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A new methodology for a rapid and high-throughput comparison of molecular profiles and biological activity of phytoextracts

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Abstract

To robustly discover and explore phytocompounds, it is necessary to evaluate the interrelationships between the plant species, plant tissue, and the extraction process on the extract composition and to predict its cytotoxicity. The present work evaluated how Fourier Transform InfraRed spectroscopy can acquire the molecular profile of aqueous and ethanol-based extracts obtained from leaves, seeds, and flowers of *Cynara Cardunculus*, and ethanol-based extracts from *Matricaria chamomilla* flowers, as well the impact of these extracts on the viability of mammalian cells. The extract molecular profile enabled to predict the extraction yield, and how the plant species, plant tissue, and extraction process affected the extract's relative composition. The molecular profile obtained from the culture media of cells exposed to extracts enabled to capture its impact on cells metabolism, at a higher sensitivity than the conventional assay used to determine the cell viability. Furthermore, it was possible to detect specific impacts on the cell's metabolism according to plant species, plant tissue, and extraction process. Since spectra were acquired on small volumes of samples (25 µL), after a simple dehydration step, and based on a plate with 96 wells, the method can be applied in a rapid, simple, high-throughput, and economic mode, consequently promoting the discovery of phytocompounds.

KEYWORDS

Cynara cardunculus, FTIR-spectroscopy, *Matricaria chamomilla*, phytocompounds, polyphenols

1 | INTRODUCTION

Due to its accessibility and potential therapeutics, natural compounds extracted from plants have been explored against a high diversity of diseases, including, infections, inflammatory processes, and cancer (Dehelean et al., 2021; de Moraes Mello Boccolini & Siqueira Boccolini, 2020). Indeed, the World Health Organization (WHO) promotes the use of medicinal herbs as remedies to support the absence of conventional treatments (WHO - World Health Organization, 2021). For example, cardoon (*Cynara cardunculus* L.) is a Mediterranean species, widely investigated for its nutraceutical and medicinal properties. The flowers are widely employed in the preparation of cheeses due to their proteases. Infusions of artichoke and cardoon leaves have been used in folk medicine, owing to their

Both authors contributed equally for the work.

hepatoprotective, choleretic and anticholestatic actions (Adzet et al., 1987; Gebhardt, 2001; Valentão et al., 2002). These physiological effects have been mostly attributed to phenolic compounds (Pandino et al., 2011, Valentão et al., 2002) Other bioactive compounds include hydroxycinnamates, and inulin (Cerulli et al., 2022; Pandino et al., 2011; Rocchetti et al., 2020), Cardoon presents antioxidant effects, (Fratianni et al., 2007; Lattanzio et al., 2009; Pérez-García et al., 2000; Pistón et al., 2014) and antidiabetic, antiproliferative, and antimicrobial actions (Falleh et al., 2008; Genovese et al., 2016; Gominho et al., 2018; Kammoun et al., 2010; Ksouri et al., 2012; Mileo et al., 2012; Raccuia & Melilli, 2007; Ramos et al., 2014; Velez et al., 2012). For example, lipophilic leaf extracts showed antiproliferative effects on breast cancer cells, and on the human colorectal cancer cell lines and its knockout variants (Fuhr et al., 2022; Ramos et al., 2014). Another example of a medicinal plant is *Matricaria chamomilla* L. (chamomilla) dried flowers and essential oils, mostly used as aromatic or as a medicinal herb as anti-inflammatory, analgesic, antimicrobial, anti-spasmodic and anticancer (Catani et al., 2021; Masłowski et al., 2021). Chamomilla contains a high diversity of interesting biologically active compounds, including sesquiterpenes, flavonoids, coumarins, vitamins, phenolic acids, and glucosides.

To further explore the active compounds, present in plants, it is relevant to evaluate the impact of the sample pretreatment (e.g., if based on dry or fresh tissues), the plant tissue and species, and the compound extraction method (Bojorquez-Rodríguez, et al., 2022; Ebrahimi et al., 2022). For example, the bioactive compounds isolated from *C. cardunculus* L. depend if the plant is dried or not previously to extraction, and on the solvent used (Falleh et al., 2008; Singh et al., 2011; Srivastava, 2009; Velez et al., 2012). Due to the high interrelationships between the plant species, plant tissue, tissue preprocessing, and extraction procedures, it is advised to optimize the best conditions for a target biological activity based on a design of experiments (DoE) (Mazzara et al., 2021; Moldovan et al., 2019; Monrad, et al., 2012). Furthermore, it is relevant to early test the extract cytotoxicity. Unfortunately, all these molecular and biological characterizations usually are conducted over diverse, time-consuming, and complex analytical techniques. A method enabling a preliminary characterization of the whole molecular compositions of extracts and the extract's potential biological activity, conducted in a rapid, economic, and high-throughput mode, could enable implementations of DoE for screening among all the above-mentioned variables. A high-throughput analysis based on Fourier Transform Mid-Infrared (FT-MIR) spectroscopy presents characteristics that may enable it to achieve those goals. MIR-spectra reflects fundamental vibrational modes of a high diversity of functional groups of biomolecules and therefore, enables to obtaining a molecular fingerprint of highly complex biological samples, as obtained from plant extracts (Nabih et al., 2023; Parlinska-Wojtan et al., 2016) or based on mammalian cell culture to evaluate cytotoxicity (Mohamed et al., 2020).

The main goal of the present study was to develop a new method, to enable the acquisition of the molecular profile of plant extracts and to predict the extract cytotoxicity in a rapid, simple, high-throughput, and economic mode. For that, the molecular profile of diverse samples was acquired by FT-MIR spectroscopy, based on micro volumes of samples (i.e., 25 mL), using a plate with 96 micro-wells, and after a simple sample dehydration step. As a model system, it was considered aqueous and ethanol-based extracts of leaves, seeds, and flowers of *C. cardunculus* and flowers from *M. chamomilla*. The molecular profile of all these extracts was compared to evaluate if the new method was able to predict the impact of the plant species, plant tissue, and extraction procedure on the extract composition. The cytotoxicity of the different extracts was evaluated based on a conventional method of mammalian cell culture, that was subsequently compared with the molecular profile of the culture medium as obtained with FT-MIR spectroscopy.

2 | MATERIALS AND METHODS

2.1 | Plant extracts

The extraction process using ethanol was carried out on 10 g of plant material (leaves, flowers, and seeds). After maceration using a mortar, 100 mL of absolute ethanol (1:10 ratio) was added, and the mixture was kept under agitation (VELP Scientifica, Italy), for 20 h, at room temperature. Subsequently, the extract was filtered using a Buchner funnel and centrifuged (B-Braun Sigma 4k10) at 5000 rpm for 5 min, at 4°C. The solvent was removed using a rota-steam (Heidolph), between 60 and 90 rpm and at a temperature between 50 and 60°C. The dry extract was resuspended using the same volume of 50 mM Tris-HCl buffer, pH 8.3, and maintained at 4°C. The aqueous extract was obtained, based on 10 g of plant tissue (flowers, leaves, and seeds), being macerated using a mortar. After that, it was added 100 mL of 50 mM Tris-HCl and maintained in agitation (VELP Scientifica) at 400 rpm for 4 h, at 4°C. Then, the extracts were centrifuged (B-Braun Sigma 4k10), at 7500 rpm, for 5 min. The supernatant was recovered, filtered, and stored at 4°C.

2.2 | Cytotoxicity assay

Experiments were carried out in 96-well microplates (Nunc, Thermo-Fisher Scientific) maintained at 37°C, 5% CO_2 and 95% humidity (BINDER CB150). Manipulations were carried in laminar vertical flow cabinet (Faster BHG2006). Each well of the microplate was seeded with 1×10^4 HEK 293T cells, in 100 µL of Dulbecco's Modified Eagle Medium high glucose with L-Glutamine (DMEM, Sigma Aldrich,) containing 10% (v/v) of inactivated Fetal Bovine Serum (FBS) (Sigma Aldrich) and 1% of Penicillin-Streptomycin 10,000 U/mL (Gibco). When cells reached ~80% confluence, were exposed to extracts, as follows: 100 µL new DMEM with FBS was added. Was also added to each well 10 µL of plant extracts in Dulbecco's Phosphate Buffer-Saline (DPBS) (Sigma Aldrich). Water-based and ethanol-based extracts (all resuspended in water) from leaves, flowers, and seeds from C. *cardunculus* and ethanol-extracts from flowers of *M. chamomilla*, were tested in quadruplicates, at the following final concentrations: 1%, 4%, and 9% (v/v). Six replicas of the controls containing only DPBS were conducted.

Cellular viability assays were conducted after 24 h, based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, according to the kit manufacturer (Kit-CT02, Sigma-Aldrich), briefly as follows: the medium from each well, on the 96-well microplate, was removed and conserved at −20°C for future analysis by FTIR spectroscopy. It was added to each well, 100 µL of complete DMEM and 10 µL of the MTT reagent. The microplate was incubated for 4 h at 37°C on a CO_2 incubator. After that, 100 µL of isopropanol with 0.04 N HCl was added to each well. After 30 min, the absorbance was read for each well at 570 nm and 630 nm in a microplate reader (Biotek Synergy 2). The A570/A630 ratio is proportional to the number of viable cells in each well. A one-Way analysis of variance (ANOVA) test was performed to assert statistical differences between sample values and control values.

2.3 | FT-MIR spectroscopy

Each sample was analysed in triplicate. For each analysis, 25 µL of the sample was transferred to a 96-well Silicon (Si) plate and then dehydrated for about 2.5 h, in a desiccator under vacuum (Vacuubrand, ME 2). Spectral data was collected using a FTIR spectrometer (Vertex 70, Bruker) equipped with an HTS-XT (Bruker) accessory. Each spectrum represented 64 coadded scans, with a 2 cm^{-1} resolution, and was collected in transmission mode, between 400 and 4000 cm^{-1}. The first well of the 96-well plate did not contain any sample and the corresponding spectrum was acquired and used as background, according to the HTS-XT manufacturer.

2.4 | Spectra pre-processing and processing

All spectra were preprocessed by atmospheric correction or second derivative or with unit vector normalization. Second-order derivative was based on Savitzky-Golay algorithm, with a filter window of 15 data points and a 2nd order polynomial fit. Principal component analysis (PCA) was conducted based on normalized second derivative spectra between 3100 and 2800 cm-1 and 1800 to 900 cm-1. Atmospheric correction was conducted in OPUS® (version 6.5, Bruker), and the remaining preprocessing and PCA were conducted by The Unscrambler® X (version 10.5, CAMO software AS).

Univariate data analysis was based on the following 28 bands from the spectra: 703, 774, 834, 920, 1034, 1080,1034, 1106, 1121, 1152, 1202, 1238, 1267, 1364, 1370, 1402, 1457, 1504, 1552, 1591, 1656, 1740, 1773, 2857, 2875, 2932, 2962, and 3296 cm^{-1}. Diverse spectral ratios between nearby bands were determined. An ANOVA was conducted to evaluate sets of experiments and conducted on Excel (Microsoft).

3 | RESULTS

3.1 | Molecular profile of extracts

The molecular profile of the following extracts was acquired by rapid and high-throughput FT-MIR spectroscopy, after a simple dehydration step: water and ethanol-based extracts from cardoon leaves, flowers, and seeds and ethanol-based extracts from chamomilla flowers (Figures 1

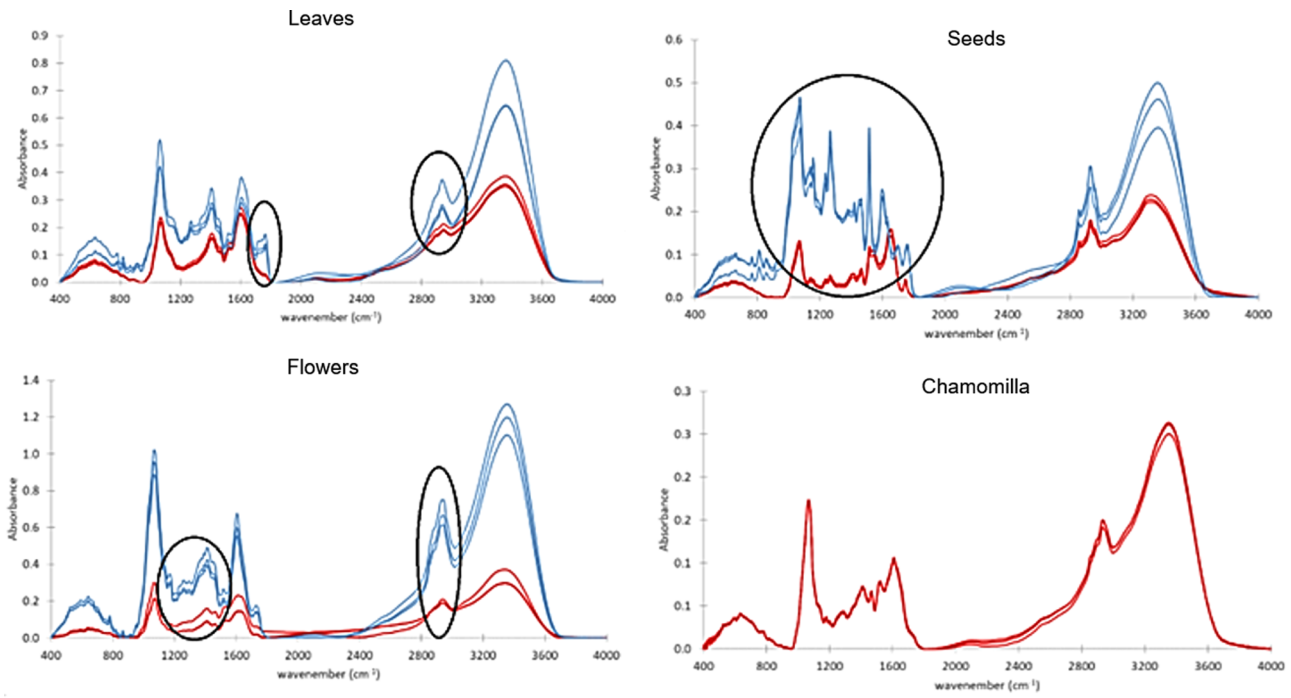

FIGURE 1 Spectra from aqueous (blue) and ethanol-based (red) extracts obtained from cardoon leaves, seeds, and flowers and ethanol extracts from chamomilla flowers. Spectral analytics were conducted in triplicate. Dashed ovals highlight spectra regions more distinct between ethanol and water-based extracts.

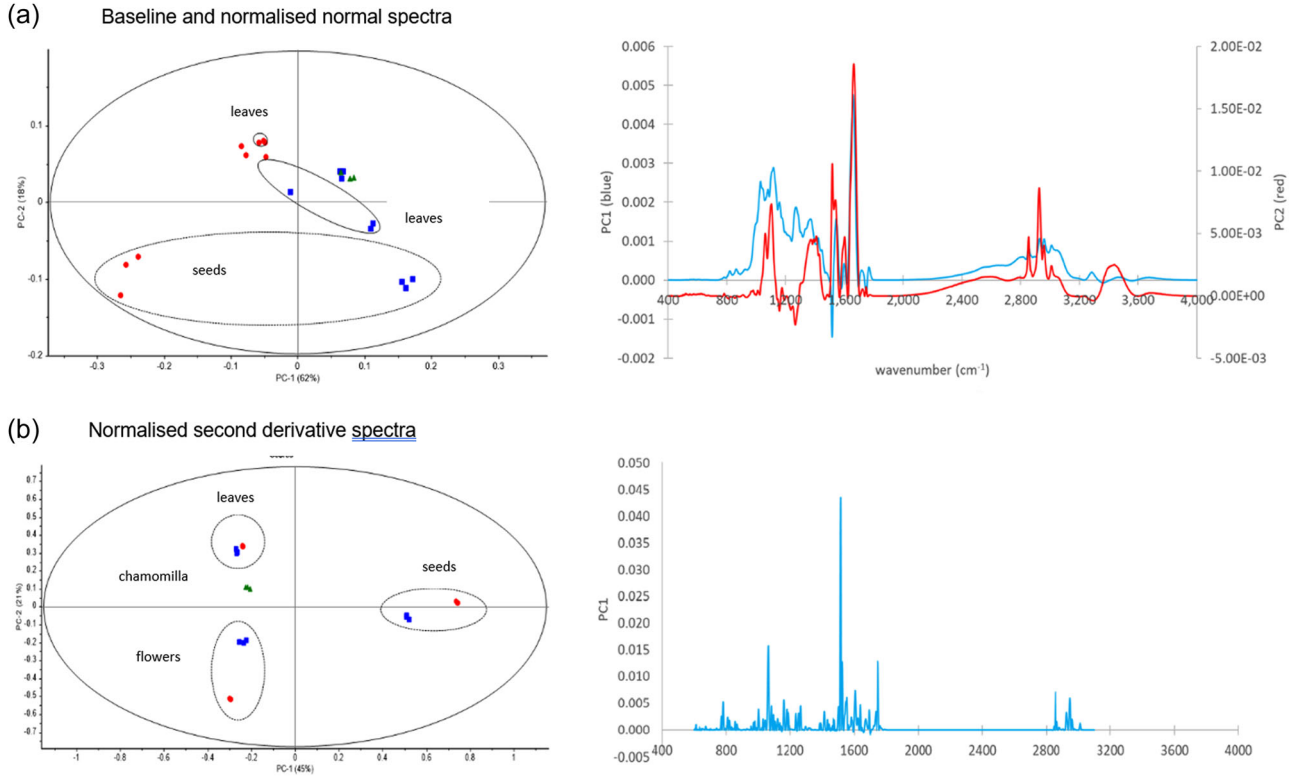

FIGURE 2 Principal component analysis (PCA) from normalized spectra (a), or from normalized second derivative spectra (b) and corresponding loading vectors, obtained from water-based (red) and ethanol-based extracts (blue) from cardoon leaves, flowers and seeds and ethanol-based extracts from chamomilla flowers (green).

& 2). The absorbance values obtained from the spectra are proportional to the quantity of absorbing substances, and therefore the spectra absorbance predicts the quantity of phytocompounds obtained in each extraction procedure, that is the extraction yield. It was observed that aqueous extracts of the cardoon leaves, seeds, and flowers presented a sum of absorbances of all spectral bands of 1.7, 2.2 and 3.3-fold higher in relation to the corresponding ethanol-based extracts, that is the aqueous-based extracts presented between 2 and 3-fold higher yields in phytocompounds in relation to the corresponding ethanol-based extracts.

The highest yield of compounds was obtained with cardoon flowers extracted with water, of 1.7 and 2.1-fold higher, than obtained with cardoon leaves and seeds, respectively. The extracts of cardoon seeds presented the lowest yield in total compounds, independently of the extraction solvent, between 50% and 80% of the obtained with leaves and flowers, respectively. The chamomilla flower extracts resulted in 62% of the compounds obtained with ethanol extracts of cardoon flowers.

All extracts presented distinct spectra, and consequently different molecular compositions (Figure 1), especially between 800 and 1800 cm^{-1}. PCA was based on normalized spectra, since normalization highlights differences in spectra due to sample composition rather than the sample quantity (Figure 2a). The second derivative spectra were also considered to resolve overlapped bands. Therefore, normalized second derivative spectra highlight different biochemical compositions between samples (Figure 2b). The fact that the PCA scores of all extracts are in distinct regions of the score plot, corroborates that all extracts presented distinct molecular compositions. PCA of normalized spectra (Figure 2a) also points out that, ethanol based-extracts presents a distinct molecular composition in relation to the corresponding water-based extracts.

The PCs loading vectors describes how much each spectral band contributes to a specific principal component. Large loadings (positive or negative) indicate that a particular band has a strong relationship with a particular principal component. The loading sign indicates whether the band and a principal component are positively or negatively correlated. The loading vector of PC1 (Figure 2a) pointed the following bands as having a positive contribution in the score's separation of ethanol based-extracts: 1029, 1078, 1118, 1157, 1278, 1372, 1410, 1546, 1611, 1654, 1694, 1773, 2852, 2915, 2939, 3010 cm^{-1}. Since polyphenols presents the strongest contribution on the region between 800 and 1800 cm^{-1} of this type of extracts and between 2850 and 3010 cm^{-1} (Lavecchia et al., 2019; Parlinska-Wojtan et al., 2016) this output is according to ethanol extracts presenting higher yields in polyphenols.

It was observed in both PCA (Figure 2a,b), that seed extracts present the most distinct composition in relation to other extracts since their corresponding scores are the most isolated. For example, analysis of PC2 loading vectors of PCA based on normalized spectra (Figure 2a), pointed out the following bands as significant to discriminate seeds extracts from the remaining extracts: 1063,

1104, 1360, 1516, 1541, 1666, 2856 and 2928 cm^{-1} and 1158 and 1268 cm^{-1}.

Interestingly, PCA based on second derivative normalized spectra, pointed that scores from each tissue plant are closer to each other, independently from the extraction process, and more distant from the other tissue plant (Figure 2b), pointing that each tissue plant presented specific molecular signatures, distinct from other tissues, independently from the extraction process. For a more detailed analysis, each plant tissue was analyzed separately (Figure 3). From loading vectors of PCA, comparing water-based extracts with ethanolic extracts, it was possible to identify the chemical functional groups that are more distinct between extracts for each plant tissue (Table 1).

3.1.1 | Cytotoxicity analysis

The aqueous and ethanol-based extracts were tested in quadruplicate at 1%, 4%, and 9% (v/v) on mammalian cell culture (with Human Embryonic Kidney cells, HEK). From the extract concentrations evaluated, the extracts that significantly affected cell viability based on the conventional MTT assay ($p < 0.05$), were the highest concentrations of cardoon water-based extracts of seeds and leaves (Table 2). This is most probably due to the higher concentration of phytocompounds in the water-based extracts in relation to the ethanol-based extracts (Figure 1), as previously pointed out. At a 10% of significance, all concentrations of aqueous extracts of cardoon leaves and the highest concentration (i.e., 9%) of ethanol-based extracts of cardoon seeds and flowers, affected cell viability.

Spectra preprocessed by atmospheric and baseline correction, from culture media of cells exposed to different extracts, are represented in Figure 4. To search for data pattern among spectra, diverse spectra PCA were conducted (Figures 4–6). The PCA (Figure 4), pointed out that the cell's metabolism was affected by all the extracts at all concentrations evaluated, since scores of controls (i.e., from cells not exposed to extracts, as represented in blue scores) are in the majority localized in a different region of the score-plot than scores from cells exposed to extracts obtained from either ethanol (red dots), and water-based extracts (green dots). Therefore, all extracts had an impact on cell metabolism even if the cell viability, as analyzed by the MTT assay, was not affected.

For simplicity, PCA in Figure 5 represents only cells incubated with the maximum extract concentrations (i.e., at 9%) and the control experiment (i.e., conduct without extracts). The distances between scores in the score-plot pointed that aqueous based-extracts had a general higher impact on cell metabolism than ethanol-based extracts since the green dots corresponding to water based-extractions are in general more distant from blue dots from the control scores, than the distance between the ethanol-based extracts and the control (red vs. blue scores). This is according to the MTT assay that pointed out the unique extracts with a significant impact (at 5% significance) in cell viability in relation to the control to be water-based extracts (Table 2).

Figure 6 represents PCA for each plant tissue at all three concentrations, that is 0%, 1%, 4%, and 9%. The most well-separated scores between different extract concentrations were obtained with cardoon water extracts. Interestingly, this extract was the unique one that at the three concentrations significantly affected cell viability at 10% significance (Table 2). The following extracts also enabled a separation of scores in PCA-plot between the three extract concentrations: water extracts of cardoon leaves and ethanol extracts of chamomilla flowers. This is according to cell viability output, since these two extracts at the highest concentration (9%) affected cell viability at 10% significance. All this corroborates the previous observations, that the molecular signature of the culture medium, reflects the impact of extracts on cells metabolism and viability.

Univariate data analysis of spectral bands was also conducted to quantify differences between the impact of extracts on cell metabolism. To further minimize the effect of baseline drifts and sample quantity, (partially already minimized by baseline correction and normalization, respectively), ratios of spectral bands, that were nearby each other, were considered. The Table S1 presents p-values obtained from comparing 23 bands ratios between group of experiments.

As expected, all the tested extracts (aqueous and ethanol-based from different tissues of both plants) resulted on diverse band ratios statistically different when compared with the control (Table S1, line A-H). Therefore, all extracts had a statistically significant impact on cell metabolism. Interestingly aqueous extracts of cardoon leaves and seeds lead to the highest number of band ratios statistically different in relation to the control (19 and 18, respectively), while the aqueous extract of cardoon flowers presented only 11 bands ratios statistically different in relation to the control. This was according to the MTT assay, which pointed out that the unique extracts that presented a significant effect on cell viability ($p < 0.05$) was the highest concentration of aqueous extracts of cardoon leaves and seeds.

It was also observed that the set of band ratios statistically different depended on the plant (i.e., cardoon vs. chamomilla), tissue plant (i.e., leaves, flowers, and seeds) and mode of extraction (i.e., aqueous vs. ethanolic). For example, the unique extract that impacted significantly ($p < 0.01$) the A1152/A1202 and A1740/A1656 band ratios in relation to the control, was the aqueous extract of cardoon leaves. These bands reflect the medium composition on polysaccharides (since 1152 cm^{-1} reflects mostly C-O and C-OH vibrations), lipids (since 1740 cm^{-1} reflects mostly C = O vibrations in esters) and peptides and proteins (since 1656 cm^{-1} reflects mostly C-O and C-N vibrations in amide I). The extracts that impacted most the spectral band ratios A1457/A1504 and A2857/A2877 were the aqueous extracts of cardoon seeds, reflecting lipids since most of these bands are from CH_2 and CH_3 vibrations. The band ratio A3296/A1656, was mostly affected by aqueous and ethanol extracts from cardoon flowers, reflecting alterations of peptides and proteins (since 1656 cm^{-1} reflects amide I vibrations), and eventually amino acids (since 3296 cm^{-1} reflects NH vibrations). The chamomilla flower

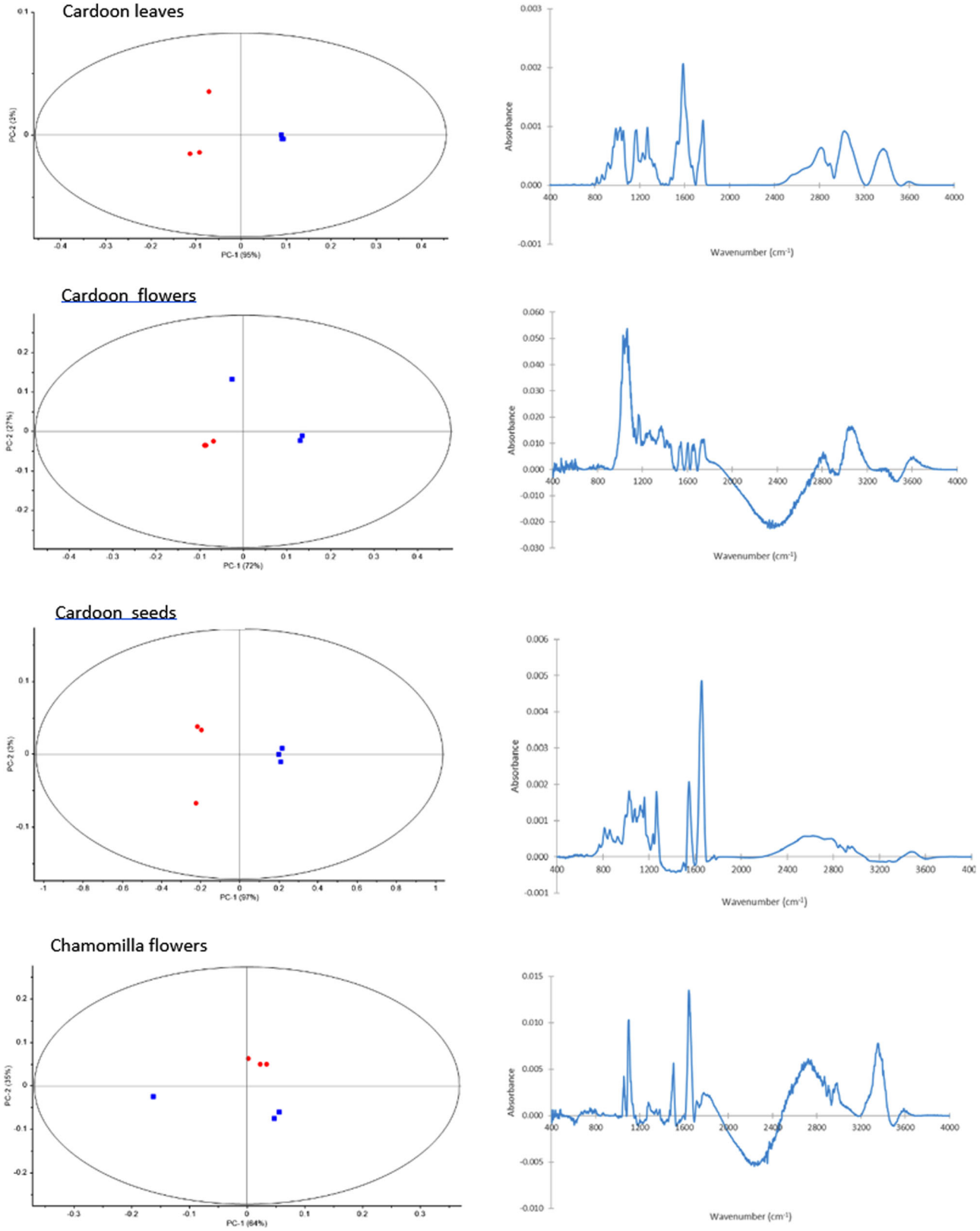

FIGURE 3 Principal component analysis (PCA) of normalized spectra (left panels) and corresponding loading vector (right panels), to compare water and ethanol-based extracts for each plant tissue.

TABLE 1 Bands presented as different between aqueous and ethanolic extracts of cardoon tissues, as observed in the loading vector of principal component analysis represented in Figure 3.

Plant material	Wavenumber present in higher proportion in ethanol extracts (cm^{-1})
Leaves	822 to 989, 1019, 1057, 1161, 1231, 1271, 1327, 1526, 1588, 1667, 1763, 2823, 2894, 3009, 3365.
Flowers	1027, 1058, 1076, 1170, 1261, 1364, 1418, 1537, 1648, 1748, 2809, 3034
Seeds	769, 814, 853, 941, 1000, 1030, 1080, 1128, 1161, 1540, 1660

TABLE 2 Cells viability, due to cells exposition to aqueous and ethanol-based extracts from cardoon leaves, flowers, and seeds and ethanol extracts of chamomilla flowers, based on quadruplicate experiments.

Extract concentration tested		Cells viability (%) average ± standard deviation	*p*-value relative to control
Control (0% extract)		100 ± 13	-
Cardoon leaves ethanol extract	9%	100 ± 14	0.481
	4%	106 ± 5	0.175
	1%	107 ± 21	0.554
Cardoon flowers ethanol extract	9%	95 ± 16	0.617
	4%	102 ± 9	0.819
	1%	109 ± 20	0.387
Cardoon seeds ethanol extract	9%	111 ± 9	0.073
	4%	97 ± 10	0.698
	1%	105 ± 16	0.579
Cardoon leaves water extract	9%	132 ± 25	0.028
	4%	104 ± 15	0.679
	1%	111 ± 20	0.556
Cardoon flowers water extract	9%	116 ± 24	0.202
	4%	89 ± 10	0.168
	1%	92 ± 4	0.277
Cardoon seeds water extract	9%	120 ± 4	0.026
	4%	120 ± 11	0.031
	1%	118 ± 16	0.089
Chamomilla flowers ethanol extract	9%	87 ± 7	0.084
	4%	88 ± 9	0.159
	1%	104 ± 21	0.716

extract was the one that affected most the ratios A1552/A1591 (due to amide II) and A1656/A1591 (due to amide I bands), from peptides and proteins on the culture media.

When comparing all the aqueous extracts of cardoon (including leaves, seeds, and flowers) with the equivalent ethanol-based extracts, it was observed a higher number of spectral ratios ($n = 19$) statistically different between them ($p < 0.01$) (Table S1, line I). Therefore, the different compositions on the ethanol-based extracts (Figure 2), for example usually presenting a higher content in polyphenols (Lavecchia et al., 2019; Marques et al., 2019; Žlabur et al., 2020) had a significant different impact on cell metabolism. Interestingly, ethanol, based extracts had a higher impact on the band ratios A703/A774, while aqueous extracts impacted ratios including bands due to CH_2 and CH_3 vibrations in lipids, as A2932/A2962 and A2857/A2962, and amide bonds from peptides and proteins such as A1552/A1591 and A1656, and phosphate groups as from A1238, among others, pointing the higher impact on cells metabolism based on aqueous extracts as previously highlighted.

Either based on aqueous extracts (Table S1, Lines J-K) or ethanol-based extracts (Table S1, Lines M-O), there were diverse spectral bands statistically different between culture medium from cells exposed to leaves and seeds extracts on the cell's metabolism, and between leaves and flowers, and seeds and flowers. The number of significant bands ratios was higher when comparing the aqueous extracts between them (with 11 to 17 statistically different ratios, $p < 0.01$) in relation to when comparing ethanol-based extracts (with 3–5 statistically different ratios, $p < 0.01$), according to previous observations (Figure 5).

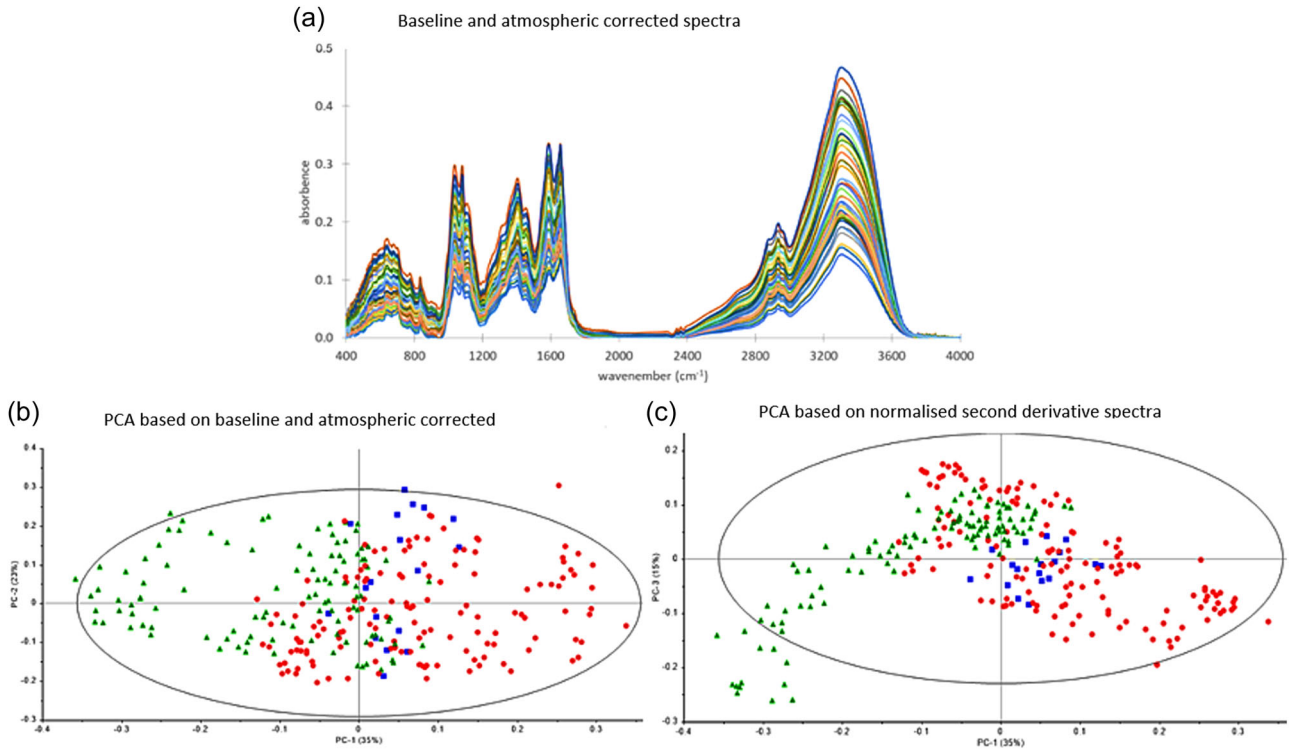

FIGURE 4 Spectra from the culture media from exposed cells, preprocessed by atmospheric and baseline correction (a) and the corresponding principal component analysis (PCA) (b), or the PCA based on normalized second derivative spectra (c). Spectra were obtained from culture media of cells incubated without extracts (blue dots), or with ethanol-based extracts (red dots) or with aqueous-based extracts (green dots), obtained from cardoon leaves, seeds, and flowers, and chamomilla flowers.

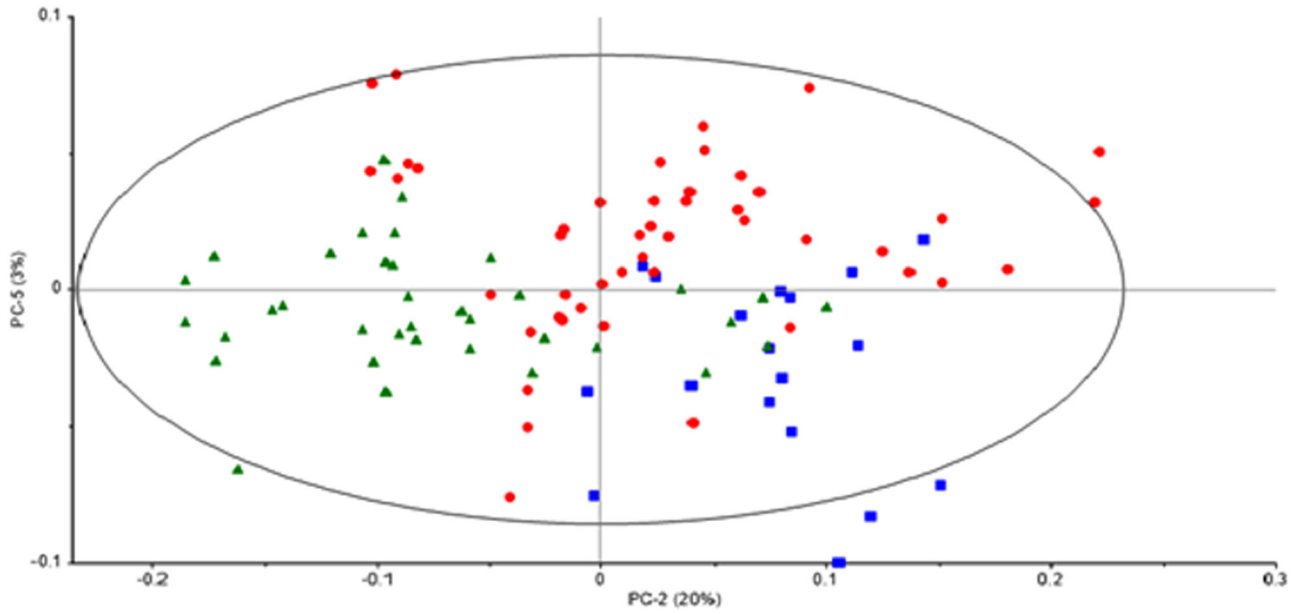

FIGURE 5 Principal component analysis (PCA) from normalized second derivative spectra obtained from culture media of cells incubated without extracts (blue squares), or with extracts at 9% of ethanol-based (red circles) or aqueous-based (green triangles).

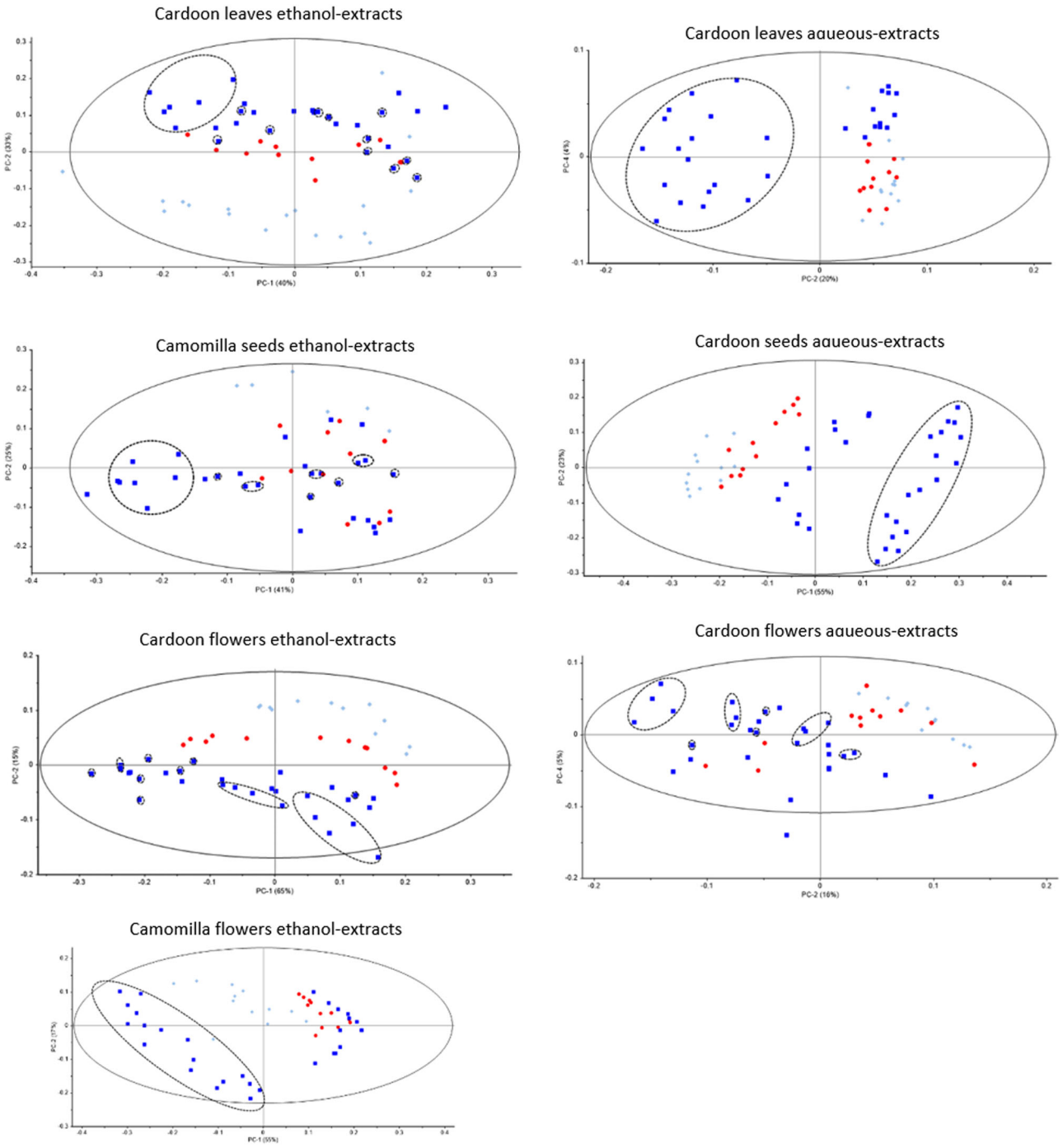

FIGURE 6 Principal component analysis (PCA) of normalized second derivative spectra obtained from culture media of cells incubated without extracts (blue squares surrounded by a dashed oval), or extracts at 1% (blue squares), 4% (red circle) or 9% (light blue diamond) obtained with ethanol or with water-based extracts.

4 | DISCUSSION

4.1 | Molecular profile of extracts

The FT-MIR spectra of aqueous and ethanolic-based extracts from cardoon leaves, seeds, and flowers and ethanol extracts from chamomilla flowers (Figure 1), pointed that the composition of the extracts was significantly different between them, especially between 800 and 1800 cm^{-1} (Figure 1), characteristic of polyphenols (Lavecchia et al., 2019). These predictions are according to the observations of other authors, that different cardoon tissues present different compositions on polyphenols (Falleh et al., 2008; Srivastava, 2009) and that are also different from chamomilla flowers (Pagliari et al., 2022).

From the spectra, it was also predicted that aqueous extracts presented 2 to 3-fold higher yields of phytocompounds in relation to ethanol-based extracts, according to other authors (Hajji Nabih et al., 2023).

The spectra PCA (Figures 2 and 3) pointed that ethanol and aqueous-based extracts presented different compositions, where the PCA loading pointed that ethanol extracts presented higher yields in polyphenols, in relation to the corresponding aqueous-based extract, according to other authors' observations (Hajji Nabih et al., 2023; Lavecchia et al., 2019; Marques et al., 2019; Žlabur et al., 2020). From the PCA of each plant tissue (Figure 3) it was also possible to predict that the ethanol-based extracts presented a different content of polyphenols in relation to the corresponding aqueous-based extracts, according to other authors that pointed ethanol extracts containing different polyphenols, including for example flavonoids and tannins, among others (Adegbaju et al., 2020; Hajji Nabih et al., 2023).

Also, from the spectra PCA, it was predicted that, each plant tissue leads to extracts with specific molecular compositions, distinct from other tissues, independently of the extraction process. This is according to other authors pointing out, for example, that aqueous and ethanolic extracts from cardoon flowers do not present terpenes and present lower yields in glycosides concerning aqueous and ethanolic extracts from chamomilla flowers (Adegbaju et al., 2020). In opposition, alkaloids were found in all extracts of cardoon flowers, but not on chamomilla extracts (Adegbaju et al., 2020).

Therefore, FT-MIR spectroscopy enabled to predict the yield in phytocompounds obtained during the extraction process and to compare the extract molecular profile obtained according to the plant species, plant tissue, and extraction process.

4.1.1 | Cytotoxicity analysis

The PCA and bands ratios of spectral bands of media of mammalian cell culture exposed to different extracts, enabled to predict the impact of the extract on the cell's metabolism, according to other authors as for example Rosa et al. (2016), that were able to predict cells metabolic state based on FT-MIR spectroscopy analysis of the media (Figures 4–6 and Table S1). It was also observed that the spectra detected the impact on the cell's metabolism even when the cell viability was not affected, as analysed by the conventional MTT assay. This highlights the high sensitivity of the FT-MIR spectroscopy in acquiring the cells' metabolic state. This is according to other authors, pointing FTIR spectroscopy advantages to monitor cytotoxicity (Fale et al., 2015; Martin et al., 2010; Rodriguez et al., 2002; Rosa et al., 2015).

Interestingly, based on the spectra of the culture media, the extract with a higher impact on the cell's metabolism, was according to data from the MTT conventional assay (Table 2), and to the fact that aqueous-based extracts presented higher quantities of phytocompounds than the corresponding ethanol-based extracts. Furthermore, based on PCA and on bands ratios, it was observed that the impact on the metabolism was affected by the plant species, plant tissue, and extraction process, highlighting the new method's high specificity.

5 | CONCLUSIONS

The direct analysis of plant extracts by FT-MIR spectroscopy, enabled to predict the phytocompounds yield obtained per extraction procedure, and that extracts from a specific plant tissue obtained from aqueous and ethanol-based extractions, presented similar compounds between them, in relation to extracts from other plant-tissue. It was also predicted from the spectra PCA, that ethanol-based extracts presented a higher yield in polyphenols, and that the types of polyphenols were different from the ones obtained in aqueous-based extracts. All these predictions were according to other authors' observations. Therefore, FT-MIR spectroscopy enabled to compare the chemical composition between extracts, that is enabled to predict the impact of variables such as plant species, plant tissues, and extraction processes on the relative extract chemical composition.

For samples with complex biological compositions, such as phytocompounds obtained from plant extractions, FT-MIR spectroscopy, applied in this format, will not enable to identify, and consequently quantify, a target molecule, as usually obtained by other techniques, for example based on gas or liquid chromatography associated to mass spectrometry or based on nuclear magnetic resonance. Despite this, and in a much rapid, simple, high-throughput and economic mode, FT-MIR spectroscopy enabled to relatively compare the extracts' molecular profile, in a very sensitive and specific mode, according to the plant species, plant-tissue and extraction process.

FT-MIR spectroscopy also enabled to predict the impact of the extract exposition on the metabolism of mammalian cells. This analysis was according to the conventional assay used to predict the cells viability, based on MTT, which besides time-consuming is very expensive. The high sensitivity of the FT-MIR-spectroscopy new method, enabled to monitor the impact on cell metabolism, even in conditions where the cell viability was not affected, while the method's high specificity enabled to predict the impact on cell metabolism according to the plant species, plant tissue, and extraction method.

All the analyses referred above were conducted over small volume samples (25 µL), after a simple dehydration step, using microplates with 96 wells. The simple and high-throughput formats, associated to a small sample volume for analysis, will promote the screening of high number of combinations of sample pre-treatments, extraction procedures, plant species and plant tissues, as based on DoE. Furthermore, the sample low volumes needed for analysis reduces the associated costs and the biological material needed for all these screening studies, while enabling increasing replicate experiments, that is enabling robust and economic optimization procedures.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in PubMed at https://pubmed.ncbi.nlm.nih.gov/.

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