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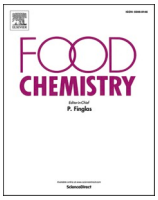

Antioxidant activity of *Zuccagnia*-type propolis: A combined approach based on LC-HRMS analysis of bioanalytical-guided fractions and computational investigation

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ABSTRACT

This study reports a combined approach to assess the antioxidant activity of *Zuccagnia*-type propolis. Fractions exhibiting the highest antioxidant activities evidenced by DPPH, a β-carotene bleaching and superoxide radical scavenging activity-non-enzymatic assays, were processed by LC-HRMS/MS to characterize the relevant chemical compounds. A computational protocol based on the DFT calculations was used to rationalize the main outcomes. Among the 28 identified flavonoids, caffeic acids derivatives were in the fraction exhibiting the highest antioxidant activity, with 1-methyl-3-(4′-hydroxyphenyl)-propyl caffeic acid ester and 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeic acid ester as major components. Results clearly showed roles of specific chemical motifs, which can be supported by the computational analysis.

This is the first report ascribing the antioxidant ability of *Zuccagnia*-type propolis to its content in specific caffeic acid derivatives, a potential source of radical scavenging phytochemicals. The proposed protocol can be extended to the study of other plant-products to address the most interesting bioactive compounds.

1. Introduction

Propolis is a sticky bee product (*Apis mellifera L.*), mainly consisting of resinous substances collected from buds and barks of several tree species and mixed with beeswax. Propolis acts as a strong biocide within the hive, and it is accounted for the low incidence of bacteria and fungi (Falcão et al., 2013). Propolis has obtained popularity in the healthy foods market for its recognized antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant, antiproliferative, immuno stimulating, anti-ulcerous, properties, and it is used as a label claim. (V. S. Bankova, De Castro, & Marcucci, 2000; Falcão et al., 2013; Sforcin & Bankova, 2011; Sforcin, Fernandes, Lopes, Bankova, & Funari, 2000). The variability of the propolis chemical composition depends on available botanical resources (Sforcin & Bankova, 2011), rather than geographical location. It is well known that resins excreted by poplar trees (*Populus* spp.) are the preferred resource of bees in the temperate regions of Europe, but also in North America and non-tropical regions of Asia. Conversely, resins from birch (*Betula* spp.) are the primary botanical source selected by bees in colder temperate climates (V. Bankova, 2005). Regarding biological activities, so far poplar-type propolis has been the most studied one. (V. Bankova, 2005; V. Bankova, Popova, Bogdanov, & Sabatini, 2002; Dezmirean, Paşca, Moise, & Bobiş, 2021; Murtaza et al., 2014; Russo, Longo, & Vanella, 2002). Afterward, other extensively studied specimens were the Brazilian "green propolis", whose antioxidant activity was attributed to prenylated coumaric acid derivatives, such as artepillin C (Fonseca et al., 2011; Gardana, Scaglianti, Pietta, & Simonetti, 2007). This is a constituent always present in a native Brazilian plant, *Baccharis dracunculifolia,* whose resin has a peculiar green color. Otherwise, propolis from Venezuela and Cuba were related to the genus *Clusia* and their antioxidant and antibacterial capacities were ascribed to prenylated benzophenones (V. Bankova et al., 2002; V. S. Bankova et al., 2000; Popova et al., 2011). In hot arid regions of Northwest Argentina,

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such as the Del Monte desert, bees collect mainly the exudates from native resinous shrubs (Vera et al., 2011a; Zampini, Salas, Maldonado, Simirgiotis, & Inés Isla, 2021). The north-western Argentinean propolis (NAP) from arid mountain regions are known to be more active than those collected in flat regions since plants growing in these high-altitude areas are subjected to both an important thermal excursion and photo-irradiation. These extreme temperatures trigger an intense production of phenolic compounds favorable to prevent oxidative stress (Alonso-Amelot, 2008; Isla, Moreno, de Álvarez, Los, & Zampini, 2021; Rice-Evans, Miller, & Paganga, 1996; Sroka, 2005). For these reasons, NAPs were introduced in the national Food Code as dietary supplements. The Argentinean legislation indicates that only ethanolic extracts of propolis containing at least 0.25% of flavonoids and 0.25% of phenolic components as dry weight can be commercialized (Instituto Argentino de Normalizacion y Certification, 2008). Several reports showed that NAPs possess antioxidant activity and a phenolic content larger than that established by this legislation (Ana Lilia Salas et al., 2015; Solórzano et al., 2012; Vera et al., 2011b). One of the studied NAP propolis is that related to the Argentinean shrub *Z. punctata*, having a very long history of medicinal use by different communities. According to ethnobotanical data, aerial parts (stems and leaves) are mainly used as infusions and baths for the treatment of mycosis, respiratory, gastrointestinal and inflammatory diseases (Vattuone, Soberon, Sgariglia, Quiroga, & Sampietro, 2013). *Z. punctata* continues to be consumed despite being included in the preliminary red list of endangered plants. Given the chemical similarities of *Zuccagnia*-type propolis with its botanical source (Agüero et al., 2010; Álvarez et al., 2023; Svetaz et al., 2004), propolis could replace the plant for medicinal uses and lead to conserve it.

Caffeic acid phenethyl ester (CAPE) has been identified as one of the common components with the major antioxidant capacity in propolis. Nevertheless, some NAPs characterized as *Zuccagnia*-type, display high levels of chalcones, flavanones, dihydrochalcones, caffeic acid derivatives (Zampini et al., 2021), and the total absence of CAPE (Solorzano et al., 2019; Eliana Rita Solorzano et al., 2017). Although the notable antioxidant activity of *Z. punctata* and related propolis was generally ascribed to some plant markers belonging to chalcones, dihydrochalcones, flavanones (2′,4′-dihydroxychalcone, 2′,4′-dihydroxy-3-methoxy chalcone 2′,4′-dihydroxydihydrochalcone, 2′,4′-dihydroxy- 3-methoxy dihydrochalcone, and 7-hydroxyflavanone) (Isla et al., 2021; Morán Vieyra et al., 2009; Ana L. Salas et al., 2020), the specific role of the active principles of the related propolis, as well as for other NAP, is still uncertain.

This study aims at evaluating the antioxidant capacity of *Zuccagnia*-type propolis by detailing the role of single components, using various bioassays combined to the chemical identification. A bioanalytical-guided fractionation is used to identify the main active compounds related to the sample antioxidant activity. The most bioactive fractions were selected and processed by liquid chromatography-high resolution tandem mass spectrometry (LC-HRMS/MS) for the characterization of the main phenolic compounds that can be associated to the antioxidant activity. Finally, to rationalize the experimental outcomes, Density Functional Theory (DFT)-based theoretical modelling was used to relate the antioxidant potential to the molecular structure of the active species and to identify those chemical motifs which promote the observed enhanced activity.

2. Materials and methods

2.1. Reagents

All reagents and solvents were of analytical grade. Ultrapure water (Millipore, Milli-Q system) was used to prepare standard solutions, dilutions and blanks.

All the following reagents were purchased from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany): 1,1-diphenyl-2-picrylhydrazyl (DPPH), dimethylsulfoxide (DMSO), butylated hydroxytoluene (BHT), phenazine methosulfate (PMS), nitroblue tetrazolium (NBT), natural product reagent (NP consisting in 1% methanolic solution of diphenylboric acid aminoethyl ester), linoleic acid, Tween 40 (polyoxy ethylene sorbitan monopalmitate), β-carotene and quercetin. Solutions of these reagents were prepared at 1 mg/mL in 50/50 (*v*/v) methanol/water or DMSO and then diluted to 100 ng/mL in 50/50 (v/v) methanol /water. Standard compounds of 7-hydroxyflavanone, 2′,4′-dihydroxy-4-methoxychalcone, 2′-hydroxychalcone, 4′-hydroxychalcone, 3,5,7-trihydroxyflavone (galangin), 4′,5,7-trihydroxyflavone (apigenin), 5,7-dihydroxyflavone (chrysin), 5,7-dihydroxy-4′-methoxyisoflavone (biochanin), 3-(3,4-Dihydroxycinnamoyl) quinic acid (chlorogenic acid) were kindly obtained by prof. Raffaella Filippini and prof. Guglielmina Froldi (University of Padua). 2′,4′-dihydroxy-3′-methoxychalcone was obtained from *Z. punctata* extract according to Agüero et al. (2010).

2.2. Propolis sample

Propolis samples were collected from apiaries located in Casa de Piedra city (Catamarca province, Argentina). The collected material was gathered to prepare a single sample, which was stored at −20 °C until use to prevent natural oxidation. The propolis was preliminary characterized as *Zuccagnia*–type propolis according to the protocol described in (Eliana Rita Solorzano et al., 2017) for the ethanolic extract, evidencing the presence of the established biomarkers, e.g. 2′,4′-dihydroxychalcone, 2′,4′-dihydroxy-3′-methoxychalcone, 7-hydroxyflavanone, 7-hydroxy-8-methoxyflavanone, 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeic acid ester (Agüero et al., 2010).

2.3. Sample preparation

All propolis samples were prepared according to Solórzano et al., 2012 (Solórzano et al., 2012; Eliana Rita Solorzano et al., 2017). Briefly, 2 g of propolis samples were cut into small pieces and extracted with 20 mL of 80% ethanol using an ultrasonic device (30 min, 80 W). Extracts were centrifuged (Sorvall RC50) for 20 min at 9000*g* (at 4 °C) and the supernatant was collected. Successive extractions of the residue were made and gathered to a final volume of 100 mL. Ethanolic extract was dried by evaporation in vacuum at 50 °C. 1 mg/mL solution was prepared by dissolving the dry extract in 80/20 (*v*/v) methanol/water and was named as propolis ethanolic extract (PEE). 100 mL of this extract underwent liquid-liquid extraction by using the same volume of a hexane/chloroform solvent and a separatory funnel (three successive extractions). The by-extracts were then dried by evaporation under vacuum at 50 °C and were named hexanoic (HEX), chloroform (CHL) and the remaining, aqueous (AQ). These extracts were re-dissolved in methanol or DMSO to obtain the solutions used for all the determinations of the antioxidant potential.

The CHL dry extract was selected for the fractionating process by using liquid-solid chromatography (column length, 80 cm; internal diameter, 3.5 cm) on silica gel 60, (0.2–0.5 mm particle size, Merck). It was put with silica gel and a little volume of methanol in a rotavapor to obtain the “plug” of the column. The fractionation was obtained by gravity using suitable mixtures of chloroform and ethyl acetate, starting from 2% of ethyl acetate and increasing this percentage after evaluating aliquots of eluate by TLC (Silica gel F254), using UV at 254 and 365 nm (UV Lamp Model UV 5 L-58 Mineralight Lamp) and by spraying with saturated ceric sulfate solution followed by heating at 100 °C. The elution was done with the aim of collecting fractions with an informative chemical profile in terms of phenolic compounds, as resulted by the TLC spots obtained testing every 20 mL of the eluate. Twelve fractions were firstly collected under a sensible gradient elution, and then those exhibiting similar TLC profiles were gathered so that seven fractions were finally obtained (F1-F7, see Fig. 1), and subjected to the antioxidant activity assays. Fractions were evaporated under vacuum at 50 °C and then solubilized with the same volume of methanol or DMSO for the following analysis. The yields of the seven fractions were 37.5 mg (F1),

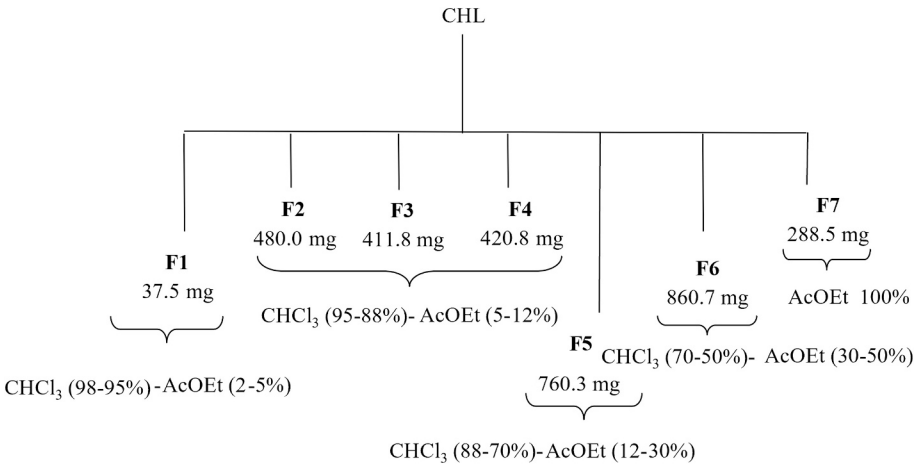

Fig. 1. Scheme of the chloroform (CHL) extract fractionation obtained by using liquid-solid chromatography (silica gel 60 column, 80 cm × 3,5 i.d. cm) and gathered from TLC characterization. Elution was made with the chloroform/ethyl acetate (AcOEt) ratios reported. The yields reported for the different fractions are expressed as mg of dry weight.

480.0 mg (F2), 411.8 mg (F3), 420.8 mg (F4), 760.3 mg (F5), 860.7 mg (F6), 288.5 mg (F7), expressed as mg of dry weight.

2.4. Antioxidant activity

2.4.1. DPPH assay

The DPPH scavenging assay was carried out according to Vivot et al. (Vivot et al., 2001) with slight modifications. Reaction mixtures containing different concentrations of PEE (2.5–80 μg dry weight extract/mL) and CHL-extract fractions were dissolved in 5 μL DMSO and 95 μL of DPPH$^{\bullet}$ solution (0.125 mg/mL) in a 96-well microtiter plate and were incubated at 25 °C for 30 min. The absorbance was then measured at 550 nm in a microplate spectrophotometer (Multiskan GO, Thermo Scientific). The radical scavenging activity of different propolis fractions was determined by comparison with a DMSO solution, using BHT as positive control. The radical scavenging capacity was expressed as SC_{50}, which indicates the sample concentration required to scavenge 50% of DPPH$^{\bullet}$ and reported in Table 1.

2.4.2. β-carotene assay

The antioxidant activity was also determined using the β-carotene bleaching method according to Peterson et al. 1999 (Peterson, Emmons, & Hibbs, 2001). One mg/mL solution of β-carotene in chloroform was evaporated to dryness in a rotary evaporator. The residue was then re-suspended in 20 μL of linoleic acid and 184 μL of Tween 40. The resulting mixture was diluted with 10 mL of 50 mM H_2O_2 and stirred vigorously to form a uniform emulsion. Then, 40 mL of distilled water were added, and 4 mL aliquots of this emulsion were transferred into test tubes containing the propolis extracts at various concentrations (2.5–80 μg dry weight extract/mL) and the control. An initial absorbance reading (at t_0) was performed at 470 nm and then after 60 min of incubation time at 50 °C. Two control samples without sample extract and with BHT were used as negative or positive controls, respectively. The antioxidant activity ($AA_{\%}$) was calculated as relative inhibition percent, by using the following equation (Peterson et al., 2001):

$$AA\% = \frac{DRc - DRs}{DRc} \times 100$$

where AA% is the antioxidant activity; DR_C is the degradation rate of β-carotene in the control sample; DR_S degradation rate of β-carotene in the sample with antioxidant.

The AA% is finally expressed in Table 1 in terms of protection index (PI_{50} values) indicating the sample concentration required to inhibit the 50% β–carotene bleaching.

Table 1
Antioxidant activities of the total ethanolic extract of propolis (PEE), hexanoic (HEX), chloroform (CHL) and aqueous (AQ) by-extracts compared to the fractions obtained from CHL by-extract. $N = 3$.

Sample	DPPH SC_{50}, (μg/mL)	β-carotene PI_{50}, (μg/mL)	SRSA SC_{50}, (μg/mL)
PEE	23.1 ± 1.4	8.4 ± 1.4	ND
HEX	270 ± 13	9.1 ± 1.6	ND
CHL	18.5 ± 0.5	5.0 ± 0.9	ND
AQ	220 ± 11	17.0 ± 3.1	ND
F1	>400	>80	>80
F2	>400	40.3 ± 6.1	>80
F3	>400	43.3 ± 6.5	>80
F4	364 ± 18	5.9 ± 0.9	>80
F5	90.4 ± 4.5	4.6 ± 0.7	>80
F6	9.0 ± 0.5	2.4 ± 0.5	13.0 ± 3.2
F7	82.0 ± 4.1	14.3 ± 0.9	28.2 ± 4.5
BHT	30.1 ± 1.2	1.9 ± 0.3	25 ± 3
Quercetin	–	–	55 ± 8

2.4.3. Superoxide radical scavenging activity-non-enzymatic assay (SRSA)

Superoxide radicals were produced by the NADH/PMS system according to Valentaõ et al., 2002 (Valentão et al., 2002). 35 μL of DMSO containing from 5 to 80 μg dry weight /mL of each sample, was mixed with 75 μL of 2 mM NADH, 75 μL of 250 μM NBT, and 37 μL of 5.4 μM PMS. All the reagents were dissolved in a phosphate buffer (19 mM, pH 7.4). The reaction mixture was incubated for 30 min at 37 °C and the absorbance was measured at 560 nm. BHT and quercetin were used as positive controls. The SC_{50} values were obtained from the inhibition curves and reported in Table 1.

2.5. LC-DAD-HRMS/MS measurements

LC-Quadrupole-Time of Flight (QTOF, resolving power 18,000 at m/z 311.0805 as FWHM) analysis was performed with a UHPLC system (Agilent Series 1200; Agilent Technologies, Palo Alto, CA, USA), consisting of vacuum degasser, autosampler, binary pump and column oven coupled to both DAD and QTOF mass analyzer (Agilent Series 6520). Five μL of sample extracts were injected into an analytical column (Kinetex pentafluorophenyl, 100 mm × 2.1 mm, 2.6 μm particle size, Phenomenex, Italy) thermostated at 30 °C. The mobile phase components were water (A) and methanol (B), both 1 mM formic acid. The mobile phase gradient profile, at a flow rate of 0.25 mL/min, was as follows, in min: $t_{0–16}$ 20–100% B; $t_{17–19}$, 100% B; $t_{19–20}$ 20% B; $t_{21–27}$ 20% B. DAD signals were recorded in the 210–600 nm range. The QTOF

system was equipped with an electrospray ionization interface (ESI), operating in dual ESI mode and negative ESI acquisition, with the following operating parameters: capillary voltage, 3500 V; nebulizer pressure, 35 psi; drying gas, 10 L/min; gas temperature, 350 °C; fragmentor voltage, 120 V; skimmer 65 V. For mass accuracy control, calibration was daily performed with the manufacturer's solution, and the signal at *m/z* 112.9856 (formic acid dimer) was set as reference mass in all chromatographic runs. MS and MS/MS mass spectra were recorded over the range 80–1000 *m/z* with a scan rate of 4 spectra/s and over the range 50–1000 *m/z* with a scan rate of 6 spectra/s, respectively. The total cycle was 1.3 s, 6 compounds per cycle, isolation width of 4 u, active mass exclusion enabled after 30 spectra and 0.3 min, and absolute and relative precursor threshold 1000 were counts and 0.001%, respectively. Collision energies for tandem MS experiments were in the range of 15–30 eV.

Raw data were processed by Agilent MassHunter Qualitative Analysis 10.0 (Agilent Technologies, Palo Alto, CA, USA) for automatic feature extraction based on the following parameters: "small molecules" as extraction algorithm, "common organic molecules" as isotope grouping, absolute peak height > 1000, and "-H" and "H_2O" as allowed negative ions and neutral losses, respectively. The number of compounds extracted was restricted to the top 100 in terms of peak heights. Precision of the measures, assessed as relative standard deviation of three replicates, was always better than 14%.

2.6. Computational methodology

For all compounds listed in Table 2, the geometry optimization was performed in gas phase without any constraint. The M06-2X functional (Zhao & Truhlar, 2008) combined with the 6-31G(d) basis set was used, as implemented in Gaussian 16 (Frisch et al., 2016). For all compounds, radicals were generated by removing a hydrogen atom from each hydroxyl group, since OH groups are the most active sites for the scavenging mechanism here considered, i.e., hydrogen atom transfer (HAT). After full geometry optimization, spin contamination was checked for these doublet species to assess the reliability of the wavefunction and the associated energy. Frequency calculations were performed at the same level of theory (M06-2X/6-31G(d)) to confirm the stationary points (all positive frequencies) and to obtain the thermodynamic corrections at 1 atm and 298 K. Subsequently, single-point energy calculations were carried out at M06-2X/6–311 + G(d,p) in the gas phase to derive more accurate energy values, and, subsequently, at the same level of theory, in benzene, chloroform and water using the continuum Solvation Model based on Density (SMD) (Antony, Sure, & Grimme, 2015; Marenich, Cramer, & Truhlar, 2009). This level of theory is defined (SMD)-M06-2X/6–311 + G(d,p)//M06-2X/6-31G(d) and was successfully applied by some of us in previous studies to assess the antioxidant activity via HAT of important organic compounds (Bortoli et al., 2019; Muraro, Polato, Bortoli, Aiolli, & Orian, 2020; Ribaudo et al., 2020; Zeppilli et al., 2023).

Benzene and water were chosen to mimic an apolar and a polar environment, respectively, (Galano, 2011) and chloroform was included to more closely model the experimental setup. The electron spin density surfaces were extracted for selected structures with Multiwfn (Lu & Chen, 2012) with a high-quality grid and the isodensity value of 0.002.

3. Results

3.1. Biological screening assays

Phenolic compounds are one of the most important pharmacologically active natural classes capable of scavenging free radicals (Halliwell, 1996). Aiming to determine the antioxidative components of

Table 2
Results from the analysis carried out on PEE and the most bioactive fractions obtained from CHL by-extract from propolis samples collected in the Catamarca province. The relative abundance of each compound in propolis extract and fractions is related to the most abundant signal identified in the PEE extract, i.e. 2′, 4′- dihydroxychalcone **1**. MW_t = theoretical molecular weight.

N	Compounds	MW_t	Formula	RT, min				
					PEE	F4	F5	F6
	Chalcones and Dihydrochalcones							
1	2′, 4′- dihydroxychalcone	240.0786	$C_{15}H_{12}O_3$	14.55	100.0	37.5	12.4	19.9
2	2′, 4′-dihydroxy-3′-methoxychalcone	270.0892	$C_{16}H_{14}O_4$	14.35	45.5	–	–	14.3
3	2′,4′,4-trihydroxy-6′-methoxychalcone	286.0841	$C_{16}H_{14}O_5$	13.96	2.8	1022.8	95.3	–
4	4′-hydroxy-2′-methoxydihydrochalcone	256.1099	$C_{16}H_{16}O_3$	13.65	91.9	247.4	–	38.4
5	2′,4′-dihydroxydihydrochalcone	240.0786	$C_{15}H_{12}O_3$	12.33	23.1	–	–	–
	Flavanones							
6	7-hydroxyflavanone	240.0786	$C_{15}H_{12}O_3$	12.37	26.1	–	–	–
7	7-hydroxy-8-methoxyflavanone	270.0892	$C_{16}H_{14}O_4$	11.98	22.9	–	18.1	–
8	7,4′-dihydroxy-5-methoxyflavanone	286.0841	$C_{16}H_{14}O_5$	9.40	3.6	–	–	–
9	3,7-dihydroxyflavone	256.0736	$C_{15}H_{12}O_4$	10.09	5.2	–	–	–
10	7,8-dihydroxyflavanone	256.0736	$C_{15}H_{12}O_4$	10.38	9.7	103.6	130.0	56.2
11	pinocembrin (5, 7-dihydroxyflavanone)	256.0736	$C_{15}H_{12}O_4$	12.94	12.4	–	–	10.6
12	3,5-dihydroxyflavanone	256.0736	$C_{15}H_{12}O_4$	13.48	16.0	–	471.6	–
13	3,7,8-trihydroxydihydroflavanone	272.0698	$C_{15}H_{12}O_5$	10.94	7.7	467.3	–	–
14	pinobanksin-5-methyl ether (3,7-dihydroxy-5-methoxyflavanone)	286.0841	$C_{16}H_{14}O_5$	10.50	7.5	275.1	268.1	74.1
	Flavones							
15	rhamnocitrin (3,5,4′-trihydroxy-7-methoxyflavone)	300.0634	$C_{16}H_{12}O_6$	14.42	2.5	–	–	–
16	galangin (3,5,7-trihydroxyflavone)	270.0528	$C_{15}H_{10}O_5$	13.96	22.2	101.9	–	–
17	3,5-dihydroxy-7,8-dimethoxyflavone	314.0790	$C_{17}H_{14}O_6$	13.80	11.1	–	–	–
18	chrysin (5,7- dihydroxyflavone)	254.0579	$C_{15}H_{10}O_4$	13.26	9.1	452.9	–	–
	Caffeic acid derivatives Acids and esters							
19	caffeic acid	180.0423	$C_9H_8O_4$	3.81	–	15.1	–	15.3
20	1-methyl-3-(4′-hydroxyphenyl)-propyl caffeic acid ester	328.1310	$C_{19}H_{20}O_5$	13.44	46.8	–	18.9	1700.9
21	1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeic acid ester	344.1260	$C_{19}H_{20}O_6$	12.30	99.3	–	22.1	2219.2
22	4'terbutyloxy phenyl p-coumaric acid ester	312.1362	$C_{19}H_{20}O_4$	14.59	5.6	30.8	40.5	–
23	1-methyl-3-(4'hydroxyphenyl)-propyl *p*-coumaric acid ester	312.1362	$C_{19}H_{20}O_4$	13.94	26.7	744.1	904.4	26.8
24	1-methyl-3-phenylpropyl caffeic acid ester	312.1362	$C_{19}H_{20}O_4$	15.58	–	431.1	60.9	–
25	caffeic acid diprenyl ester (geranyl caffeate)	316.1675	$C_{19}H_{24}O_4$	15.39	28.6	1245.9	105.6	19.0
26	1-methyl-3-(3′,4′-di hydroxyphenyl)-propyl ferulic acid ester	358.1416	$C_{20}H_{22}O_6$	13.27	10.5	–	164.6	289.9
27	Caffeic acid benzyl ester	270.0892	$C_{16}H_{14}O_4$	12.99	9.3	151.5	80.1	–
28	2-methyl-3-(3′-hydroxy-4′-methoxyphenyl)propyl caffeic acid ester	358.1416	$C_{20}H_{22}O_6$	13.41	5.3	–	–	327.8

Zuccagnia-type propolis, preliminary screening tests, by using DPPH, β-carotene and non-enzymatic techniques, were carried out for HEX, CHL, AQ and PEE. The SC_{50} and PI_{50} values obtained for all the samples are summarized in Table 1, together with those related to two reference molecules, i.e. BHT and quercetin.

Concerning the free radical scavenging activity of the stable DPPH radical, the method is based on the measurements of $DPPH^{\bullet}$ residue after a given time, which is inversely related to the free radical scavenging activity of the antioxidant components. Differently from the β-carotene bleaching assay, the DPPH assay can be performed in polar organic solvent (Gulcin & Alwasel, 2023). CHL and PEE samples showed good antioxidant activity, with SC_{50} values of 18.5 μg/mL and 23.1 μg/mL, respectively, consistent with those already reported for *Z. punctata* ethanolic extract (Barbieri, Gilabert, & Benavente, 2023) (18.83 ± 0.21 μg/mL), whilst HEX had the lowest value. Since CHL exhibited the best antioxidant effectiveness, it was selected for the chromatographic fractionation on the silica-gel column (Section 2.4). Among the collected fractions, the F6 was far apart the most effective one with an SC_{50} of 9.0 μg/mL.

The β-carotene bleaching assay determined the protective effect of the extracts towards unsaturated fatty acids, which are quite susceptible to oxidative processes. The β-carotene bleaching was monitored by reading its absorbance decrease at 470 nm, which is slowed in the presence of antioxidants. Also in this case, the CHL extract was the most promising, and the most active fractions with this assay were eluted in the low-medium polarity range (F4-F6, see Fig. 1), with PI_{50} values lower than 10 μg/mL. Again F6, which is a relatively quite polar fraction, had the lowest PI_{50} value (2.4 μg/mL) for stabilizing free radicals in micellar medium, followed by F5, and F4 (Table 1). A noticeable value of β-carotene PI_{50} antioxidant activity of the fraction F7 was ascribed to the residual elution of components belonging to F6, as indicated by the TLC results.

For the evaluation of superoxide scavenging activity based on the PMS/NADH-NBT system, the absorbance decrease at 560 nm by antioxidants indicates the consumption of superoxide anion in the reaction mixture. The more polar fractions (F6 and F7) resulted to be more effective for stabilizing the superoxide radical, having again F6 the lower SC_{50} value (13 μg/mL). F2, F3, F4 and F5 did not reach the SC_{50} value within the range of evaluated concentrations.

3.2. LC-DAD-MS/MS identification of compounds

The correlation between the antioxidant activity and the chemical composition in terms of phenolic compounds was made on selected fractions showing the best effectiveness according the three used assays. F4, F5 and F6 were thus chosen for the LC-DAD-HRMS/MS analysis, as a compromise mainly between DPPH and β-carotene results, as SRSA gave limited indications. PEE was also analyzed in order to better depict the fractionation of the compounds from the propolis extract.

For each feature, identification and structure elucidation was based on the DAD profile, mass accuracy of the $[M\text{-}H]^-$ species (mass accuracy <5 ppm), interpretation of the experimental MS/MS spectrum according to (Eliana Rita Solorzano et al., 2017), and freely available databases, i.e. MassBank (massbank.eu/MassBank), Fooddb (www.foodb.ca) and ReSpect (https://rdrr.io/github/WMBEdmands/compMS2Miner/man/ReSpect.html).

In previous papers, (Solorzano et al., 2019; Eliana Rita Solorzano et al., 2017) the bioactivity and low allergenic potential of *Z. punctata* and propolis-related were ascribed to the simultaneous presence of several compounds belonging to chalcones, dihydrochalcones, flavones, flavanones, and caffeic acid derivatives with low sensitizing potential. A list of compounds selected from those previously reported for *Z. punctata* and related propolis was used for screening purposes. Table 2 reports the relative abundance percentage of each identified component for all sample extracts and fractions; the amount of each component is expressed as relative percentage area with respect to the most abundant one of the original PEE extract, which is one of the biomarker of the *Z. punctata*-related propolis, i.e. 2′, 4′- dihydroxychalcone **1**. This choice was made in order to simply visualize the eventual enrichment factor, and possibly balancing eventual matrix effects.

Caffeic acid derivatives are defined as esters consisting of caffeic, ferulic, cinnamic or coumaric acids linked by the acidic hydroxyl group to aromatic or non-aromatic moieties, which produce diagnostic MS fragment ions. Briefly, caffeates usually generate the characteristic deprotonated ion ($[M\text{-}H]^-$) of caffeic acid at *m/z* 179 (*m/z* 179.0350) that subsequently yields a fragment at *m/z* 135 (*m/z* 135.0452) (Jaiswal, Matei, Ullrich, & Kuhnert, 2011; Medana, Carbone, Aigotti, Appendino, & Baiocchi, 2008). Accordingly to the specific fragmentation pattern and identification scheme reported in (Eliana Rita Solorzano et al., 2017), and taking also into account the LC-DAD profile, the LC-MS/MS identifications of the main caffeic acid derivatives are reported in Table S1.

The list of total extracted features, cleaned from 6 blank samples (signals present in all analysis with peak height > 1000), was used to roughly assess the total composition of the F4, F5 and F6 fractions and the relative abundance of the identified compounds (Fig. 2a). F6 was the fraction richest in signals, followed by F4 and F5, and identified compounds accounted for about 47.3, 46.6 and 42.1% of the signals extracted from each fraction, respectively. As can be seen (Fig. 2b), the fractionating process resulted not selective for F4, where the identified chalcones, dihydrochalcones, flavones, flavanones, and caffeic acid derivatives were present with different relative abundances, whilst F5 was enriched in flavanones and caffeic acid derivatives, which in turn contributed almost exclusively to F6.

Since caffeic acid derivatives represent the most abundant class in all the selected fractions, it is evident that the resulting final radical scavenging activity of each fraction is strictly dependent on the amount of the single component present in the fraction itself.

As regard the most active fraction F6, the major component (Table 2 and Fig. 2a) was identified as 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeic acid (**21**, $[M\text{-}H]^- = 343.1187$), 1-methyl-3-(4′-hydroxyphenyl)-propyl caffeic acid ester (**20**, $[M\text{-}H]^- = 327.1238$) as the second one, followed by 2-methyl-3-(3′-hydroxy-4′-methoxyphenyl)propyl caffeic acid ester (**28**, $[M\text{-}H]^- = 357.1344$) and its isomeric form, tentatively attributed to 1-methyl-3-(3′,4′-di hydroxyphenyl)-propyl ferulic acid ester (**26**). Hypothesis about the hydroxylation positions of these compounds, not previously reported from propolis or *Z. punctata,* are derived from the shikimic acid metabolic pathway, which shows catechol (ortho), pyrogallol (di-ortho) or *p*-hydroxy for mono-hydroxylated rings (V. Bankova et al., 2002; Grayer, 2006; Vera et al., 2011b) as typical positions.

3.3. DFT analysis

The radical scavenging activity of the compounds listed in Table 2 was quantified considering the most relevant reactive oxygen species (ROS) quenching mechanism for polyphenols, i.e. hydrogen atom transfer (HAT, Eq. 1).

$$AOH + ROS^{\bullet} \rightarrow AO^{\bullet} + ROSH \quad (1)$$

where AOH denotes an antioxidant with hydroxyl sites from which the hydrogen atom can be transferred to quench ROSs.

ΔG^0_{HAT} were computed at (SMD)-M06-2X/6–311 + G(d,p)//M06-2X/6-31G(d,p) referring to the reaction of Eq. 1 and considering the following biologically relevant ROSs, i.e. $HO^{\bullet}$, $HOO^{\bullet}$, $CH_3OO^{\bullet}$, $CH_2{=}CHOO^{\bullet}$, $CH_2CHCHCHOO^{\bullet}$ and $CH_3O^{\bullet}$. $HO^{\bullet}$ is the most reactive radical; the peroxyl radicals $HOO^{\bullet}$ and $CH_3OO^{\bullet}$ are much less reactive, and thus can reach remote cellular locations; finally, $CH_3O^{\bullet}$ has an intermediate reactivity between $HO^{\bullet}$ and the peroxyl radicals. In addition, the four main hydroperoxyl radicals derived from methyl linolenate (here named L1-L4) were chosen to mimic larger unsaturated peroxyl

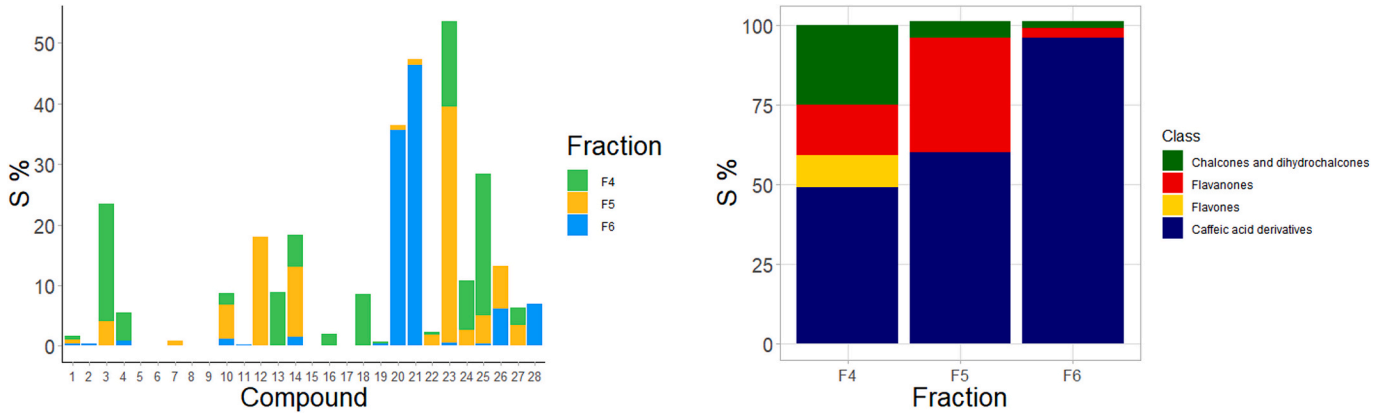

Fig. 2. Panel a) relative contribution of the single compound in the fraction, expressed as percentage of the total signals (S%) identified by LC-HRMS within the fraction. Compounds **1–5** belong to chalcones and dihydrochalcones, **6–14** to flavanones, **15–18** to flavones and **19–28** to caffeic acid derivatives; panel b) relative contribution of the class within the fraction, expressed as percentage of the total signals (S%) identified by LC-HRMS within the fraction.

radicals that can be evidenced by the β carotene assay (Porter, Caldwell, & Mills, 1995); Although the most effective mechanism for radical scavenging of flavonoids is strongly dependent not only on the antioxidant structure, but also on the medium polarity and the pH (León-Carmona, Alvarez-Idaboy, & Galano, 2012; Li et al., 2018; Michalík, Rimarčík, Lukeš, & Klein, 2019; Redzepovic, Markovic, & Tosovic, 2017; Spiegel, Andruniów, & Sroka, 2020; Zheng, Deng, Guo, Chen, & Fu, 2019), HAT is considered the most prominent method in the gas phase or in apolar media (such as benzene and chloroform) for many flavonoids molecules (León-Carmona et al., 2012; Vo et al., 2019; Zheng et al., 2019). Moreover, previous computational studies showed the importance of the OH bond dissociation energy (BDE) in determining the antioxidant capacity of a flavonoid (Alov, Tsakovska, & Pajeva, 2015; Luiz, Costa, Gomes, Silva, & Lião, 2017; Michalík et al., 2019). While this is indeed a meaningful parameter, it does not take into account the partner ROS which is scavenged. In this respect, the selectivity of a particular antioxidant is lost altogether if only the OH BDE is computed. In our approach, the particular reactivity of an antioxidant molecule towards a well-defined ROS is evaluated. Moreover, a general consensus on the best level of theory to employ in modelling these systems has not been reached yet (Michalík et al., 2019). In our study, we chose to use the M06-2X functional paired with the Pople basis set 6-31G(d) for the optimization and 6–311 + G(d,p) for the accurate calculation of the energy, since it was recently proposed as the best method to evaluate the total antioxidant activity of a molecule with QM calculations (Galano & Alvarez-Idaboy, 2013) and applied successfully in similar studies involving radical scavenging (Bortoli et al., 2019; Muraro et al., 2020, 2019; Ribaudo, Bortoli, Ongaro, et al., 2020; Ribaudo, Bortoli, Pavan, Zagotto, & Orian, 2020).With this methodology we focused on HATs from the hydroxyl phenolic groups, which were seen to have a pronounced exergonicity in similar molecules (Alberto, Russo, Grand, & Galano, 2013). The results are shown in Table S2-S10, and Fig. 3, where L denotes the average value of L1-L4 radicals as their values do not differ significantly: for all the molecules considered in this study and all the selected reactive sites, ΔG^0_{HAT} with the $HO^\bullet$ and $CH_3O^\bullet$ radicals are thermodynamically very favorable (Table S7), whereas with the peroxyl and unsaturated radicals the reactivity vary among the different structures (Tables S8 and S9).

All compounds have negative ΔG^0_{HAT} for reactions with $HO^\bullet$ and $CH_3O^\bullet$ whereas for the less reactive radicals ($HOO^\bullet$, $CH_3OO^\bullet$ and $CH_2{=}CHOO^\bullet$) this is true for most of the flavanones, flavones and caffeic acid derivatives (with a few exceptions), whereas chalcones and dihydrochalcones are calculated to have a positive ΔG^0_{HAT} with these radicals (Fig. 3). Finally, most of the compounds analyzed showed a positive ΔG^0_{HAT} with the methyl linoleate radical derivatives apart from few flavanones, flavones and some caffeic acid derivatives. Most notably this

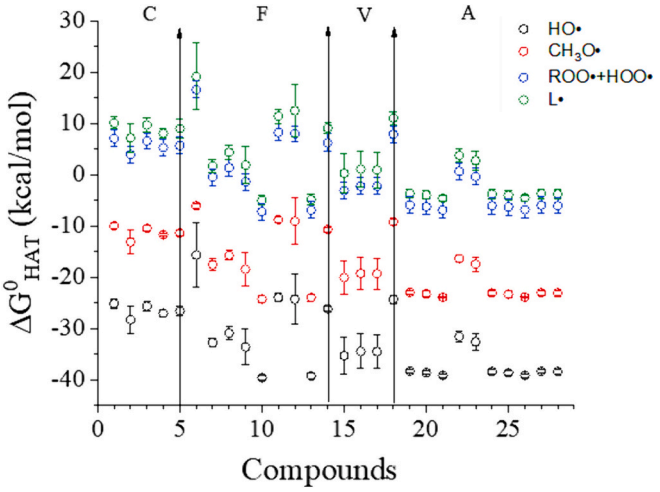

Fig. 3. ΔG^0_{HAT} averaged over four environments (gas-phase, chloroform, water and benzene) for the selected 28 compounds divided in 4 categories: C = chalcones and dihydrochalcones (**1–5**), F = flavanones (**6–14**); V = flavones (**15–18**); A = Caffeic acid derivatives acids and esters (**19–28**). $ROO^\bullet + HOO^\bullet$ comprises $HOO^\bullet$, $CH_3OO^\bullet$, $CH_2{=}CHOO^\bullet$, and $CH_2CHCHCHOO^\bullet$. L represents the average from L1-L4. The error bars highlight the variability of ΔG^0_{HAT} due to the different solvent. Level of theory (SMD)-M06-2X/6–311 + G(d,p)//M06-2X/6-31G(d,p).

latter category is the one that has the highest number of compounds showing a negative ΔG^0_{HAT} also with L1-L4 radicals (Table S10).

4. Discussion

The systematic chemical identification of the antioxidant constituents of *Zuccagnia*-type propolis is still lacking. In previous reports, the recognized biological activities have been ascribed to chalcones, dihydrochalcones, flavones and flavanones identified as biomarkers (Isla et al., 2021; Ana L. Salas et al., 2020), and limited interest has been given to the role of caffeic acid derivatives. They are compounds with a propenic chain linked to the phenols able to promote the modulation of the amount of ROS through the stabilization of the phenoxyl radical by resonance (Natella, Nardini, Di Felice, & Scaccini, 1999). Moreover, a polyphenol is a more efficient antioxidant than a phenol, due to the introduction of an electron-donating group, such as a hydroxyl group, in the ortho or para position (Göçer & Gülçin, 2011). The combined computational analysis allows to relate with accuracy the reactivity with the molecular topology.

In fact, DFT results (Tables S2-S10 and Fig. 3 and 4) show that structures with hydroxyl groups that do not have any other oxygen-containing moiety in the close proximity present very unfavorable ΔG^0_{HAT}. This effect is more pronounced if the OH group responsible for the ROS scavenging is the only substituent in the molecule (such as in **6**), while it is mitigated if other oxygen-containing groups are found in the structure (e.g., in compounds **12**, **14**, **22** and **23**). An explanation can be found by considering the spin density plots of the AO$^{\bullet}$: the reaction is more exergonic if the electron spin density arising from the radical formation can be more efficiently delocalized on an oxygen-containing moiety present in the *ortho* position to the carbon atom bearing the OH group acting as the scavenger. In fact, the calculated energies show that all the structures having a thermodynamically favorable reactivity towards all the selected radicals have a common structural feature, i.e. they possess two adjacent hydroxyl groups stabilizing the radical form obtained after the HAT process. We calculated the spin density of these structures to see this effect and found that the unpaired electron density is more delocalized in these structures contributing to their higher stability and to a more favorable radical scavenging action by HAT (Fig. 4).

Taking into account these structural considerations, the major component found in the F6 fraction, that is 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeic acid ester (**21**, $[M\text{-}H]^- = 343.1187$), accomplishes these requirements.

The relative abundance of **21** in the F6 fraction was more than twenty-fold larger than in the PEE extract and the SC_{50} values obtained for F6 fraction showed about three-fold higher scavenging effectiveness described by the DPPH test.

A more detailed representation of the relative abundance of the single molecule identified in the three fractions is shown in Fig. 2. It is worth of notice that chalcones and dihydrochalcones (**1**–**5**), markers of the *Z.p.*-type propolis, are not significantly represented in the fractions selected by DPPH and β-carotene results; only compounds **3** and **4**, 2′,4′,4-trihydroxy-6′-methoxychalcone and 4′-hydroxy-2′-methoxydihydrochalcone, were quite abundant in F4. Flavanones (**6**–**14**) and flavones (**15**–**18**) fractionation profile was distributed between F4 and F5, while caffeic acid derivatives (**19**–**28**) seemed to follow an elution gradient from F4 to F6, even if the behavior of this class was compound-dependent: compound **20**, **21**, **26** and **28** are the main constituents of the F6, which conversely was not concentrated in **22**, **23**, **24**, **25** and **27**. These last compounds, although belonging to the same class, do not contain hydroxyphenyl moiety in the ester function (**22**, **24**, **25** and **27**), have an alkyl alcohol (**22**, **25**) or are ester of the coumaric acid (**22**,**23**). The poly-hydroxylation of the aromatic rings shift the elution in the more hydrophilic fraction F6 having perhaps the lowest values of SC_{50} and PI_{50}. This evidence supported the role of some specific caffeic acid derivatives (**20**, **21**, **26** and **28**) in the scavenging activity.

The complexity of a natural substance containing multiple active radical scavengers poses a tough challenge for the theoretical investigation of *Z.p.*-type propolis as the values of ΔG^0_{HAT} of the single compound give only a partial image on the total reactivity. We have therefore summarized the contributions of each single compound and class of compounds, weighting the calculated ΔG^0_{HAT} with their relative abundance, as assessed from the LC-MS/MS analysis. This indicator, named $\Delta G^{0,}{}_{HAT}$ and shown in Fig. 5, may better support the interpretation of the thermodynamic contribute to the scavenging activity of the

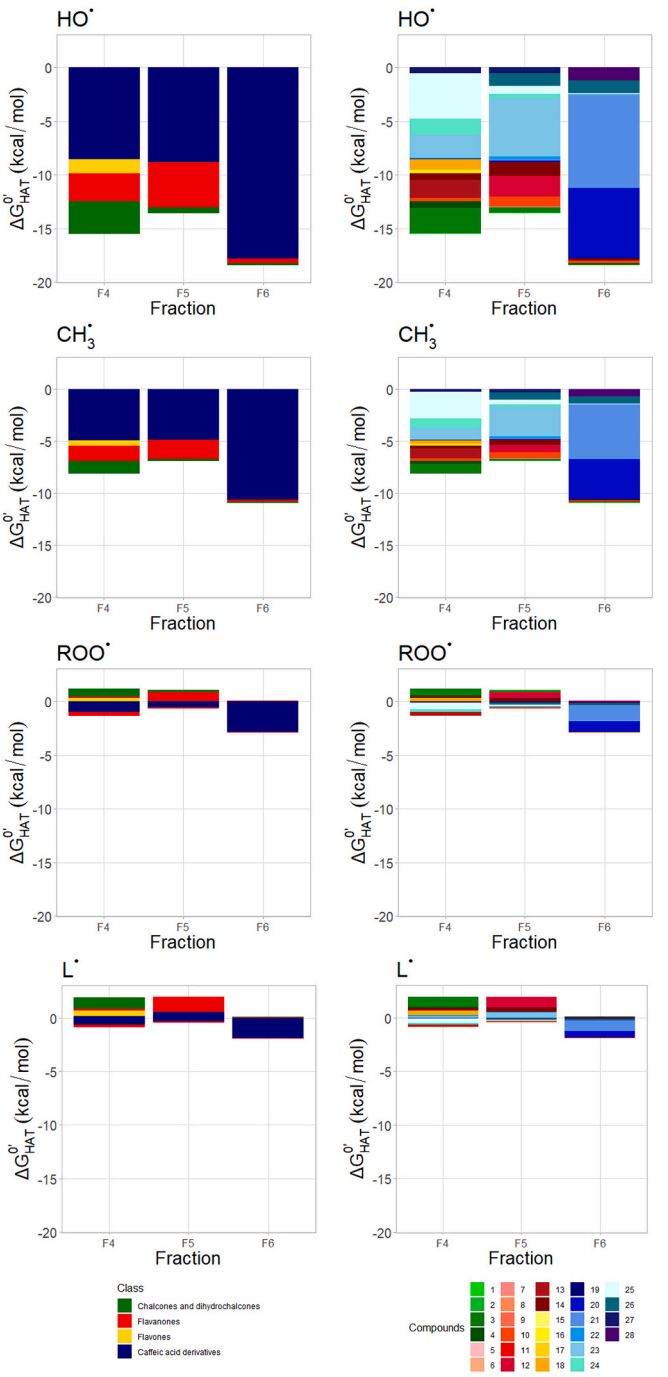

Fig. 5. Calculated $\Delta G^{0,}{}_{HAT}$ (kcal/mol) for each class of compounds and for single compounds, weighted for their relative abundance, as assessed from the LC-MS/MS analysis. ROO$^{\bullet}$ comprises HOO$^{\bullet}$, $CH_3OO^{\bullet}$, $CH_2{=}CHOO^{\bullet}$, and $CH_2CHCHCHOO^{\bullet}$, and L$^{\bullet}$ represents the average from L1-L4. Level of theory (SMD)-M06-2X/6–311 + G(d,p)//M06-2X/6-31G(d,p).

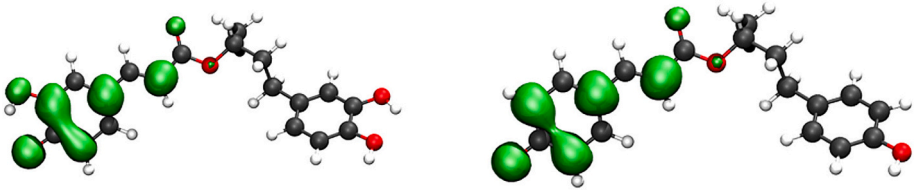

Fig. 4. Electron spin density calculated for 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeic acid ester (**a**) and 1-methyl-3-(4'hydroxy)phenyl propyl p-coumaric acid ester (**b**). Isodensity value 0.002 a.u. Level of theory M06-2X/6–311 + G(d,p)//M06-2X/6-31G(d,p).

fractions. It is clear how the F6 fraction, rich in caffeic acid derivatives (**19**–**28**) is the most thermodynamically favored in radical scavenging for all the tested free radicals. The most striking differences are found with the response of the fractions to the hydroperoxyl radicals ($ROO^{\bullet}$) and the methyl linoleate radicals ($L^{\bullet}$). As can be seen in Fig. 5, F4 and F5 fractions contain compounds that are overall not thermodynamically favored in the radical scavenging action with such models, whereas the F6 fractions clearly displays negative $\Delta G^{0,}{}_{HAT}$. Notably, also the extent of the different thermodynamic behavior assessed in this way may be considered coherent with results obtained from the DPPH and β-carotene assays, as the last one showed lower variations among fractions than those observed for DPPH (see Table 1). In particular, the high abundance of compounds **20** and **21** is the key factor that make the F6 fraction thermodynamically favorable. The origin of such favorable $\Delta G^{0,}{}_{HAT}$ can be traced to the presence of two close lying OH groups in those molecules that help stabilize the resulting radicals (see molecular formulas in Table S1).

In this regard, Ramachandra & Subbaraju, 2006 (Ramachandra & Subbaraju, 2006) synthesized 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeic acid ester (**21**) and 1-methyl-3-(4′-hydroxyphenyl)-propyl caffeic acid ester (**20**), and compared their antioxidant activity with commercially available antioxidants. Results showed that **21** and **20** were 16 fold and five-fold more active than BHT, respectively, evaluated with DPPH and nitroblue tetrazolium assays.

Other caffeic acid derivatives such as CAPE in poplar-type propolis have been reported to exhibit optimal antimicrobial and anti-proliferative activities related to its free radical scavenging properties (Banskota et al., 2002; Eid et al., 2010; Russo et al., 2002). It must be noted that although geranyl caffeate **25** is a caffeic acid derivative, its presence in the F4 fraction, despite its highest relative abundance and ΔG^0_{HAT} values comparable with **20** and **21**, resulted not efficient for the scavenging activity assessed with DPPH. Similarly, the most abundant components of *Zuccagnia*-type propolis such as chalcones, dihydrochalcones and flavanones isolated mostly in F4 and F5 do not seem to be essential for the free radical scavenging capacity, as can measured from both DPPH and β-carotene data. Indeed, for such class the ΔG^0_{HAT} values support these results. Anyway, some role may be played by the relative solubility in the test solutions, or a synergic action with other components, as suggested by the significant antioxidant activity values of PEE.

From the computational analysis, it emerges that caffeic acids are the compounds that show the most distinct scavenging ability among those investigated, as their reactions with all the selected free radicals, which comprise a wide array of free radicals present in a biological environment, were seen to be thermodynamically favored in all occurrences most noticeably with the larger and less reactive free radicals. In addition, a direct confirmation that the presence of vicinal OH groups is a required feature to have an effective scavenging activity was proved for compounds in all the investigated classes.

5. Conclusion

Zuccagnia punctata, as an endemic shrub growing in the Andean region of Argentina, has unique functional properties so that *Zuccagnia*-type propolis can represent ideal candidates to be included in healthy-effect formulations, both in nutraceutical and food products. The possibility to obtain the active principles without tearing the native plant *Zuccagnia punctata* also relies on the knowledge of role of the single compounds to the observed bioactivity.

To the best of our knowledge, this is the first report linking the free radical scavenging ability of *Zuccagnia*-type propolis to specific phenolic class and compounds guided by bioanalytical fractionation, also suggesting some associations between structures and bioactivities. From these results it is evident that the caffeic acid derivatives play the main contribute to the outstanding antioxidant activity of the *Z. punctata* propolis, being **20** and **21** the most relevant compounds enriched in the fraction with the medium-high polarity (F6). It is also possible to discriminate among the effectiveness of the different caffeic acid derivatives, as some compounds, i.e. **22**, **23** and **24**, although quite abundant in the F4-F5 fractions and in some cases even larger than **20** and **21**, did not contribute consistently to the antioxidant activity, as evidenced by the DPPH and β-carotene data of the fractions. Indeed, our computational analysis confirms the importance of the presence of vicinal hydroxyl groups, as despite most of the compounds of the caffeic acid derivatives series have a negative ΔG^0_{HAT} with all the free radicals considered, the only species showing a positive ΔG^0_{HAT} are compound **22** and **23**, which display a single OH group that makes their scavenging action thermodynamically not favored.

This proposed combined approach can be very useful to drive the evaluation of bioactive substances such as the phenolic compounds naturally present in most of the products derived by plants with a nutraceutical value.

CRediT authorship contribution statement

Eliana Rita Solorzano: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Sara Bogialli:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Marco Bortoli:** Writing – review & editing, Validation, Investigation, Data curation, Conceptualization. **Laura Orian:** Writing – review & editing, Supervision, Methodology, Investigation. **Denis Badocco:** Visualization, Data curation. **Silvia Pettenuzzo:** Validation, Formal analysis, Data curation. **Gabriella Favaro:** Visualization. **Paolo Pastore:** Writing – review & editing, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.140827.

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