**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 diverse variables that affect the composition of the obtained compounds mixtures, such as the plant species, plant tissue and the phytocompounds extraction process. Furthermore, it is relevant to evaluate the biological activity associated to the high diversity of biocompounds mixtures obtained along these processes, including cytotoxicity. The present work evaluates how Fourier Transform Infra-Red (FTIR) spectroscopy can be used to acquire in a simple, rapid, economic, and high-throughput mode the whole molecular fingerprint of aqueous and ethanolic extracts obtained from leaves, seeds and flowers of *Cynara cardunculus*, and ethanolic extracts from *Matricaria chamomilla* flowers. The impact of plant species, plant tissue, and extraction procedure on phytocompounds yield and whole molecular composition was evaluated. FTIR-spectroscopy was also applied to study the effect of each extract on animal cell metabolism, and to compare this activity of different extracts. FTIR-spectra were acquired in automatic mode based on a small sample volume (25 μL) on 96-wells microplate. The low reduced volumes will further reduce costs and the quantity of biological material needed for this type of analysis while enabling to increase the diversity of conditions screened to achieve. This type of assay can therefore promote the discovery of phytocompounds.

**Keywords:** Phytocompounds; FTIR-spectroscopy; Polyphenols; Extraction procedures; *Cynara cardunculus*, *Matricaria chamomilla*.

**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 [1]. Indeed, the World Health Organization promotes the use of medicinal herbs as remedies to support the absence of conventional treatment [2]. 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 hepatoprotective, choleretic and anticholestatic actions [3-5]. These physiological effects have been mostly attributed to phenolic compounds [5]. Other bioactive compounds include hydroxycinnamates, [6,7], and inulin [8]. Cardoon presents antioxidant effects, [9-12] antidiabetic, antiproliferative, and antimicrobial [13-21]. For example, lipophilic leaf extract showed antiproliferative effects on breast cancer cells, and on the human colorectal cancer cell lines and its knockout variants [22]. Considering all these potential effects of different cardoon tissues. Another example of a medicinal plant is *Matricaria chamomilla* L. (chamomilla) dried flowers and essential oils, mostly used as an aromatic or medicinal herb as anti-inflammatory, analgesic, antimicrobial, antispasmic and anticancer [23-25]. 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 sample pre-treatment (e.g., if based on dry or fresh tissues), the plant tissue and species, and the compound extraction conditions and method [26,27]. For example, the cardoon species, and if the plant is dried before and the solvent used for extraction affects the bioactive molecules isolated from *Cynara cardunculus* L. [17,28-30]. Furthermore, for further application, it is relevant to test the cytotoxicity of the extract. Due to the high interrelationships between plant species, plant-tissue, tissue pre-processing and extraction procedures, it is advised to optimise the best conditions for a defined biological activity based on a design of experiments (DoE) [31-33]. 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 potential biological activity, conduct in a rapid, economic and high-throughput mode, could enabled 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 to achieve those goals. MIR-spectra reflect fundamental vibrational modes of a high diversity of functional groups of biomolecules and therefore, enable to obtain a molecular fingerprint of highly complex biological samples, as obtained from plants extracts [34-36],or based on animal cells culture to evaluate cytotoxicity [36].

The main aim of the present study was to acquire by high-throughput FT-MIR spectroscopy, the molecular fingerprint of mixtures of bioactive compounds, obtained from extracts based on aqueous and ethanolic extractions of leaves, seeds, and flowers of *C. cardunculus* and flowers from *M. chamomilla.* The molecular profile of these extracts will be compared between them. The cytotoxicity of the different extracts will be evaluated based on a conventional method on animal cell culture and compared with the culture medium molecular profile acquired by FT-MIR spectroscopy. It is therefore aimed to promote the development of methods enabling to explore the potential of medicinal herbs.

**MATERIALS AND METHODS**

***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, Germany) (5000 rpm, 5 minutes, at 4 °C). The solvent was removed using a rota-steam (Heidolph), between 60-90 rpm and at a temperature between 50-60°C. The dry extract was resuspended using the same volume of 50 mM Tris-HCl buffer, pH 8.3, and maintained at 4ºC. 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, Italy) at 400 rpm for 4 h, at 4ºC. Then, the extracts were centrifuged (B-Braun Sigma 4k10, Germany), at 7500 rpm, for 5 minutes. The supernatant was recovered, filtered, and stored at 4°C.

***Cytotoxicity assay***

Experiments were carried out in 96-well microplates (Nunc, ThermoFisher Scientific, USA) maintained at 37ºC, 5% CO2 and 95% humidity (BINDER CB150, Germany). Manipulations were carried in laminar vertical flow cabinet (Faster BHG2006, Italy). Each well of the microplate was seeded with 1x104 HEK 293T cells, in 100µL of Dulbecco’s Modified Eagle Medium high glucose with L-Glutamine (DMEM, Sigma Aldrich, Germany) containing 10% (v/v) of inactivated Fetal Bovine Serum (FBS) (Sigma Aldrich, Germany) and 1% of Penicillin-Streptomycin 10,000 U/mL (Gibco, USA). 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 10µL of plant extracts in Dulbecco’s Phosphate Buffer-Saline (DPBS) (Sigma Aldrich, Germany). 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 contained only DPBS were conducted.

Cells viability assay was conducted after 24 hrs, based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, according to the kit manufacturer (Kit-CT02, Sigma-Aldrich, Germany), briefly as follows: the medium from each well, on the 96-well microplate, was removed and conserved at -20ºC for future analysis by FTIR spectroscopy. It was added to each well, 100µL of complete DMEM and 10 µL of the MTT reagent. The microplate was incubated for 4 hours at 37ºC on a CO2 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, USA). The A570/A630 ratio should be 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.

***FT-MIR spectroscopy***

Each sample was analysed in triplicate. For each analysis, 25 μL of sample was transferred to a 96-wells 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-wells plate did not contain a sample and the corresponding spectra was acquired and used as background, according to the HTS-XT manufacturer.

***Spectra pre-processing and processing***

All spectra were pre-processed 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 to 2800 cm-1 and 1800 to 900 cm-1. Atmospheric correction was conducted in OPUS® (version 6.5, Bruker, Germany), and the remaining pre-processing and PCA were conducted by The Unscrambler® X (version 10.5, CAMO software AS, Norway).

Univariate data analysis was considered 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).

**Results and Discussion**

***Extracts molecular profile***

The molecular profile of the following extracts were 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 extracts from chamomilla flowers (Fig. 1 & 2). Based on spectra, it was observed that aqueous extracts present a higher yield of compounds in relation to ethanolic extracts, according to other authors [34]. The cardoon leaves, seeds and flowers aqueous extracts 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. 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 to 80% of the yields obtained with leaves and flowers, respectively. The chamomilla flowers extracts resulted in 62% of the compounds yield obtained with ethanol-extracts of cardoon flowers.

Chart, histogram

Description automatically generated

Chamomilla

Leaves

Flowers

Seeds

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

All extracts presented distinct spectra, and consequently different molecular compositions (Fig.1), especially between 800 to 1800 cm-1 [38,39,40], characteristic of polyphenols [37]. This is according to that different cardoon tissues presenting different compositions on polyphenols [17,28], and that are also different from chamomilla flowers [38]. The comparations between extracts were further explored by spectra PCA.

PCA was based on normalised spectra, since normalization highlights differences in spectra due to sample composition rather than the sample quantity (Fig.2A). It was evaluated second derivative spectra, to resolve overlap bands. Therefore, normalized second derivative spectra highlight different biochemical composition between sample (Fig.2B). The fact that the PCA scores of all extracts are in distinct regions of the score plot, corroborates that all extracts present distinct compositions. PCA of normalized spectra (Fig.2A) also points that, ethanol extracts present a distinct molecular composition in relation to the corresponding water-based extracts. This is according to other authors pointing that ethanol based extracts usually lead, to a different content in polyphenols [34,37,42,43].

Chart, diagram

Description automatically generated

1. Baseline and normalised normal spectra

leaves

chamomilla

leaves

seeds

leaves

seeds

flowers

seeds

1. Normalised second derivative spectra

**Fig.2 -** PCA from normalized spectra (A), or from normalised 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).

The PCs loading vectors describe how much each variable contributes to a specific principal component. Large loadings (positive or negative) indicate that a particular variable has a strong relationship with a particular principal component. The loading sign indicates whether a variable and a principal component are positively or negatively correlated. The loading vector of PC1 (Fig.2A) points the following bands as having a positive contribution in the score’s separation of ethanol 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 to 1800 cm-1 of this type of extracts and between 2850-3010 cm-1 [35,37], this output is according to other authors that ethanol extracts presents higher yields in polyphenols [34,37,42,43].

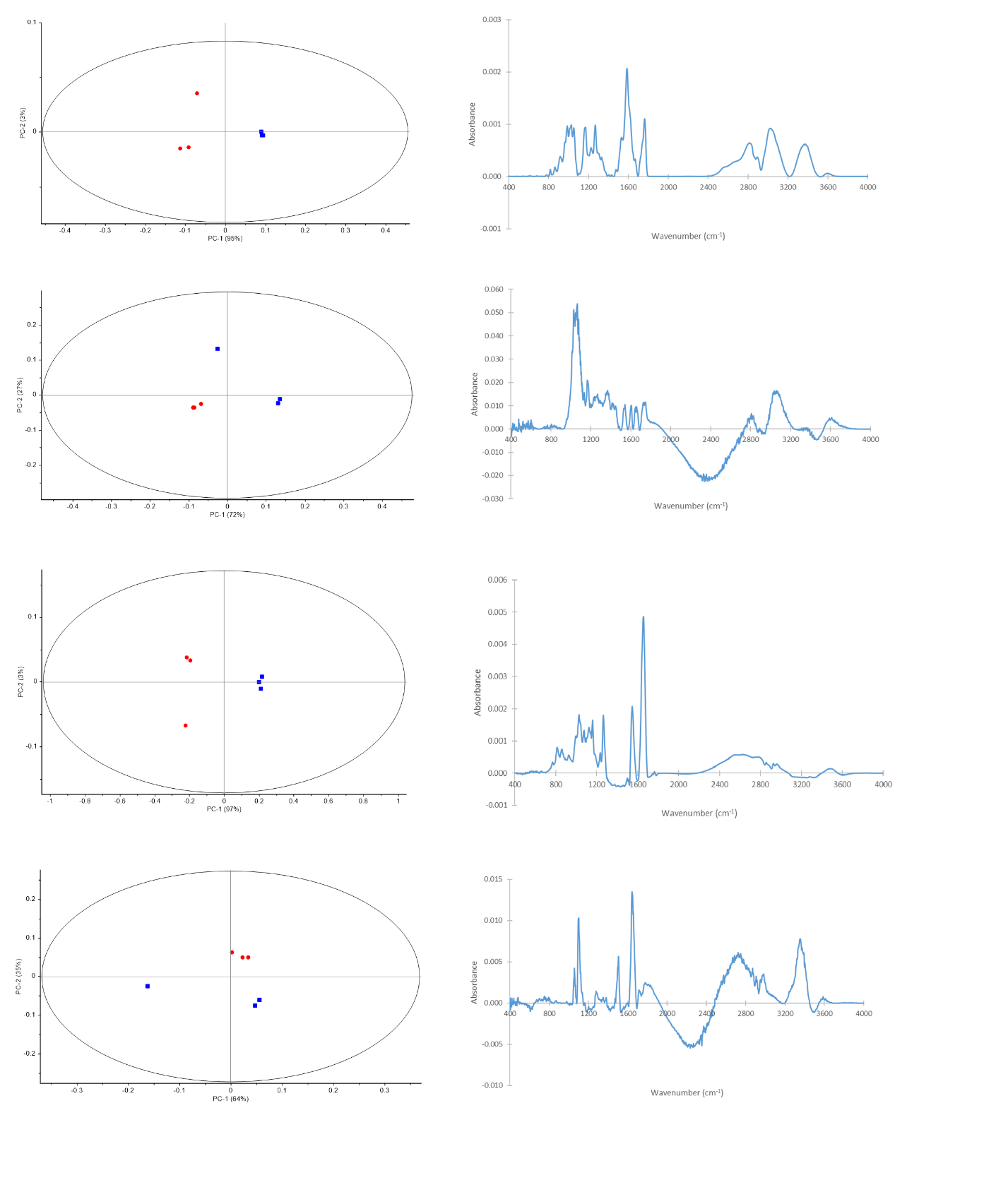
For a more detailed analysis, each plant tissue was analysed separately (Fig.3). From loading vectors of PCA, comparing water-based extracts with ethanolic extracts, it was possible to identify the chemical functional groups that are more distinct between extracts for each plant tissue (Table 1). These specific signatures are according to ethanol extracts obtaining different yields of different types of polyphenols, including e.g. flavonoids and tannins, among others [34,44].

**Table 1**- Bands presented as different between aqueous and ethanolic extracts of cardoon tissues, as observed in the loading vector of PCA represented in Fig.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** |

It was observed in both PCA (Fig.2A, 2B), that seed extracts present the most distinct composition in relation to other extracts since its corresponding scores are the most isolated. For example, analysis of PC2 loading vectors of PCA based on normalized spectra (Fig.2A), points 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, points that scores from each tissue plant are closer to each other, independently from the extraction process, and more distant from the other tissue plant (Fig.2B), pointing that each tissue plant presents specific molecular signatures, distinct from other tissues, independently from the extraction process. This is according to other authors pointed 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 [44]. In opposition, alkaloids were found in all extracts of cardoon flowers, but not on chamomilla extracts [44].

Cardoon leaves



Chamomilla flowers

Cardoon seeds

Cardoon flowers

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

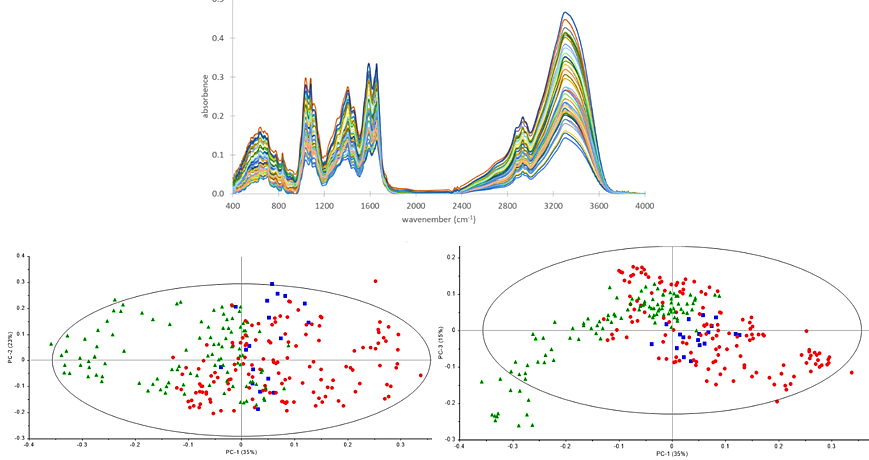
**Cytotoxicity analysis**

The aqueous and ethanol-based extracts were tested in quadruplicate at 1%, 4% and 9% (v/v) on animal cell culture (HEK). From the concentrations evaluated, the extracts that significantly affected, at 5% significance, cell viability, based on the MTT assay, 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 bioactive compounds in the water-based extracts in relation to the ethanol-based extracts, as previously pointed out (Fig.1). At 10% significance, all concentrations of aqueous extracts of cardoon leaves affected cells viability, and the highest concentration (i.e., 9%) of ethanol-based extracts of cardoon seeds and flowers.

**Table 2-** Cells viability, due to cell exposition to aqueous and ethanol-based extracts from cardoon leaves, flowers, and seeds and ethanol extracts of chamomilla flowers.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Extract concentration tested | Cells viability (%) | Average | Standard deviation | *p*-value relative to control |
| Control (0% extract) | 111 97 90 101 117 83 | 100 | 13 | - |
| 9% Leaves-ethanol | 121 92 98 91 | 100 | 14 | 0.481 |
| 4% | 104 104 102 113 | 106 | 5 | 0.175 |
| 1% | 136 86 101 103 | 107 | 21 | 0.554 |
| 9% Flowers-ethanol | 74 101 93 113 | 95 | 16 | 0.617 |
| 4% | 90 110 108 99 | 102 | 9 | 0.819 |
| 1% | 128 82 109 118 | 109 | 20 | 0.387 |
| 9% Seeds- ethanol | 119 120 101 105 | 111 | 9 | 0.073 |
| 4% | 89 102 108 89 | 97 | 10 | 0.698 |
| 1% | 129 98 93 101 | 105 | 16 | 0.579 |
| 9% Leaves-water | 115 107 144 161 | 132 | 25 | **0.028** |
| 4% | 93 114 119 89 | 104 | 15 | 0.679 |
| 1% | 115 98 93 136 | 111 | 20 | 0.556 |
| 9% Flowers-water | 88 108 123 144 | 116 | 24 | 0.202 |
| 4% | 88 78 88 101 | 89 | 10 | 0.168 |
| 1% | 98 92 88 91 | 92 | 4 | 0.277 |
| 9% Seeds – water | 115 110 131 126 | 120 | 10 | **0.026** |
| 4% | 122 109 134 115 | 120 | 11 | **0.031** |
| 1% | 135 119 122 96 | 118 | 16 | 0.089 |
| 9% Flowers-ethanol | 89 82 87 90 | 87 | 7 | 0.084 |
| 4% from chamomilla | 82 87 102 83 | 88 | 9 | 0.159 |
| 1% | 73 119 125 100 | 104 | 23 | 0.716 |

Spectra pre-processed by atmospheric and baseline correction, from culture media of cells exposed to different extracts, are represented in Fig.4. To search for data pattern among spectra, diverse spectra PCA were conducted (Fig.4-6).

**Fig.4 -** Spectra from the culture media from exposed cells, pre-processed by atmospheric and baseline correction (A) and corresponding 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 extracts obtained from water (green dots), obtained from cardoon leaves, seed, and flowers and chamomilla flowers.

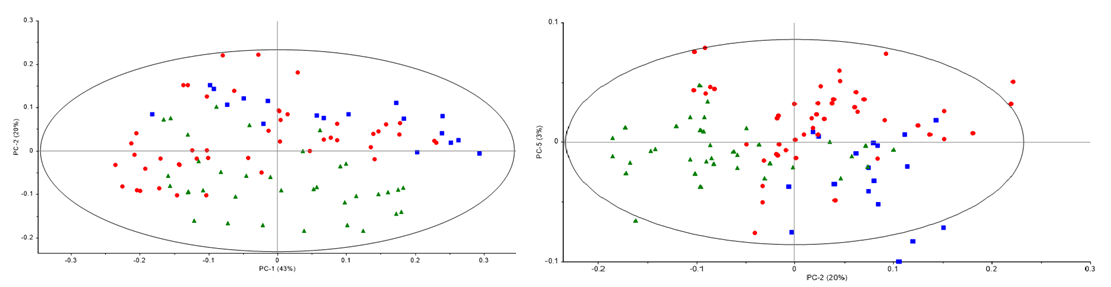
B) PCA based on baseline and atmospheric corrected spectra

C) PCA based on normalised second derivative spectra pectra

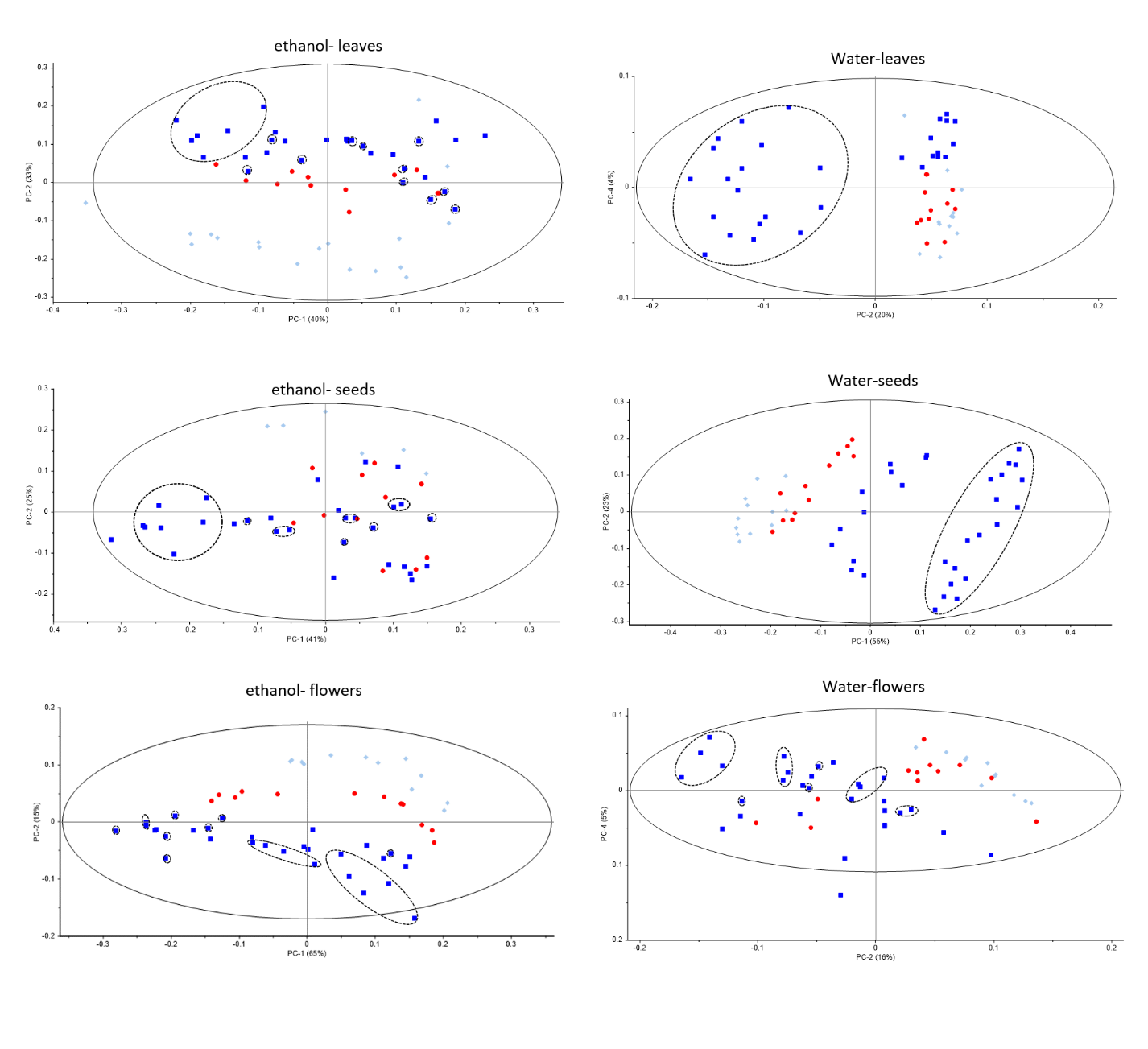
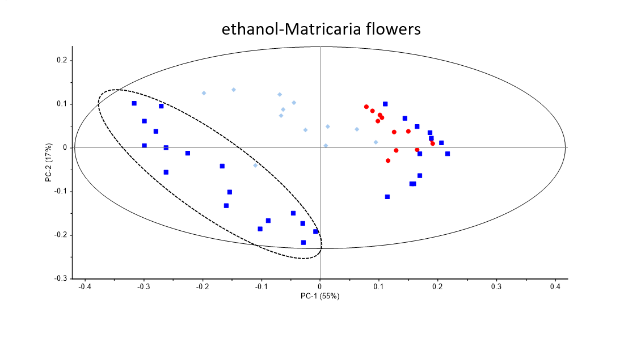
A) Baseline and atmospheric corrected spectra

Spectra PCA, represented in Fig.4, points that the cell's metabolism was affected by all the extracts at all concentrations evaluated, since score plots 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), either from water-based extracts (green dots). Therefore, all extracts had an impact on cell metabolism even if the cell viability, as analysed by the MTT assay, was not affected. This points to the high sensitivity of the FTIR spectroscopic technique in acquiring the impact of these biocompounds on the cell’s metabolism, in a much higher sensitivity than the MTT assay. This is according to other authors, pointing FTIR spectroscopy advantages to monitor cytotoxicity [45-48].

For simplicity, PCA in Fig.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 point 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 versus 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).



**Fig.5** - 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). Extracts from cardoon leaves, seeds, and flowers and from chamomilla flowers were considered.

Fig.6 represents PCA for each plant tissue at all three concentrations, *i.e.*, 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 cells 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 chamomilla flowers ethanol extracts. This is according to cells viability output, since these two extracts at the highest concentration (9%) affected cells 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.****

Cardoon leaves aqueous-extracts

Cardoon seeds aqueous-extracts

Cardoon flowers aqueous-extracts

Camomilla seeds ethanol-extracts

Cardoon flowers ethanol-extracts

Camomilla flowers ethanol-extracts

Cardoon leaves ethanol-extracts

**Fig.6 -** 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. Extracts from cardoon leaves, seed, and flowers and chamomilla flowers were considered.

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 minimised by baseline correction and normalization, respectively), ratios of spectral bands, that were nearby each other, were considered. Table 3 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 3, line A-H). Therefore, all extracts had a statistically significant impact on cell metabolism. Interestingly cardoon leaves and seeds aqueous extracts lead to the highest number of band ratios statistically different in relation to the control (19 and 18, respectively), while the cardoon flowers aqueous extract presented only 11 bands ratios statistically different in relation to the control. This was according to the MTT assay, that pointed as the unique extracts presented a significant effect on cells viability (p<0.05) the highest concentration of cardoon leaves and seeds aqueous extracts.

It was also observed that the set of band ratios statistically different depended on the plant (i.e., cardoon versus chamomilla), tissue plant (i.e., leaves, flowers, and seeds) and mode of extraction (i.e., aqueous versus ethanolic). Therefore, all the extracts had a specific impact of cells metabolism, i.e., each extracted affected a different set of metabolic pathways. 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 cardoon leaves aqueous extract. These bands reflect an impact on 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). While the extract that impacted most the spectral band ratios A1457/A1504 and A2857/A2877 was the cardoon seeds aqueous extracts, reflecting the impact on lipids in the culture medium since most of these bands are from CH2 and CH3 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 aminoacids (since 3296 cm-1 reflects NH vibrations). The chamomilla flowers 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. All this specificity, i.e., where each extract presents a specific impact on cells metabolism, is according to the fact that each extract also presented a specific molecular composition as previously observed.

When comparing all the cardoon aqueous extracts (including leaves, seeds and flowers) with the equivalent ethanol-based extracts, it was observed a higher spectral ratio (n=19) statistically different between them (p<0.01) (Table 3, line I). Therefore, the different composition on the ethanol-based extracts (Fig.2), e.g., usually presenting a higher content in polyphenols [37,42,43], had a significant different impact on cells metabolism. Interestingly, ethanol, based extracts had a higher impact on the band ratios A703/A774, associated to the fingerprint regions, which reflects a high diversity of chemical functional groups, while aqueous extracts impacted ratios including bands due to CH2 and CH3 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.

**Table 3.** Statistical significance (p-values) of ratios of spectral bands, obtained from culture media of cells exposed to cardoon and chamomilla extracts. A total of 23 ratios of spectral bands were evaluated. **Is highlighted in bold, p-values <0.01.**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| A | Ratios of spectral bands →  Set of experiments under comparison 🡣 | n | 703/ 774 | 774/ 834 | 1034/ 1080 | 1034/ 920 | 1106/ 1121 | 1152/ 1202 | 1238/ 1267 | 1364/ 1370 | 1402/ 1457 | 1457/ 1504 | 1552/ 1591 | 1656/ 1591 | 1740/ 1773 | 1740/ 1656 | 1030/ 1656 | 1080/ 1656 | 1238/ 1656 | 2857/ 2875 | 2857/ 2932 | 2857/ 2962 | 2932/ 2962 | 2962/ 3296 | 3296/ 1656 |
| B | cardoon leaves aqueous extract vs. control | 19 | 2.80E-01 | 9.40E-01 | **6.10E-04** | 8.20E-02 | **3.10E-11** | **3.00E-04** | **4.60E-03** | **7.90E-05** | 9.10E-01 | **5.60E-03** | **1.60E-06** | **2.20E-03** | **8.70E-05** | **1.50E-06** | **4.50E-05** | **5.40E-05** | **5.60E-05** | **2.70E-04** | **7.40E-03** | **9.10E-05** | **7.40E-08** | **8.20E-04** | **6.80E-03** |
| C | cardoon seeds aqueous extract vs. control | 18 | 8.00E-01 | **1.00E-03** | **2.10E-14** | 8.90E-01 | **1.70E-13** | 4.80E-01 | **4.60E-06** | **7.40E-11** | **2.70E-05** | **2.60E-10** | **4.90E-07** | **2.10E-05** | **3.10E-04** | 6.40E-02 | **1.40E-07** | **1.60E-06** | **1.50E-06** | **5.50E-12** | 7.40E-02 | **1.60E-06** | **1.30E-14** | **1.50E-09** | **1.40E-05** |
| D | cardoon flowers aqueous extract vs. control | 11 | 1.00E+00 | 5.90E-02 | **1.60E-03** | 9.90E-01 | **7.80E-08** | 4.00E-01 | 2.40E-01 | **2.40E-03** | 6.00E-02 | 6.90E-01 | **1.00E-03** | 6.60E-02 | 5.60E-01 | 9.30E-01 | **4.30E-05** | **3.90E-06** | 4.70E-02 | **3.10E-03** | 5.70E-02 | **1.50E-04** | **8.40E-11** | **2.40E-04** | **2.60E-11** |
| E | cardoon leaves ethanol extract vs. control | 12 | **9.90E-03** | **2.60E-06** | **5.10E-06** | 2.00E-01 | 2.40E-02 | 5.70E-01 | 3.90E-01 | 1.50E-01 | **8.30E-03** | 9.30E-02 | 5.90E-01 | **4.10E-03** | 1.50E-01 | 2.20E-01 | **1.30E-03** | **2.90E-03** | 1.70E-01 | **1.20E-03** | **9.10E-04** | **1.00E-03** | 2.50E-01 | **3.50E-03** | **4.30E-05** |
| F | cardoon seeds ethanol extract vs. control | 8 | 7.40E-02 | **5.20E-03** | **4.90E-08** | 6.10E-01 | **1.90E-04** | 8.80E-01 | 5.50E-01 | 1.50E-02 | **1.00E-04** | 2.10E-02 | 1.30E-01 | 9.60E-01 | 1.40E-01 | 3.30E-01 | **6.20E-03** | 2.70E-02 | 1.60E-01 | **5.60E-03** | 2.30E-02 | 1.70E-02 | 2.00E-01 | **1.90E-04** | **1.40E-03** |
| G | cardoon flowers ethanol extract vs. control | 12 | **9.10E-04** | **1.50E-09** | 3.20E-01 | 2.60E-01 | **2.80E-04** | 3.80E-01 | 4.40E-01 | **1.20E-03** | **4.60E-07** | 3.50E-01 | 3.30E-01 | 9.10E-02 | 2.40E-01 | 2.40E-01 | **9.00E-04** | **5.60E-04** | 3.70E-01 | **9.50E-04** | **1.30E-03** | **1.70E-03** | 3.80E-01 | **2.20E-03** | **6.40E-08** |
| H | camomilla flowers ethanol extract vs. control | 10 | 5.50E-02 | 6.50E-01 | **3.00E-11** | 5.30E-01 | **1.30E-10** | 3.30E-01 | 3.40E-02 | **2.60E-07** | 3.70E-02 | **7.90E-07** | **8.40E-11** | **4.80E-11** | 6.60E-01 | 5.00E-02 | 7.30E-01 | 1.70E-01 | 1.20E-01 | **2.90E-06** | **3.90E-03** | **1.80E-03** | 5.40E-02 | **5.10E-05** | 6.50E-01 |
| I | all cardoon aqueous extracts vs all cardoon ethanol extracts | 19 | **1.30E-07** | **2.20E-17** | 5.50E-01 | 2.40E-01 | **1.70E-22** | **1.00E-04** | 5.60E-01 | **8.40E-17** | **3.60E-06** | **4.70E-07** | **8.30E-32** | **1.40E-20** | **7.80E-15** | **1.30E-11** | **5.10E-08** | **2.30E-09** | **5.60E-11** | **6.30E-08** | **3.80E-03** | **3.60E-06** | **7.00E-30** | **6.00E-05** | 3.20E-01 |
| J | cardoon aqueous extracts of leaves vs seeds | 17 | 6.40E-02 | **1.10E-04** | **2.20E-16** | **3.10E-03** | **1.40E-08** | **6.70E-11** | **5.00E-15** | **3.40E-14** | **4.00E-12** | **9.70E-16** | 1.90E-01 | **4.40E-03** | 4.90E-01 | **1.00E-07** | **8.70E-05** | 1.40E-02 | 5.10E-02 | **9.50E-16** | 4.00E-02 | **6.90E-03** | **1.90E-08** | **3.30E-08** | **8.60E-03** |
| L | cardoon aqueous extracts of leaves vs flowers | 11 | 2.40E-01 | 2.80E-02 | **5.40E-09** | 1.60E-02 | **4.70E-03** | **9.60E-04** | 3.90E-02 | 9.70E-02 | **2.90E-03** | **2.60E-03** | **7.30E-10** | 2.80E-02 | **4.00E-10** | **3.30E-10** | 7.40E-01 | 9.60E-02 | **2.60E-03** | 2.20E-01 | 2.50E-01 | 1.00E+00 | **2.20E-03** | 4.30E-01 | **4.60E-10** |
| K | cardoon aqueous extracts of seeds vs flowers | 16 | 7.80E-01 | **2.10E-07** | **4.40E-20** | 8.30E-01 | **3.10E-10** | 6.80E-02 | **2.90E-10** | **2.50E-14** | **6.20E-06** | **7.70E-17** | **4.00E-10** | **7.20E-06** | **1.60E-08** | 1.40E-02 | **6.10E-03** | 8.60E-01 | **1.70E-05** | **4.10E-16** | 6.40E-01 | 2.80E-02 | **3.60E-05** | **1.70E-05** | **1.80E-05** |
| M | cardoon ethanol extracts of leaves vs seeds | 4 | 2.50E-01 | 2.30E-02 | **8.80E-03** | 1.90E-01 | 1.60E-02 | 2.60E-01 | 6.50E-01 | **8.00E-04** | 5.70E-02 | 1.60E-01 | **7.70E-03** | **5.90E-03** | 9.40E-01 | 6.10E-01 | 2.80E-01 | 1.00E-01 | 9.90E-01 | 1.90E-01 | 2.40E-02 | 5.60E-02 | 6.60E-01 | 8.50E-02 | 7.40E-02 |
| N | cardoon ethanol extracts of leaves vs flowers | 3 | 2.30E-01 | 2.10E-02 | **6.80E-07** | 7.60E-01 | 4.20E-02 | 6.60E-01 | 2.50E-02 | 9.30E-02 | **7.60E-05** | 1.40E-01 | **3.90E-03** | 5.20E-02 | 8.20E-01 | 9.60E-01 | 8.40E-01 | 3.50E-01 | 5.40E-01 | 9.30E-01 | 7.70E-01 | 7.00E-01 | 5.40E-01 | 7.60E-01 | 3.00E-02 |
| O | cardoon ethanol extracts of seeds vs flowers | 5 | 1.30E-02 | **2.10E-05** | **5.80E-08** | 2.90E-01 | 3.20E-01 | 1.50E-01 | 4.20E-02 | **7.80E-07** | 3.40E-02 | **8.70E-03** | 2.90E-01 | 1.10E-01 | 8.50E-01 | 6.20E-01 | 2.00E-01 | **7.50E-03** | 5.20E-01 | 1.30E-01 | 3.40E-02 | 1.10E-01 | 4.20E-01 | 1.40E-01 | **8.20E-05** |

Either based on aqueous extracts (Table 3, Lines J-K) or ethanol-based extracts (Table 3, 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 to 5 statistically different ratios, p<0.01), according to previous observations (Fig.5).

**Conclusions**

High-throughput FTIR-spectroscopic analysis, based on microplates, enabled to acquire in a simple and rapid mode of the molecular profile of extracts and the cells medium, as only a simple dehydration was needed before spectra acquisition and were 1 spectrum takes approximately 1 min to be acquired. The direct analysis of plant extracts by FTIR-spectroscopy, enabled to predict the biocompounds yield obtained per each extraction procedure, and per each plant material used. It was also possible to compare the molecular profile obtained per extraction procedure, between plant tissue and between plant species. The FTIR-spectroscopic analysis of culture medium of cells exposed to extracts, enabled to monitor the extracts impact on cells metabolism. This analysis was according to the cell viability assay, based on MTT, that besides time-consuming in very expensive. The high sensitivity of the FTIR-spectroscopy, also enabled to monitor the impact on cells metabolism, even in conditions where the viability of the cell was not affected, and to compare the different extracts on specific cell metabolism.

All the analysis referred above were conducted on microplates with 96 wells and the microplate automatic FTIR-spectroscopic analysis. The high-throughput formats, associated to a so small volume needed to analyse, i.e., of 25μL, will promote the screening of high number of combinations of sample pre-treatment, extraction procedures, plant species and plant tissues, as based on DoE. Furthermore, this type of system will also promote multiple replicate experiments relevant to evaluate reproducibility e.g., of extraction procedures and compounds biological activities among other factors, e.g., the environmental impact on plants composition. By using the same dimension microplate, but with a higher number of wells, e.g., 384-wells, a smaller volume for analysis can be used, e.g. 5μL. These low volumes reduce the associated costs and the biological material needed for all these screening studies, while enabling increasing replicate experiments, i.e. enabling robust optimization procedures.

**Declarations**

Competing interests: The authors declare no competing interests.

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