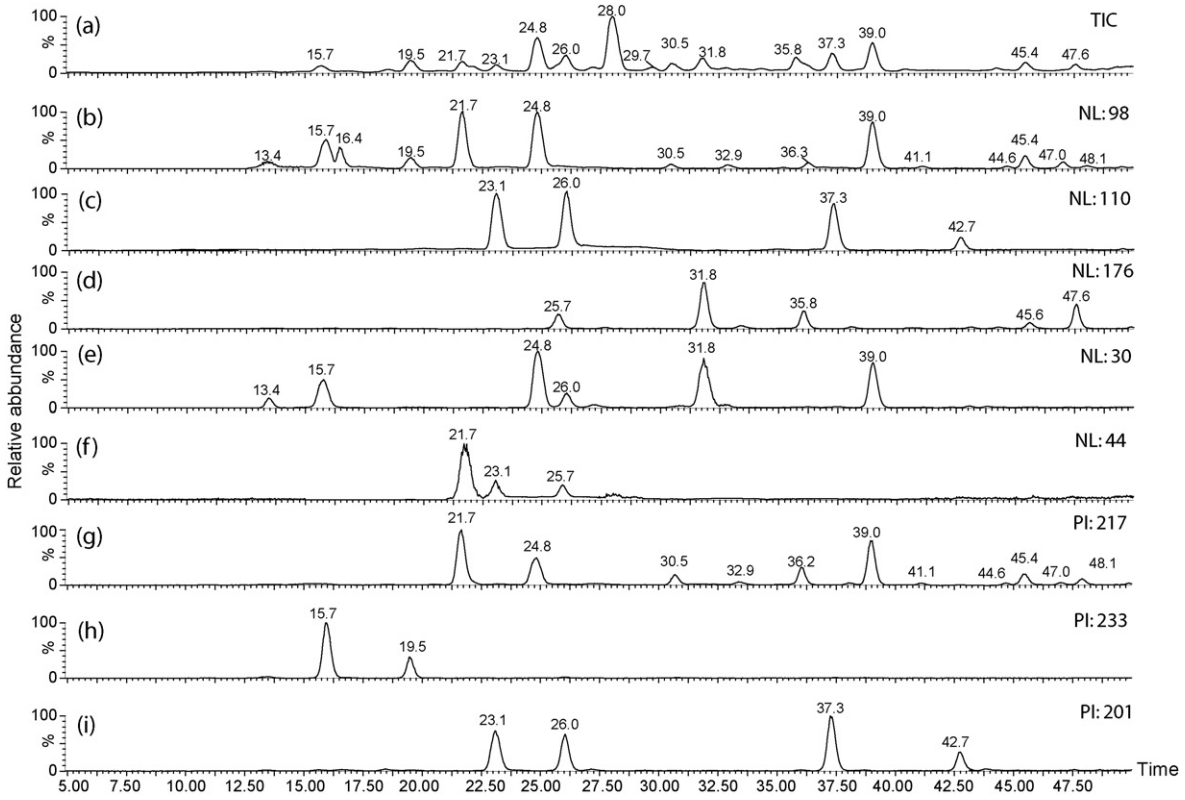


Fig. 3. LC–MS/MS analysis of *S. dominica* leaves crude acetone extract. (a) Total ion current MS chromatogram. (b–f) Chromatographic profiles achieved by a post-analytical selection of the parent ions undergoing a −98, −110, −176, −30 or −44 amu neutral loss during the fragmentation process, respectively. (g–i) Chromatographic profiles achieved by a post-analytical selection of the parent ions producing parent ions at *m*/*z* 217, *m*/*z* 233 or *m*/*z* 201 during the fragmentation process, respectively.

(Fig. 3g), indicating the presence of a single hydroxyl group on their side chain, whereas other two compounds carry two –OH on the C-11–C-21 portion, as inferred by the presence of a signal at *m*/*z* 233 in their MS/MS spectra (Fig. 3 h). No information concerning the side chain features of the compounds eluted at 13.4 and 16.4 min were achieved at this stage. The presence of four compounds characterized by a side chain without hydroxyl groups and a single double bond was inferred by the peaks with a retention time of 23.1, 26, 37.3 and 42.7 min, undergoing a −110 amu neutral loss (Fig. 3c) and generating a product ion at *m*/*z* 201 (Fig. 3i) during the fragmentation process. Finally, five peaks were detected corresponding to compounds showing Δ^{13-16} double bonds system (Fig. 3d). Information concerning the functional group linked to C-4 of some compounds were achieved selecting ions undergoing neutral loss of 30 amu (Fig. 3e), diagnostic of the presence of an alcoholic function, or of 44 amu (Fig. 3f), diagnostic of the

Table 1
Retention time and mass spectrometry data of the sesterterpenes observed in *Salvia dominica* leaves extracts.

Compound	Retention time (min)	$[M+Na]^+$	Main MS/MS fragments (*m*/*z*)
1	24.8	459.3	441.3; 429.3, 423.3; 411.3; 405.3; 361.23; 217.1
2	26.0	443.3	425.3; 413.3; 407.3; 333.3; 201.1
3	32.9	443.3	425.3; 407.3; 389.3; 345.23; 217.1
4	21.7	473.3	455.2; 437.2; 429.3; 419.2; 411.3; 375.2; 217.1
5	23.1	457.3	439.3; 421.2; 413.3; 347.2; 201.1
6	30.5	457.3	439.3; 421.2; 403.2; 359.2; 217.1
7	37.3	441.3	423.3; 405.2; 331.2; 201.1
8	36.3	457.3	439.3; 421.2; 359.2; 217.1
9	41.1	485.3	467.3; 449.3; 387.3; 217.1
10	42.7	469.3	451.3; 359.3; 201.1
11	47.0	441.3	423.3; 405.2; 343.2; 217.1
12	48.1	455.2	437.2; 419.2; 357.2; 217.1
13	39.0	441.3	423.3; 405.2; 411.3; 393.2; 343.2; 217.1
14	45.4	439.2	421.2; 403.2; 341.2; 217.1
15	44.6	439.2	421.2; 341.2; 217.1
16	31.8	441.3	423.3; 405.2; 411.3; 393.2; 265.2; 199.1
17	25.7	455.2	437.2; 419.2; 411.3; 393.2; 279.2; 199.1
18	35.8	439.3	421.2; 403.2; 263.2; 199.1
19	47.6	437.2	419.2; 261.1; 199.1
20	45.6	439.3	421.2; 263.1; 199.1
21	15.7	475.3	457.3; 445.3; 439.3; 427.3; 421.2; 403.2; 377.2; 233.1
22	19.5	473.3	455.2; 437.2; 419.2; 401.2; 375.2; 233.1
23	16.4	493.3	475.2; 463.2; 457.2; 439.2; 421.2; 395.2; 249.1
24	13.4	491.3	473.3; 455.2; 437.2; 419.2; 393.2; 249.1

presence of a carboxyl group; in the former case, six species were detected, whereas three compounds were observed in the latter. To define the C-23 function of the other compounds, it was necessary to take in account both MS and MS/MS data, also in order to evaluate the number of eliminable –OH groups present on each compound. As a result of the analysis of chromatographic and mass spectrometric, 22 of the 25 detected compounds were unambiguously identified (Table 1); on the basis of MS and MS/MS data, peak at 28 min (Fig. 3a) was assigned to rosmarinic acid (observed *m/z* value 383 $[M+Na]^+$), whose presence in *S. dominica* extracts has been previously demonstrated [10].

Compounds eluted at 13.4 min and 16.4 min showed chromatographic and mass spectrometric proprieties suggesting a sesterterpenoidic structure, but they did not correspond to any previously described compound. In Fig. 4 the MS/MS spectra of these species, named compounds **23** and **24**, respectively, are shown. Sodium adducted ion of compound **23** showed an *m/z* value of 493, and its collision induced fragmentation generated fragment ions at *m/z* 463 (−30 amu), indicating a $–CH_2OH$ group linked on C-4, and at *m/z* 395 (−98 amu) diagnostic of the presence of an hydroxyl group on C-15. Moreover, a product ion at *m/z* 249 was observed, suggesting a side chain carrying three hydroxyl groups; this hypothesis was inferred by the fragment ion at *m/z* 403, produced by the elimination of five water molecules from the parent ion. On the basis of these data, the structure reported in Fig. 5a was suggested for compound **23**. A molecular weight of 468 amu was measured for compound **24** ($[M+Na]^+$ ion at *m/z* 491). Its MS/MS spectrum showed signals at *m/z* 393 and *m/z* 249, indicating that this compound carries the same side chain of **23**. Also in this case, five eliminable hydroxyl group are present, as inferred by the signal at *m/z* 401, but no ion produced by the elimination of the group linked to C-4 was detected. These data, together with the mass difference between this compounds and compound **23** (−2 amu), let to suggest the structure reported in Fig. 5b for compound **24**.

In order to evaluate the content in sesterterpenes in different *S. dominica* organs, the described LC–MS/MS method was used. In

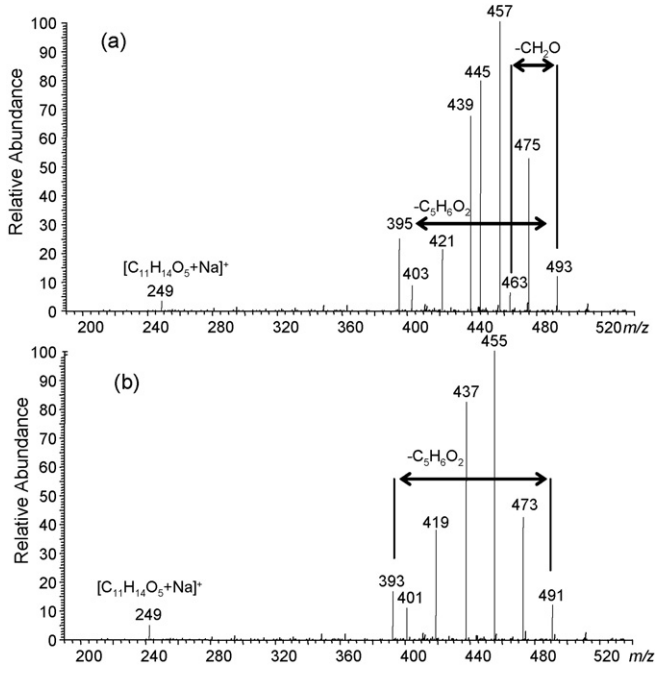


Fig. 4. MS/MS spectra of unknown compounds. (a) MS/MS spectrum of compound eluted at 16.4 min (compound **23**) in the LC–MS/MS analysis reported in Fig. 3. (b) MS/MS spectrum of compound eluted at 13.4 min (compound **24**) in the LC–MS/MS analysis reported in Fig. 3.

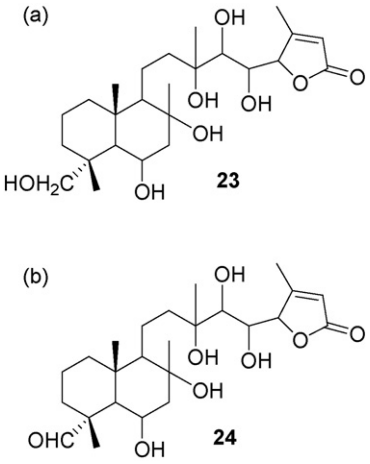


Fig. 5. Attempted structures for compounds **23** and **24**.

Fig. 6, the TIC chromatograms obtained by the LC–MS/MS analyses of crude acetone extracts of *S. dominica* leaves (Fig. 6a), leaf galls (Fig. 6b) and roots (Fig. 6d) extracts are shown. Different peaks observed in each chromatographic profile were identified, comparing their retention times and mass spectrometric data with those reported in Table 1. To correctly compare the amount of each compound into the different extracts, selected ion chromatograms were obtained for each species, the peaks of interest were integrated and the measured values were normalized using amentoflavone as an internal standard (IS). The obtained results are reported in Fig. 7. A method validation was performed to assess its suitability for quantitative analyses. The experimental results showed that the ratio peak area/internal standard area was linearly correlated to the injected amount within a particular range (Table 2). All the calibration curves showed good linear regression ($R_2 > 0.997$) and the LOD was less than 0.10 μg per injection. Therefore, quantitative data achieved by this method were considered accurate enough to allow further evaluations.

As expected, a low total amount of sesterterpenes were found into the roots, whose principal component resulted to be the bioactive diterpene aethiopinone (co-eluting with compound **15** in peak at 42.8 min, Fig. 6c, observed *m/z* value 319 $[M+Na]^+$), an antibacterial agent widely present into the roots of different plants belonging to *Salvia* genus [20–22]. However, the most hydrophobic components were present into the roots extract at a significant level, and the abundance of compounds **9**, **10**, **11**, and **20** were higher then those observed into the other parts. Chromatographic profiles obtained for the leaves and the leaf galls seem to be comparable, but differences in terms of abundance of some specific compounds were observed. In particular, com-

Table 2
Linear regression evaluated for 10 of the *Salvia dominica* sesterterpenes.

Compound	Calibration curve	Correlation coefficient	Linear range (μg)	LOD (ng)	LOQ (ng)
1	$y = 65.39x + 2.23$	0.9985	0.2–40	10	40
2	$y = 63.06x + 3.51$	0.9972	0.4–60	25	100
7	$y = 62.81x + 3.27$	0.9987	0.4–60	25	100
13	$y = 64.15x + 3.18$	0.9984	0.2–40	10	60
14	$y = 64.68x + 2.25$	0.9983	0.4–60	25	100
16	$y = 62.96x + 4.45$	0.9985	0.5–60	25	125
18	$y = 66.11x + 2.38$	0.9987	0.2–40	10	60
19	$y = 62.05x + 3.23$	0.9976	0.5–60	40	150
21	$y = 68.06x + 1.41$	0.9991	0.1–40	10	40
22	$y = 67.88x + 1.22$	0.9987	0.2–40	10	40

y and *x* stand for the peak area/internal standard ratio and the amount (μg) of the analytes, respectively.

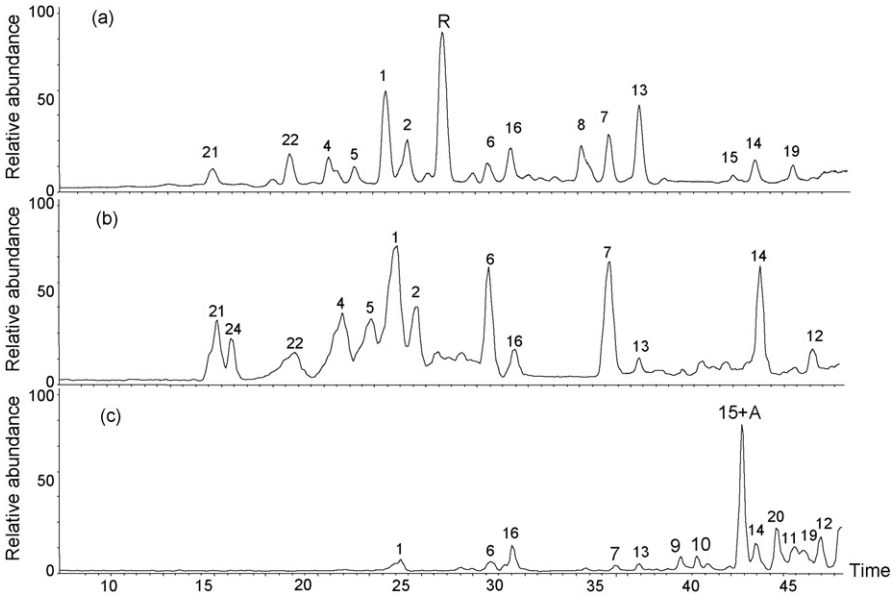


Fig. 6. Chromatographic analysis of different *S. dominica* extracts. Total ion LC–MS/MS chromatograms of (a) the leaves, (b) the leaf galls and (c) the roots extracts. Peaks numbering is in agreement with that used in Table 1; R: rosmarinic acid; A: aethiopinone.

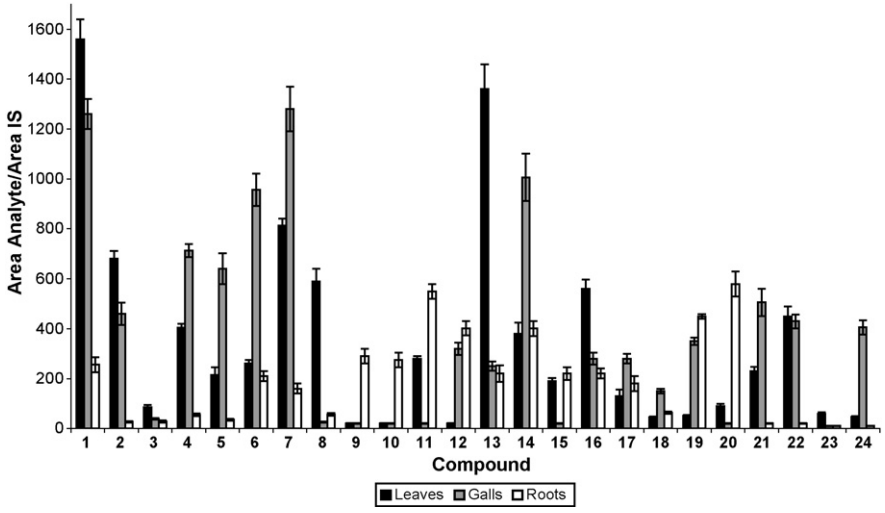


Fig. 7. Quantitative analysis of compounds **1**–**24** in different *Salvia dominica* extracts.

pounds **8**, **13**, **15** and **23** were more abundant into the leaves then into the galls, whereas higher amount of compounds **4**, **5**, **6**, **7**, **14** and **24** were detected into the galls extract; interestingly, all the compounds prevalently present into the leaves carry an alcoholic function on C-4, an those prevalently present into the galls have an aldehydic or carboxyl group on the same position, thus suggesting a relationship between oxidation level of the bicyclic portion of these compounds and their storage site. A clear evidence of this relationship could be deduced by a direct comparison between the different amount of compounds **1**, **4** and **6** detected in leaves and galls extracts. These compounds share the whole chemical structure, with the only exception of the functional group on C-4: compound **1**, carrying an alcoholic function on C-4, is principally present into the leaves whereas compounds **4** and **6**, where a carboxylic or an aldehyde function is present, are preferentially found into the galls. Similar results can be achieved also comparing the amounts of compounds **2**, **5** and **7**, or those of compounds **13** and **14** retrieved into the different extracts.

4. Conclusions

In this study we optimized an LC–MS/MS method to identify and quantify, in a single step, the sesterterpenoidic components of crude extracts from different parts of *S. dominica*. The use of this approach allowed us to completely characterize 22 already known compounds [8–10], in terms of chromatographic and mass spectrometric proprieties. Moreover, two new sesterterpenes were detected in the aerial part extract, and their chemical structures were suggested on the basis of MS and MS/MS spectra. To the best of our knowledge, this is the first mass spectrometry-based approach aimed to a systematic analysis of plant sesterterpenes.

Our results demonstrated that both leaves and leaf galls contains relevant amounts of sesterterpene lactones, but some differences were detected between those two organs, in terms or the relative abundances of some compounds. In particular, the most hydrophilic and highly oxidized compounds were primarily detected into the leaf galls extract. This finding is in agreement with the results obtained by the studies performed on six

monophagous *Pontania* species [23] and *Tibouchina pulchra* [24] leaf galls.

Our previous studies on *S. dominica* sesterterpene lactones indicated that compound **5** was the most active one; indeed, this molecule showed an affinity for TTL, the biological target identified for these compounds, at least 10 times higher then all the other compounds [10]. The present research demonstrated that this compound is primarily accumulated into the leaf galls, thus indicating these abnormal outgrowths as suitable source for these bioactive compounds. Since leaf galls from *Salvia* plant have been grown *in vitro*, retaining their ability in producing bioactive secondary metabolite under suitable environmental conditions [25,26], the development of a liquid stationary culture of *S. dominica* galls could provide a new low cost source for the production of effective TTL inhibitors.

References

[1] R.A. Keyzers, M.T. Davies-Coleman, Anti-inflammatory metabolites from marine sponges, Chem. Soc. Rev. 34 (2005) 355–365.
[2] U. Lauer, T. Anke, W.S. Sheldrick, A. Scherer, W. Steglich, Antibiotics from basidiomycetes. XXXI. Aleurodiscal: an antifungal sesterterpenoid from *Aleurodiscus mirabilis* (Berk. & Curt.) Hohn, J. Antibiot. 42 (1989) 875–882.
[3] Y. Liu, L. Wang, S. Zhang, Sesterterpenoids, Nat. Prod. Rep. 24 (2007) 1401–1429.
[4] S. Foster, V.E. Tyler, Tyler's Honest Herbal, 4th ed., The Haworth Press, Binghamton, New York, 2000, pp. 327–329 and 414–415.
[5] T. Gulacti, U. Ayhan, T. Timothy, C. Tao-Che, Sesterterpenes and other constituents of *Salvia yosgadensis*, Phytochemistry 42 (1996) 1089–1092.
[6] B. Bozan, N. Ozturk, M. Kosar, Z. Tunalier, K.H.C. Baser, Chemistry of Natural Compounds (Translation of Khimiya Prirodnykh Soedinenii), vol. 38, 2002, pp. 198–200.
[7] A. Ulubelen, in: S.E. Kintzios (Ed.), The genus Salvia. Medicinal and Aromatic Plants-Industrial Profiles, Harwood Academic Publishers, Amsterdam, 2000.
[8] M. Firoz Matloubi, A. Raheba, A. Maktoob, H.M. Bilayet, D. van der Helm, Structure and absolute stereochemistry of salvimirzacolide, a new sesterterpene from *Salvia mirzayanii*, J. Nat. Prod. 61 (1998) 279–281.
[9] G. Cioffi, A. Bader, A. Malafronte, F. Dal Piaz, N. De Tommasi, Secondary metabolites from the aerial parts of *Salvia palaestina* Bentham, Phytochemistry 69 (2008) 1005–1012.
[10] F. Dal Piaz, A. Vassallo, L. Lepore, A. Tosco, A. Bader, N. De Tommasi, Sesterterpenes as tubulin tyrosine ligase inhibitors. First insight of structure-activity relationships and discovery of new lead, J. Med. Chem. 52 (2009) 3814–3828.
[11] H.S. Barra, C.A. Arce, C.E. Argarana, Argarana, Posttranslational tyrosination/detyrosination of tubulin, Mol. Neurobiol. 2 (1988) 133–153.
[12] K. Ersfeld, J. Wehland, U. Plessmann, H. Dodemont, V. Gerke, K. Weber, Characterization of the tubulin-tyrosine ligase, J. Cell. Biol. 120 (1993) 725–732.
[13] C. Erck, L. Peris, A. Andrieux, C. Meissirel, A.D. Gruber, M. Vernet, A. Schweitzer, Y. Saoudi, H. Pointu, C. Bosc, P.A. Salin, D. Job, J. Wehland, A vital role of tubulin-tyrosine-ligase for neuronal organization, Proc. Nat. Acad. Sci U.S.A. 102 (2005) 7853–7858.
[14] A. Mialhe, L. Lafanechere, I. Treilleux, N. Peloux, C. Dumontet, A. Bremond, M.H. Panh, R. Payan, J. Wehland, R.L. Margolis, D. Job, Tubulin detyrosination is a frequent occurrence in breast cancers of poor prognosis, Cancer Res. 61 (2001) 5024–5027.
[15] C. Kato, K. Miyazaki, A. Nakagawa, M. Ohira, Y. Nakamura, T. Ozaki, T. Imai, A. Nakagawara, Low expression of human tubulin tyrosine ligase and suppressed tubulin tyrosination/detyrosination cycle are associated with impaired neuronal differentiation in neuroblastomas with poor prognosis, Int. J. Cancer 112 (2004) 365–375.
[16] K. Soucek, A. Kamaid, A.D. Phung, L. Kubala, J.C. Bulinski, R.W. Harper, J.P. Eiserich, Normal and prostate cancer cells display distinct molecular profiles of α-tubulin posttranslational modifications, Prostate 66 (2006) 954–965.
[17] R.D. Charan, T.C. McKee, M.R. Boyd, Thorectandrols C, D, and E, new sesterterpenes from the marine sponge *Thorectandra* sp., J. Nat. Prod. 65 (2002) 492–495.
[18] F.M. Moghaddam, R. Amiri, M. Alam, M.B. Hossain, D. van der Helm, Structure and absolute stereochemistry of salvimirzacolide, a new sesterterpene from *Salvia mirzayanii*, J. Nat. Prod. 61 (1998) 279–281.
[19] A. Rustaiyan, A. Niknejad, L. Nazarians, J. Jakupovic, F. Bohlmann, Sesterterpenes from *Salvia hypoleuca* Authors, Phytochemistry 8 (1982) 1812–1813.
[20] M. Hernández-Pérez, R.M. Rabanal, M.C. de la Torre, B. Rodríguez, Analgesic, anti-inflammatory, antipyretic and haematological effects of aethiopinone, an o-naphthoquinone diterpenoid from Salvia aethiopis roots and two hemisynthetic derivatives, Planta Med. 61 (1995) 505–509.
[21] A. Ulubelen, S. Oksüz, U. Kolak, C. Bozok-Johansson, C. Celik, W. Voelter, Antibacterial diterpenes from the roots of *Salvia viridis*, Planta Med. 66 (2000) 458–462.
[22] E. Walencka, S. Rozalska, H. Wysokinska, M. Rozalski, L. Kuzma, B. Rozalska, Salvipisone and aethiopinone from *Salvia sclarea* hairy roots modulate staphylococcal antibiotic resistance and express anti-biofilm activity, Planta Med. 73 (2007) 545–551.
[23] T. Nyman, R. Julkunen-Tiitto, Distribution of metabolites in galled and non-galled foliar tissues of *Tibouchina pulchra*, Proc. Natl Acad. U.S.A. 97 (2000) 13184–13187.
[24] L.B. Motta, J.E. Kraus, A. Salatino, M.L.F. Salatino, Manipulation of the phenolic chemistry of willows by gall-inducing sawflies, Biochem. Syst. Ecol. 33 (2005) 971–981.
[25] Y. Zhang, J. Song, B. Zhao, H. Liu, Crown gall culture and production of tanshinone in *Salvia miltiorrhiza*, Chin. J. Biotechnol. 11 (1995) 137–141.
[26] J. Song, Y. Zhang, J. Qi, G. Lu, Chin, Selection of a high tanshinone-producing crown gall strain and production of tanshinone in the strain, J. Biotechnol. 13 (1997) 207–210.