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Article

Preservation of phenolic compounds on dried leaf infusion of *Bauhinia forficata* Link

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Bauhinia forficata Link aqueous extract is usually recommended as a phytomedicine to reduce blood glucose levels and its biological activity has been linked to the presence of phenolic compounds from *B. forficata* preparations. Several drying processes are used in the production of dry herbal extracts, which may influence the chemical composition and efficacy of final herbal medicines. Due to significant chemical changes, defining appropriate drying processes is essential for phytopharmaceutical drug development. In view of this, we analyzed dried *B. forficata* leaf infusion (BFLI) extracts by HPLC-UV-MS^n, followed by molecular networking analysis to evaluate the chemical profiles from dried extracts yielded by freeze- and spray-drying processes. The main metabolites detected included 11 ferulic/isoferulic acid derivatives and 13 glycosylated flavonoids. The qualitative chemical profiles were alike for both drying processes, whereas the relative abundance of some flavonoids was higher using spray-drying. Taken together, our results showed that freeze- and spray-drying preserved the phenolic profile of BFLI and suggested that spray-drying may be the most suitable to obtain its dried products. Along with studying the chemical profiles of dried herbal extracts, evaluating the influence of drying processes on the quality and chemical profiles of final products is pivotal and may benefit future research.

Keywords: Fabaceae. Drying process. HPLC-UV-MS^n. Flavonoids. Ferulic acid derivatives.

INTRODUCTION

Bauhinia forficata Link (Fabaceae) is largely used in folk medicine as a diuretic, tonic, to reduce glycosuria, and its aqueous extract is usually recommended as a phytomedicine to reduce blood glucose levels of diabetic patients (Russo *et al*., 1990; Cechinel, 2000; Tonelli *et al*., 2022). The hypoglycemic activity of *B. forficata* extracts has been related to polyphenols and flavonoids such as kaempferitrin, kaempferol, and quercetin, which are described as major compounds in *B. forficata* preparations (Pinheiro *et al*., 2006; Menezes *et al.,* 2007; Ferreres *et al*., 2012; Salgueiro *et al.* 2013).

It is well known that the increase in the number of reports of adverse reactions due to variations in the chemical composition of herbal products has attracted the attention of many regulatory agencies for the standardization of herbal formulations (Bhatta, Janezic, Ratti, 2020; Oliveira *et al.,* 2020). Thus, the evaluation of the chemical profile of *B. forficata* leaf extracts is essential to guarantee the quality from pharmaceutical preparations produced with this herbal medicine.

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Drying techniques such as freeze- and spray-drying are preferably used in the production of dry herbal extracts with the goal of preserving the active compounds, as well as assure stability, safety of use, and prolong storage. In contrast, significant physical and chemical changes in the composition can occur in the drying processes that applied higher temperatures, resulting in the loss or degradation of heat-sensitive compounds. Furthermore, phenolic compounds are easily susceptible to enzymatic degradation and are highly unstable during storage and processing (Sollohub, Cal, 2010; Kasper, Winter, Friess, 2013).

In the last few years, researchers have investigated the influence of drying processes on the chemical properties of *B. forficata* hydroalcoholic extracts (Oliveira, Bott, Souza, 2006; Souza, Oliveira, 2006; Souza *et al*., 2015). However, it is still not known whether drying processes affect the phenolic composition of *B. forficata* leaf aqueous extract (infusion). Therefore, the aim of this work was to evaluate the impact of freeze- and spray-drying processes on the chemical profile from *B. forficata* leaf infusion (BFLI) by liquid chromatography coupled to mass spectrometry (LC-MS)-based molecular networking analysis to aid the annotation of metabolites and comparison of their relative abundances after each drying process.

MATERIAL AND METHODS

Infusion preparation and drying processes

B. forficata leaves were collected in the Botanic Garden of Medicinal Plant Ordem e Progresso in Jurucê, a district of the city of Jardinópolis (São Paulo, Brazil). A voucher specimen was deposited at the Medicinal Plants Herbarium of the University of Ribeirão Preto (HPM–UNAERP, Ribeirão Preto, SP, Brazil) under number 1793. The utilization of *B. forficata* samples in this study was authorized by the Council for the Genetic Heritage Management (CGEN)/Ministry of Environment (Sisgen/ register no. A093CD8) through the office of the National Council for Scientific and Technological Development (CNPq). Dried leaves (2 kg) were powdered and added to boiling water (10 L) and allowed to stand in a capped glass container for 1 hour. The infusion was filtered through analytical filter papers under vacuum. The aqueous phase was divided into two portions; one portion (3.25 L) was submitted to freeze-drying (Flexi-Dry MP™ Freeze Dryer, FTS Systems, USA) to produce freeze-dried BFLI; whereas the other (1.61 L) was subjected to spray-drying (SD-Basic Laboratory Scale Spray Dryer, LabPlant, United Kingdom) to produce spray-dried BFLI. For the freeze-drying process, the aqueous phase was frozen at -40 °C and freeze dried under vacuum at 140 $L.min^{-1}$ for 48 hours. The spray-drying process was carried out with an inlet temperature of 160 ± 1 °C, outlet air temperature of ± 80 °C, and pump flow rate of 8 $mL.min^{-1}$. The resulting dried powders were stored at -4 °C until further analysis.

High-performance liquid chromatography (HPLC) coupled to ultraviolet diode array detection (UV-DAD) and electrospray ionization mass spectrometry (ESI-MS)

Samples were prepared at a concentration of 1 mg/mL of each freeze- or spray-dried powder of BFLI in MeOH:H_2O (1:1, v/v). The samples were vortex agitated and filtered by a 0.45 µm PTFE filter before analysis. The analyses were obtained in a high-performance liquid chromatography (HPLC) system (Shimadzu Prominence LC-20A, Shimadzu Corporation, Kyoto, Japan) equipped with diode array detector (DAD) and coupled with a mass spectrometer with electrospray ionization source and analyzer ion trap (AmaZon SL, Bruker Daltonics, Billerica, MA, USA). A C18 chromatographic column (Luna, 5 µm, 250 x 4.6 mm; Phenomenex, Macclesfield, United Kingdom) was used and a total of 20 □L of each sample was injected. The mobile phase was composed of ultrapure water and MeOH (both containing 1% acetic acid) and a flow rate of 1 $mL.min^{-1}$ was applied. The gradient of elution of the mobile phase and all other chromatographic parameters were the same as those described by Ferreres *et al*. (2012). UV spectral data were recorded in the range 240-400 nm. Mass spectrometry data were acquired in negative and positive ionization modes, separately, employing the following parameters: capillary voltage, 3.5 kV; end plate offset, 500 V; nebulizer, 50 psi; dry gas (N_2) flow, 9 $L{\cdot}min^{-1}$; dry

temperature, 300 °C; auto MS/MS acquiring data between *m/z* 50 and 1200, average of three spectra; enhanced resolution for scan mode and UltraScan mode for MS/MS; spectra rate acquisition, three spectra/s; exclusion of a particular ion after three spectra and released after 30 s. The mass spectrometer was controlled by Hystar software (Bruker Daltonics Inc., Billerica, MA, USA), and chromatograms and mass spectra were visualized using Data Analysis software (Shimadzu Corporation, Kyoto, Japan).

Annotation of the chemical compounds

LC-MS data from positive and negative ionization modes were converted to .mzXML format by MSConvert software (Proteowizard Software Foundation, USA) and processed by MzMine™ (version 2.51, BMC Bioinformatics, United Kingdom). The following parameters were set for data processing: mass detection using the centroid algorithm, scan MS level 1 (noise level, 1.0E5) and scan MS level 2 (noise level, 1.0E4); Automatied Data Analysis Pipeline (ADAP) chromatogram builder (min group size in # of scans, 5; group intensity threshold, 1.0E5; min highest intensity, 1.0E6; *m/z* tolerance, 0.3 *m/z* or 200 ppm); chromatogram deconvolution using wavelets (ADAP) algorithm (S/N threshold, 10; S/N estimator, intensity window SN; min feature height, 1.0E6; coefficient/area threshold, 5; peak duration range, 0.2 – 2.00; RT wavelet range, 0.02 – 0.20) (*m/z* center calculation – median; *m/z* range for MS2 scan pairing, 0.3; RT for MS2 scan pairing, 0.2); isotopic peak grouper (*m/z* tolerance, 0.3 *m/z* or 200 ppm; retention time tolerance in minutes, 0.2; maximum charge, 2; representative isotope, most intense); and alignment using the join aligner (*m/z* tolerance, 0.3 *m/z* or 200 ppm; weight for *m/z*, 50; retention time tolerance, 0.3 (abs); weight for retention time, 50). After data processing, peaks with MS^2 scan were exported for GNPS (Global Natural Products Social Molecular Networking) analysis as a .csv quantification spreadsheet and as a .mgf file.

The output data from positive and negative ionization modes were uploaded to the GNPS platform (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp) and the "advanced analysis tools for feature networking" were used to generate the molecular networking (Nothias *et al*., 2020; Wang *et al*., 2016). The following parameters were employed: precursor ion mass tolerance, 2.0 Da; fragment ion mass tolerance, 0.5 Da; minutes pairs cos, 0.6; minimum matched fragment ions, 4; maximum shift between precursors, 500 Da; network topK 10; maximum connected component size, 100; library search minutes matched peaks, 4; score threshold, 0.7; search analogues, don't search; maximum analog search mass difference, 100 Da; top results to report per query, 1; minimum peak intensity, 0; filter precursor window, filter; filter library; filter peaks in 500 Da window, filter; normalization per file, no norm; aggregation method for peak abundances per group, sum. Finally, molecular networking was generated and analyzed with Cytoscape (version 3.7.2, Institute for Systems Biology, Seattle, WA, USA). Nodes in the molecular network were represented as pie charts, which were used to compare relative abundances of metabolites in freeze- and spray-dried samples.

Hit compounds pointed by the GNPS spectral library were compared with additional information (UV spectra and MS fragmentation patterns) and then annotated. Sugar moieties indicated by the GNPS library were characterized as hexosides, pentosides, deoxyhexosides, etc. (i.e., stereochemistry was not considered in the compound annotation). Isomers were differentiated based on retention times, since the exact positions of the sugar moieties could not be determined by the techniques implemented here (i.e., positions of the sugar moieties were not assigned for the annotated compounds).

All raw LC-MS^n data and MzMine exported files were deposited and can be accessed via the MassIVE submission (MSV000089571; Creative Commons CC0 1.0 Universal license).

RESULTS AND DISCUSSION

The chemical profiles of freeze- and spray-dried BFLI were characterized mainly by the presence of phenolic compounds, including 11 hydroxyferulic/isoferulic acids (FA) derivatives and 13 glycosylated flavonoids (Table I).

TABLE I - Annotated compounds in freeze- and spray-dried BFLI

Peak	Compound	Rt (min)	UV (nm)	Negative mode *(m/z)*		Positive mode *(m/z)*	
				MS $[M-H]^-$	MS/MS	MS $[M+H]^+$	MS/MS
1	Hydroxy-FA hexoside	4.3	299, 325	371	371→ 353, 209, 191 209→ 191, 173, 85	-	-
2	Hydroxy-FA hexoside	5.2	299, 325	371	371→ 353, 209, 191 209→ 191, 173, 85	-	-
3	Hydroxy-FA deoxihexoside	5.6	299, 325	355	355→ 337, 209, 191, 129 191→ 173, 129, 85	-	-
4	Hydroxy-FA glucuronic acid	6.3	299, 326	385	385→ 367, 209, 191, 173 191→ 173, 147, 129, 85	-	-
5	Hydroxy-FA hexoside	6.5	299, 327	371	371→ 353, 209, 191 209→ 191, 85	-	-
6	Hydroxy-FA hexoside	7.0	299, 325	371	371→ 353, 209, 191 209→ 191, 85	-	-
7	Hydroxy-FA deoxihexoside	7.2	299, 325	355	355→ 337, 209, 191 191→ 147, 129, 85	-	-
8	Hydroxy-FA deoxihexoside	8.1	299, 325	355	355→ 337, 209, 191,147 191→ 173, 129, 85	-	-
9	Hydroxy-FA glucuronic acid	8.3	299, 325	385	385→ 367, 209, 191, 173, 129 191→ 85	-	-
10	Hydroxy-FA deoxihexoside	9.2	299, 325	355	355→ 337, 209, 191, 173,129 191→ 173, 147, 129, 85	-	-
11	Hydroxy-FA glucuronic acid	10.1	299, 325	385	385→ 367, 209, 191, 173 191→ 173, 147, 129, 85	-	-
12	Kaempferol *O*-di-hexosyl-deoxyhexoside	15.0	265, 345	755	755→ 593, 285 593→ 285	757	757→ 611, 595, 449, 287 449→ 287
13	Quercetin O-hexosyl-deoxyhexosyl-hexoside	17.6	265, 355	771	771→ 609, 300 300→ 271, 257, 179, 151	773	773→ 611, 465, 303 303→ 285, 257, 229, 165, 153

TABLE I - Annotated compounds in freeze- and spray-dried BFLI

Peak	Compound	Rt (min)	UV (nm)	Negative mode *(m/z)*		Positive mode *(m/z)*	
				MS $[M-H]^-$	MS/MS	MS $[M+H]^+$	MS/MS
14	Quercetin *O*-di-hexoside	19.0	266, 355	625	625→ 463, 301 301→ 271, 255	627	627→ 465, 303 303→ 285, 257, 247, 229, 165, 153
15	Quercetin *O*-deoxyhexosyl-hexosyl-deoxyhexoside	20.3	260, 360	755	755→ 711, 609, 301 301→ 271, 255, 179, 151	757	757→ 611, 449, 303 303→ 257, 247, 229, 165, 153
16	Kaempferol *O*-hexosyl-deoxyhexosyl-hexoside	21.3	265, 350	755	755→ 593, 575, 429, 285 285→ 257	757	757→ 595, 449, 287 287→ 269, 241, 231, 213, 153
17	Kaempferol O-di-hexoside	22.6	265, 350	609	609→ 285 285→ 257, 151	611	611→ 449, 287 287→ 241,213,165, 153, 133
18	Kaempferol *O*-hexosyl-di-deoxyhexoside	23.7	265, 350	739	739→ 593, 575, 393, 285 285→ 257, 227, 169, 151, 107	741	741→595, 449, 287 287→ 269, 241, 213, 165, 153, 133, 121
19	Rutin*	25.5	265, 353	609	609→ 301 301→ 271, 229, 193, 179, 151	611	611→ 465, 449, 303 303→ 285, 257, 247, 165, 153
20	Luteolin *O*-hexosyl-deoxyhexoside	29.2	260, 345	593	593→ 285 285→ 267, 257, 229, 169, 151	595	595→ 449, 287 287→ 185, 165, 153, 133, 121
21	Quercetin *O*-deoxyhexoside	30.0	265, 355	447	447→ 301 301→ 271, 255, 229, 179,151	449	449→ 303 303→ 285, 257, 173, 165, 153

TABLE I - Annotated compounds in freeze- and spray-dried BFLI

Peak	Compound	Rt (min)	UV (nm)	Negative mode *(m/z)*		Positive mode *(m/z)*	
				MS $[M-H]^-$	MS/MS	MS $[M+H]^+$	MS/MS
22	Kaempferol *O*-hexosyl-deoxyhexoside	30.5	265, 347	593	593→ 285 285→ 267, 257, 229, 175, 163, 135	595	595→ 287 287→ 241, 213, 197, 165, 153, 121
23	Kaempferol *O*-pentoside	31.3	265, 347	417	417→ 327, 285 285→ 257, 163, 135	419	419→ 287 287→ 213, 153
24	Kaempferol O-deoxyhexoside	34.3	265, 345	431	431→ 285 285→ 257, 163, 135	433	433→ 287 287→ 241, 213, 153, 133

FA: ferulic or isoferulic acid; * confirmed by co-injection of standard.

The basic skeletons of the annotated FA derivatives and flavonoids are presented in Figure 1. All of the annotated metabolites were detected in freeze- and spray-dried BFLI. Therefore, the drying conditions used here did not significantly modify the qualitative chemical profile of dried BFLI with respect to the phenolic constituents.

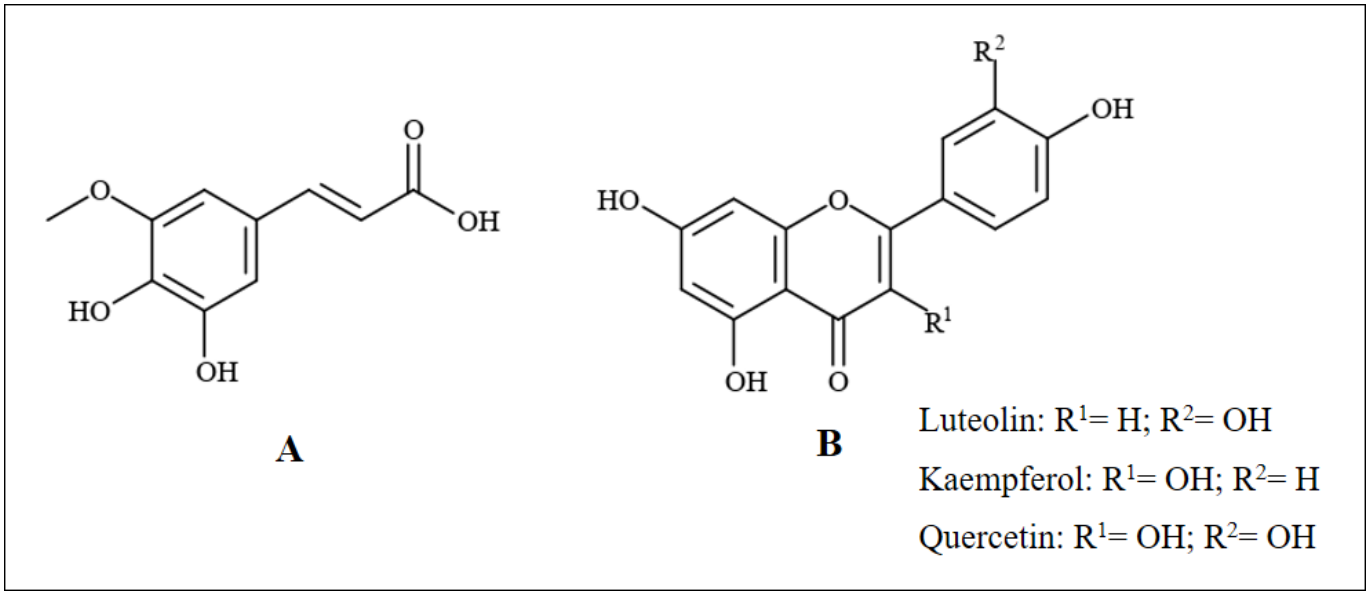

FIGURE 1 - Basic skeletons of hydroxyferulic/isoferulic acids (**A**) and flavonoids (**B**).

FA derivatives are hydroxycinnamic acids commonly found in plants and they exhibit potential therapeutic effects in the pharmaceutical field, and may occur free, dimerized, or conjugated with sugars (Paiva *et al*., 2013). Regarding the LC-MS-based molecular networking analysis, FA derivatives were detected mostly in negative ionization mode. Comparing the relative abundance of the annotated metabolites in the molecular networking (Figure 2), we observed that freeze- and spray-dried BFLI exhibited alike relative abundances for the annotated FA derivatives.

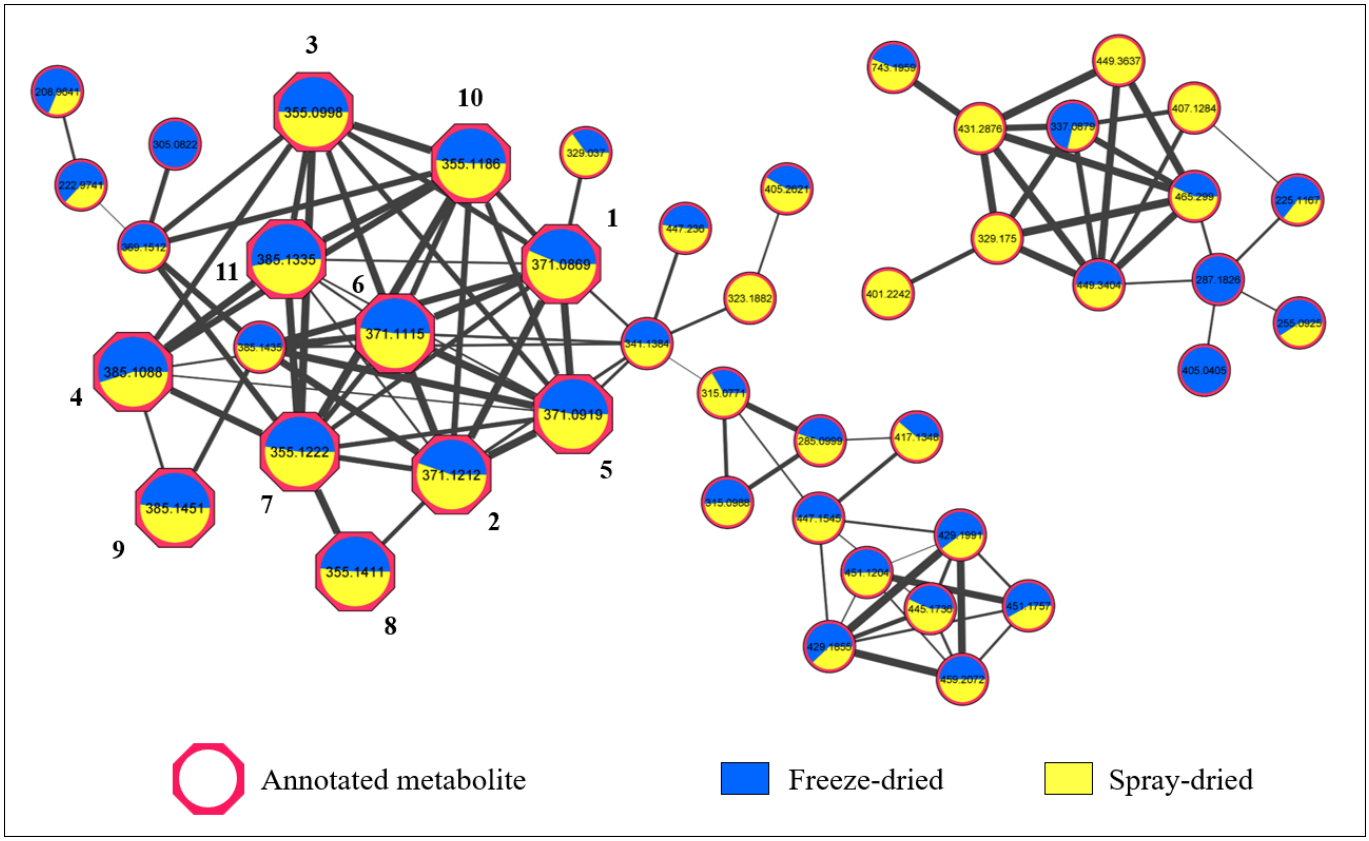

FIGURE 2 - Molecular networking of freeze- and spray-dried BFLI constructed by LC-MS data obtained in negative ionization mode. Each node represents a metabolite, which was detected in freeze-dried (blue) and/or spray-dried (yellow) samples. The annotated ferulic/isoferulic acid derivatives are highlighted as larger octagonal nodes.

Flavonoids are phenolic compounds chemically characterized by a 15-carbon phenyl-benzopyrone skeleton. According to the patterns of hydroxylation and glycosylation, as well as degree of unsaturation and oxidation, flavonoids are classified into several classes (e.g., flavones, flavonols, flavanones, flavanonols, isoflavonoids, flavan-3-ols, chalcones, aurones, and anthocyanins) and occur as aglycones, glycosides and/or methylated derivatives (Kumar, Pandey, 2013; Dias, Pinto, Silva, 2021). In this study, freeze- and spray-dried BFLI were characterized mainly by the presence of glycosylated flavonols (i.e., kaempferol and quercetin conjugated with sugar moieties). Additionally, a glycosylated flavone (luteolin conjugated with a sugar group) was also detected in both samples (Figures S1 and S2, Supplementary Material).

The molecular networking generated with LC-MS data obtained in positive ionization mode was constituted mainly by annotated flavonoids (Figure 3). The relative abundances of some flavonoids were equivalent in freeze- and spray-dried samples. However, other flavonoids, including quercetin *O*-hexosyl-deoxyhexosyl-hexoside

(**13**), quercetin *O*-di-hexoside (**14**), kaempferol *O*-di-hexoside (**17**), quercetin *O*-deoxyhexoside (**21**) and kaempferol *O*-deoxyhexoside (**24**), exhibited higher relative abundances in the spray-dried sample.

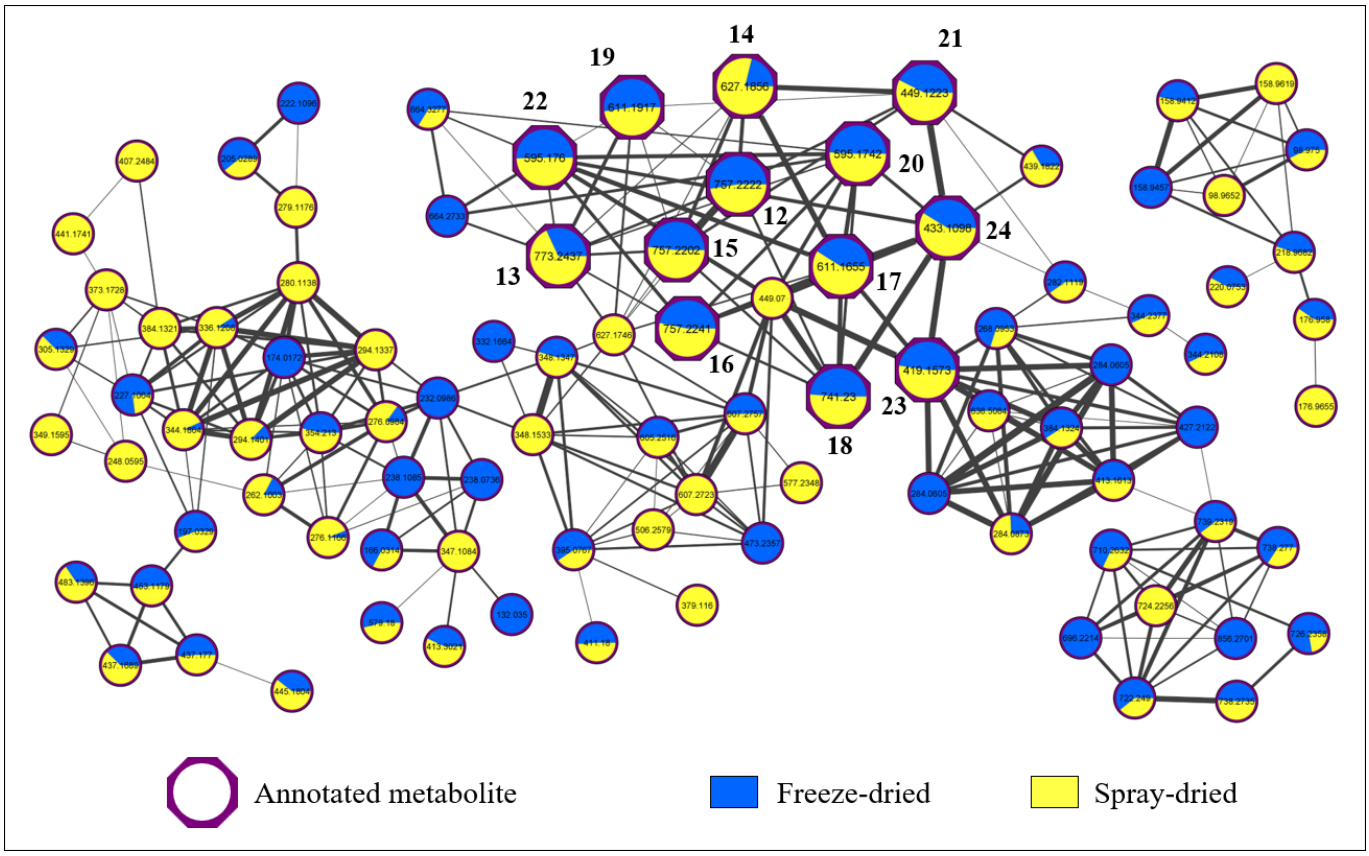

FIGURE 3 - Molecular networking of freeze- and spray-dried BFLI constructed by HPLC-MS data obtained in positive ionization mode. Each node represents a metabolite, which was detected in freeze-dried (blue) and/or spray-dried (yellow) samples. The annotated flavonoids are highlighted as larger octagonal nodes.

These findings are consistent with previous reports that described free and glycosylated forms of kaempferol and quercetin as the most frequent secondary metabolites isolated from *B. forficata* leaves (Ferreres *et al.*, 2012; Farias, Mendez, 2014; Miceli *et al.,* 2016). Compared with the chemical composition reported for the aqueous *B. forficata* extracts (Menezes *et al.,* 2007; Salgueiro *et al.,* 2013), the analytical technique employed here increased the variety of detected flavonoids and allowed characterization of FA derivatives on freeze- and spray-dried BFLI. Figures S3 and S4 (Supplementary Material) show the peak area of compounds annotated from freeze- and spray-dried BFLI obtained in positive and negative ionization modes.

Although the freeze-drying process is a preferred method for drying liquid samples containing thermally sensitive compounds, such as phenolic substances (Bhatta, Janezic, Ratti, 2020; Oliveira *et al.*, 2020), our results showed that spray-dried infusion of *B. forficata* leaves exhibited higher relative abundance of some flavonoids compared to the freeze-dried infusion. Additionally, the spray-drying process was carried out within two hours, while the freeze-drying process required 48 hours for water removal. These results suggest that spray-drying may be the most suitable drying process to obtain dried aqueous extracts of *B. forficata* leaves, whereas, both drying processes showed similar relative abundance for the FA derivatives. Similar findings were observed in a

study developed by Gomes *et al.* (2018), which reported slightly high concentrations of polyphenolic compounds in spray-dried papaya product than the freeze-dried product.

Horszwald, Julien, Andlauer (2013), also described a higher content of total flavonoids in *Aronia* powders in spray-drying samples than freeze-drying. Thus, the spray-drying process proved to be very advantageous for drying *Aronia* commercial juice, due to its faster drying, reducing the hydrolysis process and consequently preserving the integrity of the glycosylated compounds.

The application of high temperature during spray-drying processes has been related to deactivation of oxidative and hydrolytic enzymes, consequently avoiding the loss of polyphenolic components (Sukrasno *et al.,* 2011; Horszwald, Julien, Andlauer, 2013; Gomes *et al.,* 2018).

Drying conditions used in this work did not extensively modify the qualitative phenolic profile of freeze- and spray-dried BFLI. Thus, our results showed that both freeze- and spray-drying processes qualitatively preserved the phenolic compounds of BFLI and were suitable to produce dried aqueous extract of *B. forficata* Link. Along with the preservation of the phenolic compounds of dried herbal extracts, evaluating the influence of drying processes on the quality of the final products is pivotal and may benefit future researches.

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