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Integration of dry-column flash chromatography with NMR and FTIR metabolomics to reveal cytotoxic metabolites from *Amphoricarpos autariatus*

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ABSTRACT

Metabolomics generate a profile of small molecules from plant extracts, which could be directly responsible for bioactivity effects. Using dry-column flash chromatography enabled a rapid and inexpensive method for the very efficient separation of plant extract with a high resolution. This separation method coupled to NMR and FTIR-based metabolomics is applied to identify bioactive natural products. OPLS multivariate analysis method, was used for correlation the chemical composition of the plant extracts, *Amphoricarpos autariatus*, with the results of cytotoxic activity against Human cervical adenocarcinoma cell line (HeLa) and epithelial lung cancer cell line (A549). In this way, the highest contribution to the cytotoxic activity was recorded for the guaianolide sesquiterpene lactones named amphoricarpolides. The compounds indicated as bioactive after metabolomics analysis were tested, and their cytotoxic activity were confirmed.

1. Introduction

A large number of plant metabolites has provided an incomparable chemical source of the pharmaceutical products [1]. The two major fields of chemical research on biological active small molecules, metabolomics and natural product discovery, have the similar goals of identifying and characterizing small molecules, either in their isolated active state (natural product chemistry) or as mixtures (metabolomics) [2]. Plant metabolomics is a rapidly developing technology which has the goal to provide exceptionally rich data on the biochemical composition of plant materials [3]. The main purpose of plant metabolomics is to analyze as much as possible metabolites both qualitatively and quantitatively in medicinal plant samples [4].

Basically metabolomics analysis consists of three steps. The first step is the preparation of the sample including extraction process, the second is the acquisition of data using analytical chemical methods and the third and final step is data mining using appropriate chemometric methods [5,6].

Sample preparation is a critical step with important consequences for the compounds isolated and the accuracy of the results. The choice of the extraction method is an essential step to obtain the real metabolic profile [1]. To avoid possible degradation during the sample preparation, it is important to keep the procedure as simple and fast as possible [5].

The natural products exist in plant matrix as a very complex mixture, from which the product of interest are isolated and purified. For the isolation of natural products from plant material, the good selection of appropriate techniques and approaches are essential. Among the all chromatographic techniques, dry-column flash chromatography (DCFC) is a very efficient in both crude as well fine separations of the natural products mixture [7]. DCFC is a fast and cost effective chromatography approach. It is very useful technique for quick separation of large quantities of samples [8]. The instrumentation is uncomplicated compared with modern expensive flash chromatography [8–10].

DCFC procedure is similar to many other types of column chromatography in that one “packs” a column, loads the sample, and elutes the column by mobile phase. In the modern flash chromatography techniques, nitrogen or compressed air was used to push the mobile phase through the stationary phase in a tightly closed glass column or prepacked cartridges [10,11]. For the DCFC, the eluent is rapidly soaked through glass column by vacuum. The column consists of a “dry” bed of silica gel placed in a sintered glass funnel, eluted by suction, and is

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drained dry after each fraction [12].

By using different analytical platforms, different extracts or fractions of plant material and measure the bioactivity of these samples one may be able to identify signals in the metabolomics (chromatographic or spectral) data that correlate to the activity. These signals can then be linked to compounds. Advantage of this approach is that it can identify active compounds in the mixtures, without need to previous isolation of pure compounds [13].

The measurement of metabolites can be successfully achieved using nuclear magnetic resonance (NMR) spectroscopy techniques [2,14]. NMR in plant metabolomics has a wide range of applications and it has been used for quantitation of plant metabolites in mixtures [4]. NMR-based metabolomics is quickly developing towards becoming a routinely used tool. It is a robust and reliable technique requiring minimal sample preparation, thus allowing high-throughput analysis [14] and can be utilized *in vivo* [2]. One-dimensional (1D) ^{1}H NMR is the most widely used NMR approach in metabolomics because its excellent reproducibility, and thus suitability for public data bases for data mining [2,13]. Short measurement time, fast pre-analytical sample preparation and the possibility of elucidating structures of known or unknown compounds in a complex mixture using advanced two dimensional (2D) NMR methods are further advantages [1,13,14]. Another advantage to using NMR-based approaches is that the technique is non-destructive and allows further analysis to be performed.

Fourier transform infrared spectroscopy (FTIR) as the application of most available, simple, and rapid method can be an efficient alternative for the measurement of metabolites [15]. FTIR spectroscopy is also non-destructive and easy analytical technique that only requires small amounts of sample to provide information about the sample and functional groups of molecules [16]. Compared with other techniques it is simple to use, with low operational costs and high sensitivity. It is a very rapid biochemical fingerprinting technique, and combined with appropriate multivariate statistical methods, FTIR spectroscopy may be an ideal solution for resolving many analytical problems [17].

Once the NMR or FTIR data are acquired for a metabolomics study, the first step is to transform the data into a suitable format for multivariate data analysis [13]. PCA is one of the most popular data analysis techniques for exploring metabolite differences in NMR spectra. In essence PCA allows the reduction of a large number of variables to a smaller number of principal components [3,13]. Multivariate data analysis tools such as PLS and OPLS are also used to find possible correlations between the metabolite profile and bioactivity [1].

Sesquiterpene lactones represent a large and important group of compounds, both to humans and to the plants themselves [18]. Such compounds act as phytoalexins; molecules produced in reaction to microbial attack, antifeedants to deter herbivores, allelochemicals and UV protection agents [18]. These compounds have a broad range of biological activities as antimicrobial activity, anti-migraine, digestive, analgesic [19]. They have big role of treatment of cardiovascular diseases and malaria [18]. The benefits of sesquiterpene lactones are focused on their anticancer potential [20]. They are mostly found in leaves and flowering heads of plants, mainly in genera from the large Asteraceae family comprising genus *Amphoricarpos* as well. Phytochemical investigation of *Amphoricarpos* species revealed the fact that this species are rich source of sesquiterpene lactones of guaianolide type so-called amphoricarpolides [21–23]. There are couple of studies describing the biological activity of *Amphoricarpos* species. Hitherto the cytotoxic activity of *A. neumayeri* [24] and the antifungal activity of leaf surface constituents of *A. autariatus* [25]. has been reported. A rather high antimicrobial potential and strong antioxidant activity of *Amphoricarpos* taxa has been reported as well [26].

Herein is presented an integration of the old fashioned dry-column flash chromatography, enabling fast fractionation of crude plant extract of *A. autariatus* with modern NMR and FTIR spectroscopy techniques. Multivariate analysis of spectral and cytotoxic activity assays data led to rapid identification of bioactive compounds. The choice of *A. autariatus* as a model plant was based on the fact that preliminary investigation of the crude extract resulted in positive cytotoxic activity. Moreover, in our previous investigation regarding cytotoxic activities of the major lactone constituents of the extract of the genus, those with 11,13-double bond conjugated with the lactone carbonyl, exhibited considerable cytotoxic activities against HeLa cell lines [25].

2. Experimental

2.1. Chemicals and reagents

General. Dry-column flash chromatography (DCFC): silica gel (SiO_2, < 0.08 mm; Merck). TLC: Precoated plates; silica gel 60 F_{254} (Merck); visualization by UV lamp and spraying with conc. H_2SO_4, followed by heating. 1D-NMR spectra: Bruker Avance III 500 spectrometer (at 500.26 MHz for ^{1}H); δ in ppm rel. to signal of DMSO-d_5 at 2.50 ppm.

2.2. Plant material

The aerial parts of *Amphoricarpos* taxa were collected in Montenegro at canyon of river Tara, during the flowering, in June 2016. According to Blečić and Mayer [27], collected sample belongs to *A. autariatus ssp. autariatus*. The voucher specimens were identified by prof. Petar Marin, and deposited at the Herbarium of the Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade (17116).

2.3. Extraction of plant material

Air-dried leaves of sample *A. autariatus* (79.00 g) were grounded into a powder in a blender. The powder was sonicated with CH_2Cl_2-MeOH (1:1, 1 l) at r.t. for 15 min. Reextraction were repeated twice.

2.4. Dry-column flash chromatography

After filtration and evaporation of the solvent, the combined extracts yielded 4.5603 g. Fractionation was performed in a sintered glass column (21.8 cm length, 3.20 cm diameter) packed with silica gel (80.00 g). Column was equilibrated four times with 50 mL of CH_2Cl_2. The extract (m = 1.00 g) of dried powdered *A. autariatus* was adsorbed on silica gel (4.00 g), dissolving the sample in CH_2Cl_2, adding silica gel to create a slurry, evaporating the solvent, and applied on top of the column. The elution was started with pure CH_2Cl_2. The polarity of the solvent was gradually increased by addition of MeOH (up to 20%). Thirteen fractions (100 mL each) were collected. This separation procedure using 1.00 g of plant extract and new silica gel column was repeated three times, affording a total of 52 fractions.

2.5. Fractions dissolving

After evaporation to dryness fractions were transferred to a 2 mL-microtube and dissolved in DMSO-d_6 to the final concentration of 60 mg/mL. The mixture was vortexed at room temperature for 30 s, sonicated for 15 min, and then centrifuged at 13,400 rpm for 5 min. The supernatants were submitted to NMR measurements and cytotoxic activity tests on Human cervical adenocarcinoma cell lines and epithelial lung cancer cell lines. After NMR measurements and determination cytotoxic activity fractions were freeze-dried to remove DMSO-d_6 for the FTIR measurements.

2.6. In vitro cytotoxicity assay

Human cervical adenocarcinoma cell line (HeLa) and epithelial lung cancer cell line (A549) were grown in RPMI-1640 medium at 37 °C in humidified atmosphere with 5% CO_2. The cells were seeded into 96-well microtiter plates at density of 2000 cells/well. After 24 h, they

were treated with five different concentrations of examined extracts (12.5, 25, 50, 100 and 200 μg/mL), or reference compounds, while control cells were grown in culture medium only. After 72 h of incubation, the cell survival was determined by MTT test, as described previously [28]. All experiments were done in triplicates, and the data are presented as mean ± standard deviation (SD) of the results obtained in three independent experiments. The absorbance was measured at 570 nm using Multiskan EX reader (Thermo Labsystems Beverly, MA, USA) [28].

2.7. NMR measurement

All NMR spectra were recorded on a Bruker Avance III 500 NMR spectrometer operating at a proton NMR frequency of 500.26 MHz, equipped with a 5 mm BBO probehead. The spectra were referenced to the signal of DMSO-d_5. The chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. For the ^{1}H NMR spectra, 32 k data points were collected, using presaturation–"zgpr" pulse program with 64 scans, spectral width of 9014.4 Hz, and relaxation delay 1.5s. For the 2D experiments, the parameters were as follow:

COSY: relaxation delay 1.5 s, spectral width 7003 Hz in both dimensions, 2048 data points for f2, 8 scans and 1024 increments.

NOESY: the same as COSY but with relaxation delay 2 s, 32 scans, and mixing time 0.8 s.

HSQC: relaxation delay 2 s, spectral width 7003 Hz for f2 and 22644 Hz for f1, 2048 data points for f2, 8 scans and 1024 increments.

HMBC: the same as HSQC with delays optimized for coupling constant of 7.7 Hz, but with 40 scans.

2.8. FTIR measurement

FTIR spectra were recorded on the Thermo Scientific Nicolet 6700 FTIR spectrometer, using the attenuated total reflectance (ATR) technique from the Smart accessory with diamond crystal (Smart Orbit, Thermo Scientific, Madison, WI, USA). Spectral data were collected in the mid-IR range (4000–600 cm^{-1}) with 64 scans and 2 cm^{-1} resolution. A background spectrum (32 scans) was recorded before every sample spectrum.

2.9. Data analysis

The 1D NMR spectra were processed using TopSpin software (version 3.5, Bruker Biospin, Germany). The ^{1}H NMR spectra were manually phased and baseline corrected, and then binned using MestReNova software version 6.0.2 (Mestrelab Research, Santiago de Compostela, Spain). The spectra were reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 0.4–12.0, and exported to ASCII files. Orthogonal partial least squares to latent structures (OPLS) methods were performed with SIMCA software (version 14, Umetrics, Umeå, Sweden). The spectral data were normalized to the total area, mean centered, and scaled to Pareto. The region of δ 3.12–3.64 and δ 2.48–2.56 were excluded from the analysis prior to normalization because of the residual water and DMSO-d_5 signal, respectively.

IR spectra were baseline corrected and smoothed using OMNIC software (version 7.0, Thermo Scientific, USA), and exported to SPC files. Orthogonal partial least squares to latent structures (OPLS) methods were performed with SIMCA software (version 14, Umetrics, Umeå, Sweden). The spectral data were preprocessed using standard normal variate, and scaled to Pareto.

3. Results and discussion

Crude extract of *Amphoricarpos* was successfully fractionated by DCFC using gradient elution with CH_2Cl_2/MeOH solvent system. The separation procedure using new amount of plant extract was repeated three times, affording total of 52 fractions. The experimental procedure was simple, with low solvent and time consumption thus decreasing the overall separation cost.

The cytotoxic activity of *A. autariatus* fractions were evaluated against two human cancer cell lines: human cervical adenocarcinoma (HeLa) and human lung adenocarcinoma (A549). The cells survival was determined by MTT test, after 72 h of incubation as described in the experimental part.

Treatment of examined cell lines resulted in dose-dependent cytotoxicity. The most effective fractions against HeLa cells were those with IC_{50} values *c.a.* 9 μg/mL. A549 cells were the most sensitive on the fractions exhibiting IC_{50} values *c.a.* 15 μg/mL. The results of cytotoxic activity are summarized in Table S1.

To correlate chemical composition of fractions, with the results of cytotoxic activity testing, an orthogonal partial least squares to latent structures (OPLS) analysis was applied. The ^{1}H NMR spectral data were used as the *X* variables, and IC_{50} values from the cytotoxic activity as the *Y* variables. The advantage of an orthogonal model is its facilitated interpretation due to separation of the systematic variation in *X* into two parts: one linearly related to *Y* and one orthogonal to *Y* [29].

After binning, centering and pareto scaling of the ^{1}H NMR spectral, OPLS models with one predictive and three orthogonal components was obtained for both cell lines The high values of R^2 and Q^2, close to 1 indicated extraordinary goodness of fit and predictive ability of the models (Table 1).

The NMR based OPLS models were validated by permutation test. The satisfactory results obtained since regressions of Q^2 lines intersected the vertical axis at below zero, and all Q^2 and R^2 values of permuted *Y* vectors were lower than original ones. According to further validation using CV-ANOVA, both NMR based OPLS models were significant with $p < 0.05$ (Table 1). In the score plots (Fig. 1) a clear separation between the samples with different cytotoxic activities obtained along predictive component, in both models. The selection of the most influential variables was based on two parameters: variable influence on projection (VIP) scores of the predictive components, and the loadings scaled as a correlation coefficient (p(corr)). Variables with the highest VIP score (above 1.5), and the |p(corr)| values above 0.5 were considered as important for the correlation. The important variables were listed in Table S2, and marked by red color on the loading plots (Fig. 1).

In addition, FTIR spectra of *A. autariatus* fractions were collected and correlated with cytotoxic activity. As the *X* variables, the FTIR spectral data were used, and as the *Y* variables IC_{50} values from the cytotoxic activity. After standard normal variate correction and pareto scaling of the FTIR data, an OPLS model with one predictive and three orthogonal components was obtained, for HeLA cells and an OPLS model with one predictive and two orthogonal components for

Table 1
Parameters of the OPLS models.

OPLS model	Number of components (predictive + orthogonal)	R^2	Q^2	*p* (CV-ANOVA)	F (CV-ANOVA)
NMR HeLa	1 + 3	0.932	0.855	$3.01*10^{-15}$	31.05
NMR A549	1 + 3	0.922	0.866	$6.00*10^{-16}$	34.01
FTIR HeLa	1 + 3	0.885	0.827	$5.34*10^{-14}$	25.66
FTIR A549	1 + 2	0.838	0.804	$2{,}18*10^{-14}$	30.83

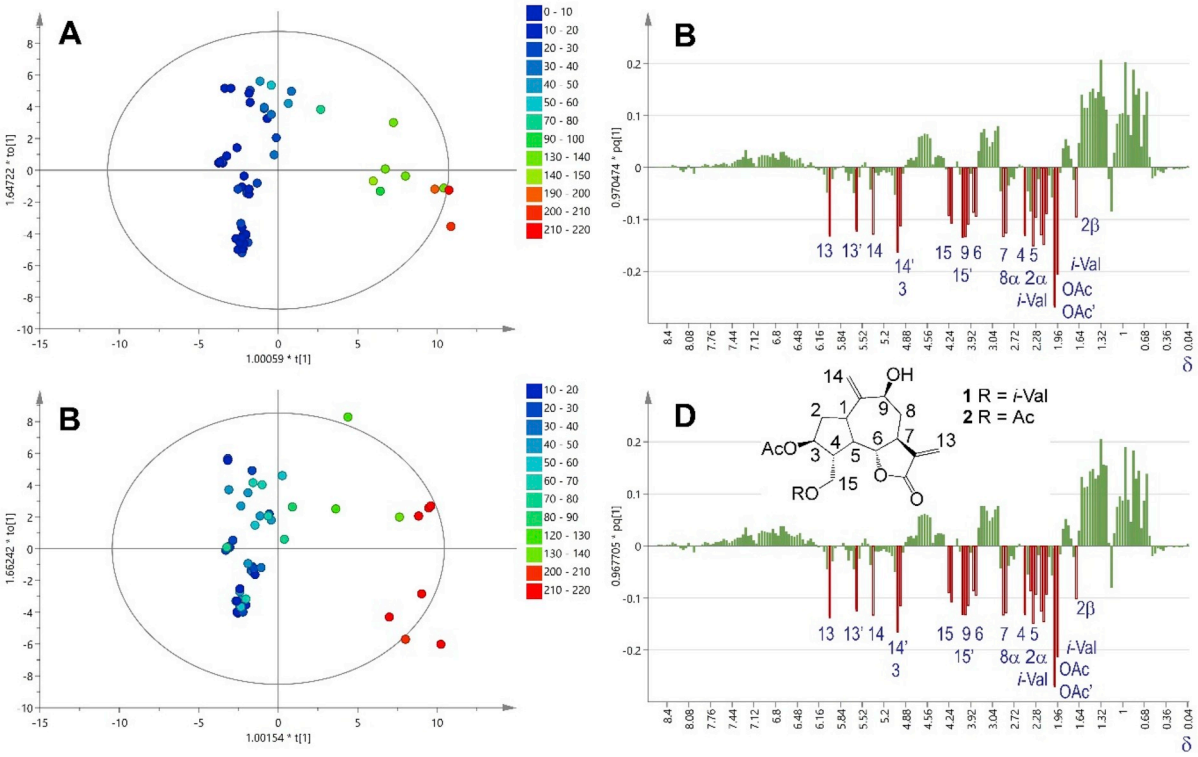

Fig. 1. OPLS score (A, C) and loadng plot (B, D) of the *A. autariatus* fractions. The scores are correlated according NMR data to the IC_{50} value of the cytotoxicity testing for HeLa and A549 cells, respectively.

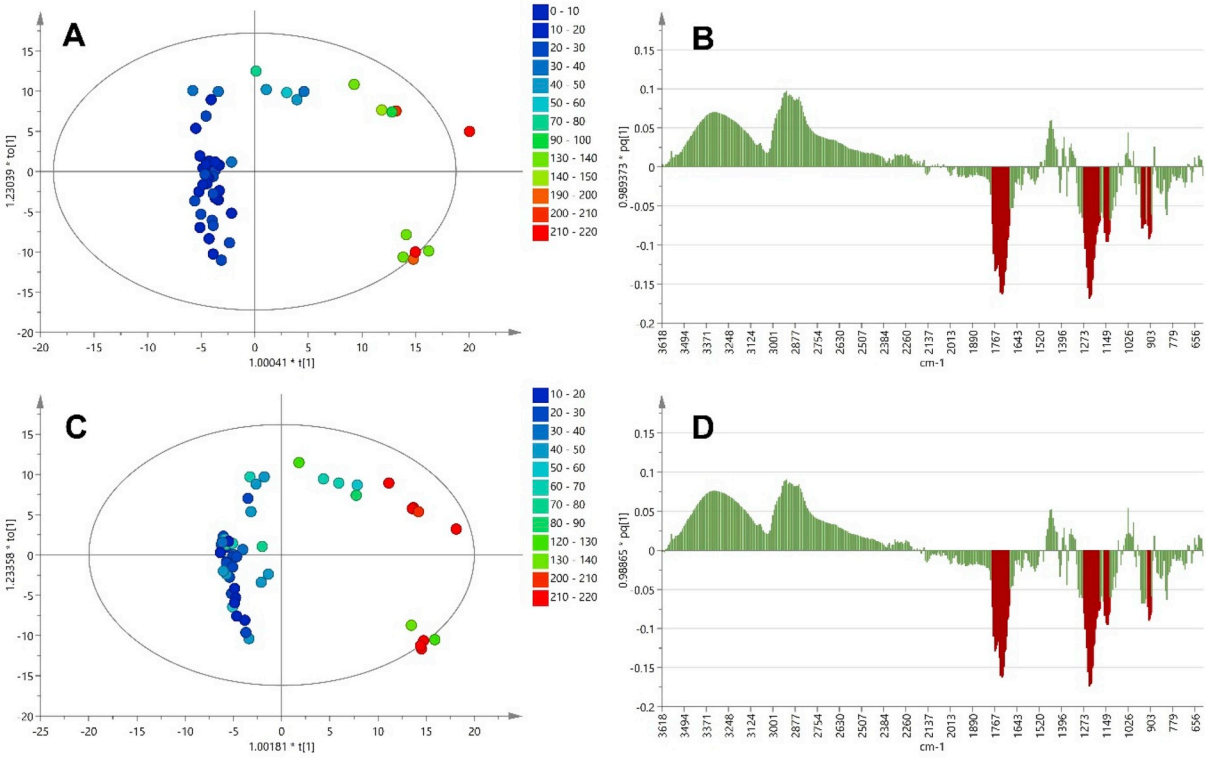

Fig. 2. OPLS score (A,C) and loadng plots (B, D) of the *A. autariatus* fractions. The scores are correlated according FTIR data to the IC_{50} value of the cytotoxicity testing for HeLa and A549 cells, respectively.

A549 cells. The high values Q^2 and R^2, both models showed excellent goodness of fit and predictive ability (Table 1) Validation of FTIR based OPLS models were also performed by permutation test, and they met the criteria already explained for NMR based methods. The CV-ANOVA validation of both FTIR based OPLS models showed they were significant with $p < 0.05$ (Table 1). In the score plot obtained a clear

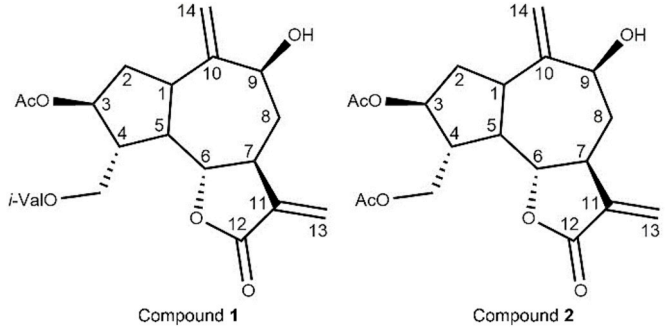

Fig. 3. Sesquiterpene γ-lactones identified as bioactive compounds; **1**: 3-*O*-acetyl-15-*O*-isovaleroyl-9β-hydroxyamphoricarpolide, **2**: 3,15-di-*O*-acetyl-9β-hydroxyamphoricarpolide.

separation between the samples with different cytotoxic activity (Fig. 2). The importance of variables was determined on the basis of the same criteria already described for NMR based models. The important variables with the VIP score above 1.5, and the |p(corr)| values above 0.5 were listed on Table S3 and marked by red color on the loading plots (Fig. 2).

Variables with the highest VIP and |p(corr)| values from OPLS models were regarded as the signals of bioactive compounds which were present in fractions with the highest cytotoxic activity (Tables S2 and S3, supplementary material). For the structure elucidation of the bioactive compounds 1D and 2D NMR techniques were shown to be the most powerful tool. Additionally, the FTIR spectra revealed functional groups of the bioactive compounds.

The crowded regions in the ^{1}H NMR spectra due to overlapping of the complex signals were resolved by an extensive analysis of 2D methods, including COSY, NOESY, HSQC, and HMBC (supplementary material). The ^{1}H NMR chemical shifts, multiplicity of the signals, and coupling constants were extracted from H,H-*J*-resolved spectra. Further, the ^{1}H NMR resonances of the bioactive compounds were further confirmed by comparison with the information from our in-house NMR spectroscopic database of plant secondary metabolites.

According to the NMR analysis, the highest contribution to the cytotoxic activity was recorded for the fractions containing signals of sesquiterpene γ-lactones with characteristic guaianolide sceleton (compound **1** and **2,** Fig. 3.). The ^{1}H NMR data of these two identified bioactive compounds are presented in Table 2 and ^{1}H NMR spectra in the supplementary material.

The results obtained by correlating FTIR data with cytotoxic activity confirmed the results obtained by NMR measurements. The wavenumbers corresponding to the variables with the highest VIP scores were compared to those of the reference compounds. On this way, all the wavenumbers defined from IR based OPLS models as signals of bioactive compounds, were also found in the FTIR spectra of pure sesquiterpene lactones **1** and **2**.

Thus, the bands at *c.a.* 1763 and 1728 cm^{-1} were attributed to γ-lactone and acetate carbonyl group stretching vibrations, respectively. The strong bands at 1240 and 1142 cm^{-1} were attributed to C-O stretching vibrations of acetate esters and γ-lactone, respectively.

These results showed that the sesquiterpene γ-lactones may play a major role in cytotoxic activity of the studied extract. This is not unexpected since it is known that the conjugated α-methylene-γ-lactone group, could be responsible for the cytotoxic functions (via Michael addition).[l1]

In order to finally prove the result obtained from the OPLS models, two identified constituents of the active fractions (**1** and **2**) were tested for cytotoxic activity on HeLa and A549 cell lines. Both reference compounds exhibited considerable cytotoxic activity, corresponding to the activity obtained from the most effective fractions. Thus, compound **1** showed IC_{50} values of 7.1 ± 0.3 μg/mL (17 μM) and 18 ± 2 μg/mL (44 μM) for HeLa and A549 cell lines, respectively. Compound 2 exhibited IC_{50} values of 9 ± 2 μg/mL (24 μM) for HeLa, and 30 ± 3 μg/mL (83 μM) for A549 cell lines. The results obtained for the bioactivity testing of the reference compounds confirmed the findings obtained from the OPLS correlations.

4. Conclusion

Coupling DCFC chromatography technique to NMR an FTIR based metabolomics and multivariate data analysis revealed the possibility of achieving the identification of biological active compounds, without need for their prior isolation. This procedure provides a fast method for identification of biologically active compounds combining chromatography and NMR or FTIR spectroscopy techniques with bioassays and multivariate data analysis. This approach was successfully applied to correlate constituents from *A. autraiatus* with cytotoxic activity. According to the obtained results guaianolide sesquiterpene lactones were identified as the most influential compounds in the OPLS models, with the highest cytotoxic activity in the studied extract. The FTIR data indicated that the bioactive molecules possessed γ-lactone moiety which was responsible for the strong activity, while NMR data enabled structure elucidation of the bioactive molecules.

The possibility to get information regarding components responsible for the particular biological activity of the complex plant extract via coupling of the old fashioned dry-column flash chromatography with the modern NMR and FTIR spectroscopy and multivariate data analysis has been demonstrated in this paper. Furthermore, the metabolomics approach facilitated the identification of bioactive compounds and allowed to skip bio-guided fractionation.

Table 2
NMR and FTIR data of the sesquiterpene γ-lactones identified as bioactive compounds.

Identified compound	NMR chemical shifts (δ)	FTIR wavenumbers (cm^{-1})
Compound **1**	6.02 (d, *J* = 3.5) (H-13), 5.63 (d, *J* = 3.1) (H-13′), 5.38 (s) (H-14), 5.02 (s) (H-14′), 4.99 (br q, *J* = 7.5) (H-3), 4.24 (dd, *J* = 3.7; 11.3) (H-15), 4.05 (dd, *J* = 6.4; 11.3) (H-9), 4.00 (dd, *J* = 4.6; 10.44) (H-15′), 3.89 (dd, *J* = 8.8; 10.2) (H-6), 2.92 (m) (H-7), 2.87 (br q, *J* = 7.7), 2.46 (m) (H-8α), 2.37 (m) (H-4 + H-2α), 2.33 (m) (H-2α + H-4), 2.22 (br q, *J* = 10.2) (H-5), 2.16 (d, *J* = 6.8) (*i*-Val), 2.03 (s) (OAc), 1.98 (m, *J* = 6.7) (i-Val), 1.69 (dt, *J* = 6.6; 13.6) (H-2β), 1.18 (br q, *J* = 12.5) (H-8β), 0.99 (d, *J* = 6.7) (*i*-Val)	3378.3 -OH st; 1762.8 C=O st γ-lactone; 1727.8 C=O st ester; 1665.5; 1643.7 C=C st; 1241.0 C-O st as ester acetate; 1142.5 C-O st γ-lactone; 1023.6 C-O st sy ester
Compound **2**	6.03 (d, *J* = 3.4) (H-13), 5.63 (d, *J* = 3.1) (H-13′), 5.38 (s) (H-14), 5.04 (s) (H-14′), 4.99 (br q, *J* = 7.4) (H-3), 4.24 (dd, *J* = 4.9; 11.3) (H-15), 4.05 (dd, *J* = 6.7; 11.3) (H-9), 4.04 (dd, *J* = 4.5; 11.2) (H-15′), 3.88 (dd, *J* = 8.9; 10.2) (H-6), 2.92 (m) (H-7 + H-1), 2.87 (br q, *J* = 7.9) (H-1), 2.45 (m) (H-8α), 2.35 (m) (H-4 + H-2α), 2.32 (m) (H-2α + H-4), 2.22 (q, *J* = 10.1) (H-5), 2.03 (s) (OAc), 1.98 (s) (OAc'), 1.69 (td, *J* = 6.8; 13.6 (H-2β), 1.19 (br q, *J* = 12.3), (H-8β)	3376.6 -OH st; 1760.7 C=O st γ-lactone; 1728.6 C=O st ester; 1664.6; 1644.3 C=C st; 1240.2 C-O st as ester acetate; 1142.4 C-O st γ-lactone; 1025.4 C-O st sy ester

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Appendix A. Supplementary data

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