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Contribution of the phenolic composition to the antioxidant, anti-inflammatory and antitumor potential of *Equisetum giganteum* L. and *Tilia platyphyllos* Scop.

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Naturally-occurring phytochemicals have received pivotal attention in the last few years, due to the increasing evidence of biological activities. Thus, in the present study, the antioxidant, anti-inflammatory and antitumor potentials of hydroethanolic extracts rich in phenolic compounds obtained from *Equisetum giganteum* L. and *Tilia platyphyllos* Scop. were assessed and directly correlated with their content of phenolic compounds, by using HPLC-DAD-ESI/MS analysis. *T. platyphyllos* showed the higher bioactive potential, evaluated in terms of antioxidant (radical scavenging effects – 105 μg mL^{-1}, reducing power – 123 μg mL^{-1}, β-carotene bleaching inhibition – 167 μg mL^{-1}, and lipid peroxidation inhibition – 56 μg mL^{-1}), anti-inflammatory (225 μg mL^{-1} inhibited 50% of nitric oxide production) and antitumor (breast – 224 μg mL^{-1}; lung – 247 μg mL^{-1}; cervical – 195 μg mL^{-1} and hepatocellular – 173 μg mL^{-1} carcinoma cells) activity, without having cytotoxic effects (>400 μg mL^{-1}). These biological properties were positively correlated with its content and composition of phenolic compounds. Flavonoid contents were markedly higher than the content of phenolic acids, in both samples, being respectively 50.4 mg g^{-1} and 11.65 mg g^{-1} for *T. platyphyllos*, and 21.7 mg g^{-1} and 4.98 mg g^{-1} for *E. giganteum*. Moreover, while in *E. giganteum* extract, kaempferol-*O*-glucoside-*O*-rutinoside was the most abundant flavonoid, in *T. platyphyllos* extract protocatechuic acid and (–)-epicatechin were the most abundant phenolic acid and flavonoid, respectively. In relation to their content of phenolic acids, protocatechuic and caffeic acids existed in higher abundance in *T. platyphyllos* and *E. giganteum* hydroethanolic extracts, respectively. However, it would be interesting to evaluate the *in vivo* efficacy of both plant extracts to unveil the involved modes of action and to establish effective therapeutic doses.

1. Introduction

Natural products, among them plant-derived preparations, revealed to be a rich source of bioactive compounds, such as alkaloids, terpenes, steroids and phenolic compounds, are substances responsible for multiple biological effects.[1–8]

The latest scientific advances have shown that most of the bioactive phytochemicals are secondary metabolites from which through their consumption promissory phytopharmacological effects may be reached.[5–8] In the case of phenolic compounds, antioxidant, anti-diabetic, anti-inflammatory, antimicrobial, antitumor, anti-ageing, cardioprotective, hypolipidemic, neuroprotective and hepatoprotective effects have been observed.[5,9–11] However, the botanical class, plant family, species and origin affect the final chemical composition and consequently the bioactivity of plant-derived preparations; cultivation conditions, harvesting time, storage and processing techniques, and extraction conditions also play a determinant role.[12–14]

Equisetum giganteum L., commonly known as "giant horsetail" (English), "cavalinha" (Portuguese), "cola de caballo" (Spanish), is a native plant from Central and South America that belongs to the Equisetaceae family.[15] The herb of this plant is largely used in dietary supplements as a diuretic,

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hemostatic, anti-inflammatory and anti-rheumatic agent.[16,17] To the author's best knowledge, no studies are available describing its antioxidant or antitumor effects, and only one study could be found regarding the *in vivo* anti-inflammatory potential of aqueous extracts derived from stems, through using antigen-induced arthritis.[17] The phenolic composition of this plant has been described, including the presence of caffeic acid derivatives and flavonoids (mainly quercetin and kaempferol derivatives).[15,18] However, it should be highlighted that different types and proportions of solvents (namely, ethanol : water) were used by the authors in the extraction procedures.

Tilia platyphyllos Scop., commonly known as "linden" or "lime" (English), "tília" (Portuguese) and "tilo" (Spanish) is a well-known plant species that belongs to the Malvaceae family, native to Central and Southern Europe, from which very interesting biological activities have also been observed.[19] Diuretic, stomachic, antispasmodic, sedative, antioxidant, hepatoprotective and anti-neuralgic properties are some of them.[19] *In vitro*[20] and *in vivo*[19] antioxidant activities of the aqueous extract obtained, respectively, from leaves and flowers were previously described, in the last case by using animal models with ethanol-induced oxidative stress. The most abundant phenolic compounds in this species have been referred to as flavonoids (namely quercetin and kaempferol derivatives).[20,21] Once again, and on the same lines as *E. giganteum*, different types of solvents (namely, ethanol : water) were used by the authors in the extraction procedures, and therefore different classes and relative abundances of phenolic compounds present were determined.

Thus, the present study aims to correlate the phenolic composition of both the mentioned plant species with their antioxidant, anti-inflammatory and antitumor potential, evaluated using hydroethanolic extracts.

2. Experimental section

2.1. Samples and preparation of hydroethanolic extracts

Aerial parts (namely, leaves, flowers and inflorescences) of *Equisetum giganteum* L. and *Tilia platyphyllos* Scop. were provided by Cantinho das Aromáticas, organic farmers from Vila Nova de Gaia (Portugal). The dried samples were reduced to powder form and subjected to an extraction procedure at room temperature (≈25 °C), in three independent experiments, as follows: 1 g of each sample was extracted under magnetic stirring for 1 h in the dark with 30 mL of ethanol : water (80 : 20, v/v), filtered and re-extracted under the same conditions. Then, ethanol was removed using a rotary evaporator (Büchi R-210, Flawil, Switzerland), while water was removed by lyophilisation. Finally, stock solutions of each hydroethanolic extract were prepared: 5 mg mL^{-1} in ethanol/water (80 : 20, v/v) for antioxidant activity assays, 8 mg mL^{-1} in water for antitumor and anti-inflammatory assays, from which several dilutions were prepared and 2.5 mg mL^{-1} in ethanol/water (80 : 20, v/v) for phenolic compound analysis (the extract was filtered through a 0.45 μm Whatman syringe filter and transferred to amber color HPLC vial).

2.2. Analysis of phenolic compounds

HPLC-DAD-ESI/MS^n analyses were performed using a Hewlett-Packard 1100 chromatograph (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA), with a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station and connected *via* the cell outlet to a MS detector API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) through an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Separation was achieved on a Waters Spherisorb S3 ODS-2 C_{18} (3 μm, 4.6 × 150 mm) column at 35 °C, using a gradient elution; identification and quantification of the phenolic compounds were performed as described in ref. 22. The chromatograms were recorded at several wavelengths, characteristic of different classes of polyphenols, such as 280 nm for phenolic acids, flavan-3-ols and flavanones, 330 nm for hydroxycinnamic acids and 370 nm for flavonols and flavones. The calibration curves for each available phenolic standard (5–100 μg mL^{-1}, Extrasynthese, Genay, France) were constructed: (+)-catechin ($y = 135x + 33$; $R^2 = 0.999$; LOD, 0.17 μg mL^{-1}; LOQ, 0.72 μg mL^{-1}); caffeic acid ($y = 359x + 488$; $R^2 = 0.998$; LOD, 0.19 μg mL^{-1}; LOQ, 0.65 μg mL^{-1}); chlorogenic acid ($y = 304x - 248$; $R^2 = 1$; LOD, 0.20 μg mL^{-1}; LOQ, 0.68 μg mL^{-1}); (−)-epicatechin ($y = 163x + 40$; $R^2 = 0.999$; LOD, 0.15 μg mL^{-1}; LOQ, 0.78 μg mL^{-1}); kaempferol-3-*O*-glucoside ($y = 236x + 70$; $R^2 = 1$; LOD, 0.11 μg mL^{-1}; LOQ, 0.38 μg mL^{-1}); kaempferol-3-*O*-rutinoside ($y = 183x + 97$; $R^2 = 1$; LOD, 0.13 μg mL^{-1}; LOQ, 0.43 μg mL^{-1}); naringenin ($y = 540x + 161$; $R^2 = 0.994$; LOD, 0.20 μg mL^{-1}; LOQ, 0.64 μg mL^{-1}); protocatechuic acid ($y = 258x + 328$; $R^2 = 0.998$; LOD, 0.14 μg mL^{-1}; LOQ, 0.52 μg mL^{-1}); quercetin-3-*O*-glucoside ($y = 336x + 358$; $R^2 = 0.998$; LOD, 0.21 μg mL^{-1}; LOQ, 0.71 μg mL^{-1}) and quercetin-3-*O*-rutinoside ($y = 281x + 374$; $R^2 = 0.998$; LOD, 0.22 μg mL^{-1}; LOQ, 0.75 μg mL^{-1}). The quantification of the peak areas was performed based on the UV signal and baseline to valley integration with the baseline projection mode and results were expressed as mg per g of the extract.

2.3. Evaluation of the antioxidant activity

Four different assays were used to assess the *in vitro* antioxidant potential of both plant extracts: scavenging effects on DPPH (2,2-diphenyl-1-picrylhydrazyl, Alfa Aesar, Ward Hill, MA, USA) radicals (RSA), reducing power measured by ferricyanide Prussian blue assay (RP), β-carotene bleaching inhibition (CBI) and lipid peroxidation inhibition (LPI), by thiobarbituric acid reactive substances (TBARS) assay, following a procedure previously described.[23] All results obtained were expressed as EC_{50} values, corresponding to the extracts' concentration that provides 50% of antioxidant activity, or 0.5 of absorbance in the case of a reducing power assay. Three independent experiments were performed in each situation, for both plant extracts and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-

2-carboxylic acid, Sigma-Aldrich, St Louis, MO, USA) was used as a positive control.

2.4. Evaluation of the anti-inflammatory activity

To assess the anti-inflammatory activity, a macrophage-like cell line RAW264.7 (European Collection of Animal Cell Culture, Salisbury, UK) was used and cultured, following a procedure previously described.[24] The nitrite levels produced were determined by measuring the optical density at 515 nm, in a microplate reader, and the obtained results were compared with the standard calibration curve. Dexamethasone (50 μM, Sigma-Aldrich, St Louis, MO, USA) was used as the positive control. Three independent experiments were carried out and the final results were expressed in EC_{50} values, *i.e.* the extract concentration that inhibited 50% of the NO production.

2.5. Evaluation of the cytotoxic and antitumor activity

The cytotoxicity was evaluated using four tumor cell lines, HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer), using a protocol previously described,[25] where the cell growth inhibition was measured at 540 nm (ELX800 microplate reader, Bio-Tek Instruments, Inc.; Winooski, VT, USA) using the sulforhodamine B assay (SRB, Sigma-Aldrich, St Louis, MO, USA).

For non-tumor porcine liver cells (PLP2) freshly harvested porcine liver was used in order to obtain the cell culture, designated as PLP2. The growth inhibition was evaluated using the SRB assay previously described.[25]

Three independent experiments were carried out for both assays, and the obtained results were expressed in GI_{50} values, *i.e.* the extract concentration that inhibited 50% of the net cell growth and ellipticine (Sigma-Aldrich, St Louis, MO, USA) was used as the positive control.

2.6. Statistical analysis

Three samples were used for each preparation and all the assays were carried out in triplicate. Results were expressed as mean values and standard deviation (SD) and analysis was performed through a Student's *t*-test with $p = 0.05$, using a SPSS v. 23.0 program (IBM Corp., Armonk, New York, USA); a Pearson's correlation analysis was also carried out with a 95% confidence level.

3. Results and discussion

3.1. Detailed analysis of the phenolic composition

Tables 1 and 2 present the phenolic compounds' characteristics, tentative identities and their relative abundance in *E. giganteum* and *T. platyphyllos*, respectively. The chromatographic profile of *E. giganteum* and *T. platyphyllos* can be observed in Fig. 1A and B. *Equisetum giganteum* presented fourteen phenolic compounds (two phenolic acids and twelve flavonol glycoside derivatives), while *T. platyphyllos* presented a more complex matrix regarding its phenolic composition, revealing the presence of thirty-three compounds (three phenolic acids, six flavan-3-ol derivatives, twenty flavonol glycoside derivatives, two flavanone and flavone derivatives). Compounds 2^{Tp} (protocatechuic acid), 4^{Eg} (caffeic acid), 3^{Tp} (catechin), 4^{Tp} (5-*O*-caffeoylquinic acid), 6^{Tp} (epicatechin), 15^{Tp} (quercetin-3-*O*-rutinoside; rutin), 17^{Tp} (quercetin-3-*O*-glucuronide), 10^{Eg} and 20^{Tp} (quercetin-3-*O*-glucoside; isoquercitrin), 23^{Tp} and 11^{Eg} (kaempferol-3-*O*-rutinoside) and 13^{Eg} (kaempferol-3-*O*-glucoside) were positively identified by comparison with authentic standards, as well as their MS fragmentation pattern, retention time and UV-vis characteristics. Kaempferol-3-*O*-glucoside has been reported in hydroethanolic extracts of aerial parts of *E. giganteum*,[15,16] and also as a quercetin-hexoside.[16] Protocatechuic acid, catechin, epicatechin and

Table 1 Phenolic compounds' identification and quantification in the *Equisetum giganteum* extract

Compound	R_t (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m*/*z*)	MS^2 (*m*/*z*)	Tentative identification	Quantification (mg g^{-1} extract)
1^{Eg}	7.2	330	341	179 (100)	Caffeic acid hexoside	0.131 ± 0.003
2^{Eg}	9.4	352	625	463 (100), 301 (32)	Quercetin-3,7-di-*O*-glucoside	Tr
3^{Eg}	11.5	348	755	593 (100), 447 (10), 285 (38)	Kaempferol-*O*-glucoside-*O*-rutinoside	7.6 ± 0.2
4^{Eg}	11.9	330	179	135 (100)	Caffeic acid	4.86 ± 0.03
5^{Eg}	14.1	348	651	489 (33), 447 (17), 285 (17)	Kaempferol-*O*-acetylglucoside-*O*-glucoside	0.087 ± 0.005
6^{Eg}	14.5	352	667	505 (100), 301 (42)	Quercetin-*O*-acetylglucoside-*O*-glucoside	Tr
7^{Eg}	15.8	348	651	489 (38), 447 (55), 285 (56)	Kaempferol-*O*-acetylglucoside-*O*-glucoside	5.01 ± 0.05
8^{Eg}	18.8	336	739	593 (77), 431 (10), 285 (27)	Kaempferol-*O*-rhamnoside-*O*-rutinoside	0.67 ± 0.04
9^{Eg}	19.2	348	593	447 (41), 431 (51), 285 (58)	Kaempferol-*O*-glucoside-*O*-rhamnoside	1.86 ± 0.02
10^{Eg}	21.2	358	463	301 (100)	Quercetin-3-*O*-glucoside	Tr
11^{Eg}	23.1	348	593	285 (100)	Kaempferol-3-*O*-rutinoside	1.95 ± 0.03
12^{Eg}	23.8	350	635	489 (13), 431 (55), 285 (31)	Kaempferol-*O*-acetylglucoside-*O*-rhamnoside	1.77 ± 0.06
13^{Eg}	24.7	350	447	285 (100)	Kaempferol-3-*O*-glucoside	1.81 ± 0.03
14^{Eg}	30.1	350	489	285 (100)	Kaempferol-*O*-acetylglucoside	0.88 ± 0.03
					Total phenolic acids	4.98 ± 0.03
					Total flavonoids	21.7 ± 0.4
					Total phenolic compounds	26.6 ± 0.3

Tr – traces.

Table 2 Phenolic compounds' identification and quantification in the *Tilia platyphyllos* extract

Compound	R_t (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg g^{-1} extract)
1^{Tp}	5.1	328	353	191 (100), 179 (44), 161 (5), 135 (38)	3-*O*-Caffeoylquinic acid	0.64 ± 0.03
2^{Tp}	6.1	260, 294sh	153	109 (100)	Protocatechuic acid	6.3 ± 0.1
3^{Tp}	7.2	280	289	245 (12), 203 (20), 187 (10), 161 (5), 137 (5)	(+)-Catechin	1.6 ± 0.1
4^{Tp}	8.1	328	353	191 (100), 179 (5), 135 (6)	5-*O*-Caffeoylquinic acid	4.7 ± 0.1
5^{Tp}	9.0	280	577	451 (26), 425 (37), 407 (100), 289 (69), 287 (7)	Procyanidin dimer B2	4.2 ± 0.1
6^{Tp}	11.1	280	289	245 (8), 203 (22), 187 (11), 161 (7), 137 (7)	(−)-Epicatechin	6.41 ± 0.01
7^{Tp}	13.1	280	865	739 (10), 713 (18), 577 (34), 575 (21), 425 (14), 407 (25), 289 (15), 287 (20)	Procyanidin trimer	2.54 ± 0.02
8^{Tp}	14.2	280	1153	865 (6), 863 (17), 577 (61), 575 (17), 289 (42), 287 (10)	Procyanidin tetramer	3.9 ± 0.1
9^{Tp}	15.5	280	1441	1153 (9), 865 (9), 577 (31), 289 (96), 287 (30)	Procyanidin pentamer	2.4 ± 0.2
10^{Tp}	16.3	356	609	463 (9), 447 (27), 301 (12)	Quercetin-*O*-hexoside-*O*-deoxyhexoside	0.13 ± 0.01
11^{Tp}	16.7	356	609	463 (20), 447 (45), 301 (31)	Quercetin-3-*O*-glucoside-7-*O*-rhamnoside	1.4 ± 0.1
12^{Tp}	17.8	286, 336sh	449	287 (100)	Eriodictyol-*O*-hexoside	1.3 ± 0.1
13^{Tp}	18.3	358	579	447 (64), 433 (10), 301 (33)	Quercetin-O-pentoside-O-rhamnoside	0.65 ± 0.05
14^{Tp}	19.2	348	593	447 (41), 431 (54), 285 (33)	Kaempferol-3-*O*-glucoside-7-*O*-rhamnoside	1.52 ± 0.02
15^{Tp}	19.5	356	609	301 (100)	Quercetin-3-*O*-rutinoside (rutin)	0.96 ± 0.02
16^{Tp}	19.8	352	593	446 (53), 301 (39)	Quercetin-3,7-di-*O*-rhamnoside	1.1 ± 0.1
17^{Tp}	20.2	350	477	301 (100)	Quercetin-3-*O*-glucuronide	0.21 ± 0.05
18^{Tp}	20.5	348	563	431 (66), 417 (26), 285 (39)	Kaempferol-*O*-pentoside-*O*-deoxyhexoside	0.76 ± 0.05
19^{Tp}	20.6	358	463	301 (100)	Quercetin-3-*O*-galactoside (hyperoside)	1.01 ± 0.02
20^{Tp}	21.1	356	463	301 (100)	Quercetin-3-*O*-glucoside (isoquercitrin)	6.36 ± 0.04
21^{Tp}	22.5	360	505	463 (12), 301 (100)	Quercetin-3-*O*-acetylglucoside	0.090 ± 0.004
22^{Tp}	22.9	284, 334sh	433	271 (100)	Naringenin-*O*-hexoside	0.90 ± 0.05
23^{Tp}	23.1	346	593	285 (100)	Kaempferol-3-*O*-rutinoside	1.03 ± 0.01
24^{Tp}	23.4	346	577	431 (72), 285 (78)	Kaempferol-3,7-di-*O*-rhamnoside	1.15 ± 0.03
25^{Tp}	23.7	356	433	301 (100)	Quercetin-*O*-pentoside	1.8 ± 0.1
26^{Tp}	24.8	350	447	301 (100)	Quercetin-3-*O*-rhamnoside (quercitrin)	3.5 ± 0.1
27^{Tp}	26.1	348	447	285 (100)	Kaempferol-*O*-hexoside	0.55 ± 0.03
28^{Tp}	26.8	352	417	285 (100)	Kaempferol-*O*-pentoside	0.38 ± 0.03
29^{Tp}	29.2	346	431	285 (100)	Kaempferol-*O*-rhamnoside	0.39 ± 0.03
30^{Tp}	30.7	350	431	285 (100)	Kaempferol-*O*-rhamnoside	0.45 ± 0.04
31^{Tp}	32.5	332	283	269 (100)	Methyl apigenin	2.07 ± 0.01
32^{Tp}	35.0	316	593	447 (10), 285 (79)	*trans*-Tiliroside	1.38 ± 0.03
33^{Tp}	35.3	326	593	447 (10), 285 (79)	*cis*-Tiliroside	0.27 ± 0.03
					Total phenolic acids	11.65 ± 0.05
					Total flavonoids	50.4 ± 0.4
					Total phenolic compounds	62.0 ± 0.4

quercetin-3-*O*-glucoside have also been found in commercial samples of *T. platyphyllos* from Syria and Tuscany.[21]

Besides the already mentioned phenolic acid derivatives, 3-*O*-caffeoylquinic acid (peak 11^{Tp}) was also tentatively identified based on its MS^2 fragmentation pattern, according to the systematic keys provided by Clifford *et al.*[26] for the identification of chlorogenic acids. Phenolic acid derivatives represent 19% of the total phenolic composition of *T. platyphyllos*.

The sample of *T. platyphyllos* presented four compounds, 5^{Tp} ($[M-H]^-$ at m/z 577), 7^{Tp} ($[M-H]^-$ at m/z 865), 8^{Tp} ($[M-H]^-$ at m/z 1153) and 9^{Tp} ($[M-H]^-$ at m/z 1441), with characteristics of proanthocyanidins (UV spectra with λ_{max} 280 nm), being assigned as a procyanidin dimer, trimer, tetramer and pentamer, respectively. A comparison with our library database allowed identifying peak 5^{Tp} as procyanidin dimer B2, also reported by Negri *et al.*[27] in hydroethanolic extracts from leaves of *Tilia cordata*. These compounds have been reported as major compounds in samples of *T. platyphyllos* by Karioti *et al.*[21] In the present study, these compounds were also present in high amounts, representing 34% of the total

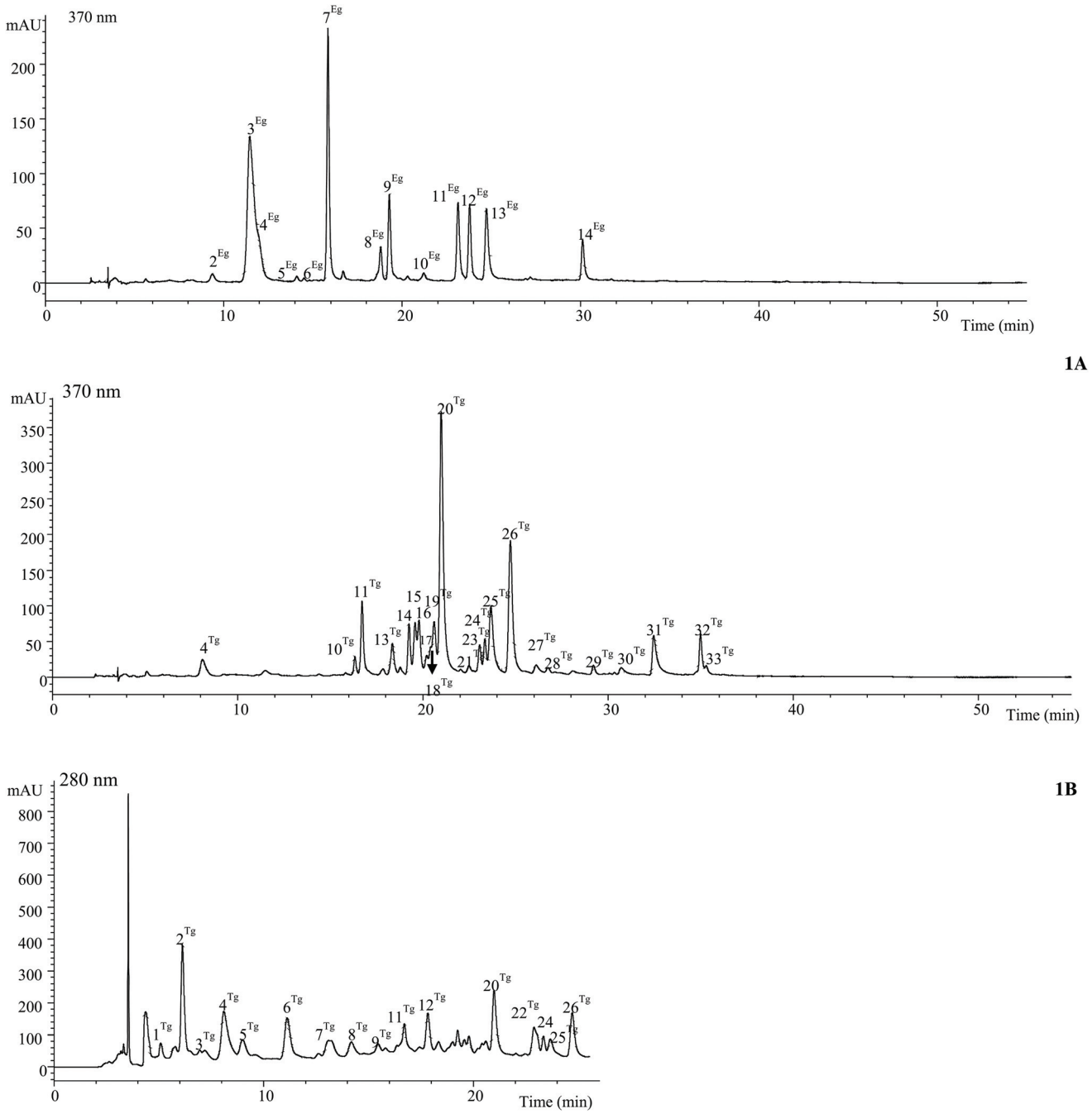

Fig. 1 Phenolic compound profiles of *E. giganteum* recorded at 370 nm (A) and *T. platyphyllos* recorded at 370 and 280 nm (B). The numbering corresponds to the peak number presented in Tables 1 and 2.

phenolic content, including (+)-catechin, (−)-epicatechin being the most abundant compound found (6.4 mg g^{-1} extract).

Peaks 10^{Tp}, 11^{Tp}, 13^{Tp}, 16^{Tp}, 19^{Tp}, 21^{Tp}, 25 and 26^{Tp} were identified as quercetin derivatives owing to the product ion observed at *m*/*z* 301 and their UV spectra (λ_{max} around 350–358 nm). Peaks 10^{Tp} and 11^{Tp} both presented the same pseudomolecular ion ($[M - H]^-$ at *m*/*z* 609) as peak 15^{Tp} (quercetin-3-*O*-rutinoside), but a different fragmentation pattern as in those compounds product ions from the alternative loss of hexosyl (*m*/*z* at 447; −162 u) and deoxyhexosyl (*m*/*z* at 463; −146 u) were observed, indicating their location at different positions of the aglycone. Compounds with similar features have been reported in *T. platyphyllos*[21] and *T. cordata*[27] and identified as quercetin-3-*O*-glucoside-7-*O*-rhamnoside, an identity that was tentatively assumed for peak 11^{Tp} in our sample owing to its greater concentration, whereas peak 10^{Tp} remained just assigned as quercetin-*O*-hexoside-*O*-deoxyhexoside. Peak 16^{Tp} showed a pseudomolecular ion $[M - H]^-$ at *m*/*z*

593, yielding two fragment ions from the consecutive loss of two deoxyhexosyl residues; it was tentatively identified as quercetin-3,7-di-*O*-rhamnoside, a compound commonly reported in *Tilia* species[27–30] including *T. platyphyllos*,[21] whereas peak 13^{Tp} ($[M - H]^-$ at *m*/*z* 579) was tentatively assigned as quercetin-*O*-pentoside-*O*-rhamnoside, as reported in *T. cordata* by Negri *et al.*[27] Compounds 19^{Tp} ($[M - H]^-$ at *m*/*z* 463), 25^{Tp} ($[M - H]^-$ at *m*/*z* 433) and 26^{Tp} ($[M - H]^-$ at *m*/*z* 447), presented MS^2 fragments corresponding to distinct losses of hexosyl (−162 u), pentosyl (−132 u) and deoxyhexosyl (−146 u) moieties, and an elution order coherent with the type of sugar substituents, according to their expected polarity. Although the position and nature of the sugar moieties could not be identified, an identity as quercetin-3-*O*-rhamnoside (quercitrin) was assumed for compound 26^{Tp}, previously described in *T. platyphyllos*[21] and other *Tilia* species.[27,29,30] Similarly, peak 19^{Tp} was tentatively assigned as quercetin-3-*O*-galactoside (hyperoside) owing to its previous identification in *Tilia tomentosa*[30] and *T. cordata*;[31] that identity would also be supported by its close elution to quercetin-3-*O*-glucoside (peak 20^{Tp}), since galactosides typically elute before the equivalent glucosides in RP-HPLC.[32] Peak 21^{Tp} ($[M - H]^-$ at *m*/*z* 505), with a molecular weight 42 u (acetyl residue) higher than compounds 19^{Tp} and 20^{Tp} was tentatively identified as quercetin-3-*O*-acetylglucoside, assuming that it could derive from the more abundant precursor.

Peaks 14^{Tp}, 18^{Tp}, 24^{Tp}, 27^{Tp}, 28^{Tp}, 29^{Tp}, 30^{Tp}, 32^{Tp} and 33^{Tp} were identified as kaempferol glycosides based on their UV spectra (λ_{max} around 348 nm) and due to the production of an MS^2 fragment ion at *m*/*z* 285. Tentative identities of these compounds were assigned based on their pseudomolecular ions using a similar reasoning as for quercetin derivatives. Thus, peaks 14^{Tp} ($[M - H]^-$ at *m*/*z* 593) and 24^{Tp} ($[M - H]^-$ at *m*/*z* 577) should correspond to kaempferol-3-*O*-glucoside-7-*O*-rhamnoside and kaempferol-3,7-*O*-dirhamnoside, respectively, compounds that have been reported in *T. platyphyllos*[21] and other *Tilia* species.[27–30] Peaks 32^{Tp} and 33^{Tp} possessed the same pseudomolecular ion ($[M - H]^-$ at *m*/*z* 593) as peaks 14^{Tp} and 24^{Tp} (kaempferol-3-*O*-rutinoside), but eluted at a longer retention time. The presence of tiliroside (kaempferol-3-*O*-(6-*p*-coumaroyl)-glucoside; 6″-*O*-(4-hydroxycinnamoyl)-astragalin) has been consistently reported in different species of *Tilia*,[27,29–31,33] including *T. platyphyllos*.[21] The observation in their fragmentation of a product ion at *m*/*z* 447, from the loss of *p*-coumaroyl residue (146 u) would also be coherent with that identity, as well as with the late elution, since the presence of the hydroxycinnamoyl residue implies a decrease in polarity. The detection of two peaks could be explained by the existence of *trans*- and *cis*-isomers of the *p*-coumaric acid, as also noted by Karioti *et al.*[21] The most abundant peak 32^{Tp} can be assigned as *trans*-tiliroside taking into account that the form *trans* is the usual one for *p*-coumaric acid. Peak 27^{Tp} ($[M - H]^-$ at *m*/*z* 447) presented the same pseudomolecular ion as kaempferol-3-*O*-glucoside (astragalin), a compound that was commonly detected in *Tilia* spp.,[21,27,29,31] but it eluted at a different retention time, as compared with a commercial standard; thus, the detected compound was just assigned as kaempferol-*O*-hexoside. Peaks 29^{Tp} and 30^{Tp}, with the same pseudomolecular ion ($[M - H]^-$ at *m*/*z* 431) are coherent with kaempferol bearing it as a deoxyhexosyl moiety. Two undefined kaempferol rhamnosides were found in *T. tomentosa* by Ieri *et al.*,[30] while the presence of kaempferol-3-*O*-rhamnoside was reported in different *Tilia* spp.[21,27,29] that could correspond to one of the compounds detected in our study. However, since no sufficient information to assign that identity to any of the two detected peaks could be obtained, both compounds remain identified as kaempferol-*O*-rhamnoside. The remaining two kaempferol derivatives, *i.e.*, peaks 18^{Tp} and 28^{Tp}, were respectively assigned as kaempferol-*O*-pentoside-*O*-hexoside and kaempferol-*O*-pentoside, respectively, based on their mass spectral characteristics. As far as we are aware, none of these compounds has been previously reported in *Tilia* spp. Flavonols accounted for 41% of the content of total phenolic compounds, quercetin-3-*O*-glucoside being the main molecule of this group.

Compounds 12^{Tp} ($[M - H]^-$ at *m*/*z* 449) and 22^{Tp} ($[M - H]^-$ at *m*/*z* 433) presented UV spectra characteristics of flavanones and MS^2 fragments at *m*/*z* 287 (eriodictyol) and at *m*/*z* 271 (naringenin), respectively, indicating the loss of an hexosyl residue (162 u), so that they were tentatively assigned as eriodictyol-*O*-hexoside and naringenin-*O*-hexoside. Finally, peak 31^{Tp} showed a pseudomolecular ion $[M - H]^-$ at *m*/*z* 283 that released an MS^2 fragment at *m*/*z* 269 (apigenin, $[M - H - 14]^-$) indicating the loss of a methyl group; this observation together with its UV spectrum, characteristic of a flavone, suggested that it might correspond to a methyl apigenin. To the best of our knowledge, neither flavanones nor flavones have been described in *T. platyphyllos*.

Equisetum giganteum mainly presented kaempferol derivatives, accounting for 81% of the total phenolic content. Thus, in addition to peaks 11^{Eg} and 13^{Eg}, positively identified as kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucoside, peaks 3^{Eg}, 5^{Eg}, 7^{Eg}, 8^{Eg}, 12^{Eg} and 14^{Eg} were also assigned as kaempferol-derived compounds based on their UV spectra (λ_{max} around 348 nm) and the observation of a product ion at *m*/*z* 285; tentative identities were attributed from their molecular weight and fragmentation patterns.

Compound 3^{Eg} ($[M - H]^-$ at *m*/*z* 755) MS^2 fragments revealed the alternative loss of hexosyl (*m*/*z* at 593; −162 u) and deoxyhexosyl-hexoside (*m*/*z* at 447; −308 u) residues, indicating the location of each residue on different positions of the aglycone. The positive identification of kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucoside may suggest the presence of glucosyl and rutinosyl as sugar substituents, so that the compound was tentatively assigned as kaempferol-*O*-glucoside-*O*-rutinoside. This molecule was the most abundant phenolic compound (7.6 mg g^{-1} extract) present in this sample. Similarly, compound 8^{Eg} ($[M - H]^-$ at *m*/*z* 739) was assigned as kaempferol-*O*-rhamnoside-*O*-rutinoside.

Peaks 5^{Eg} and 7^{Eg}, with the same $[M - H]^-$ at *m*/*z* 651 and similar fragmentation pattern, with the production of fragment ions at *m*/*z* 489 and 447, from the alternative loss of hexosyl (−162 u) and acetylhexosyl residues (−204 u), were assigned as

kaempferol-*O*-acetylglucoside-*O*-glucoside. The observation of two compounds might be explained by different substitution positions of either each of the two residues on the aglycone or the acetyl moiety on the glucose. Compound 9^{Eg} ($[M - H]^-$ at *m*/*z* 593) showed the same pseudomolecular ion as kaempferol-3-*O*-rutinoside but a different fragmentation pattern. The observation of product ions at *m*/*z* 447 (−146 u) and 431 (−162 u) revealed the alternative loss of deoxyhexosyl and hexosyl residues, allowing its identification as kaempferol-*O*-glucoside-*O*-rhamnoside, a compound that was reported in extracts of the aerial parts of *Equisetum telmateia*.[34] Peak 12^{Eg} ($[M - H]^-$ at *m*/*z* 635) presented a molecular weight 42 u greater than peak 9^{Eg} and MS^2 fragment ions at *m*/*z* 489 (−146 u, loss of a rhamnosyl residue) and 431 (−204 u, loss of an acetylhexosyl residue), being identified as kaempferol-*O*-acetylglucoside-*O*-rhamnoside. Peak 14^{Eg}, with an $[M - H]^-$ at *m*/*z* 489 yielding a unique fragment ion at *m*/*z* 285, from the loss of an acetylhexosyl residue, was assigned as kaempferol-3-*O*-acetylglucoside, a compound also reported by Correia *et al.*[34] in *E. telmateia*.

Peaks 2^{Eg} and 6^{Eg} were assigned as quercetin derivatives based on their absorption spectra and the production of a fragment ion at *m*/*z* 301. Based on their fragmentation features and following a similar reasoning as for the previous kaempferol derivatives, they were assigned as quercetin-di-*O*-glucoside and quercetin-*O*-acetylglucoside-*O*-glucoside, respectively. Peak 2^{Eg} was tentatively identified as quercetin-3,7-di-*O*-glucoside, owing to the previous description of such a compound in *E. giganteum* by Francescato *et al.*[15] and Alavarce *et al.*[16]

Finally, peak 1^{Eg} was identified as a caffeic acid hexoside according to its UV and mass characteristics.

With the exception of quercetin-3-*O*-glucoside and kaempferol-3-*O*-rutinoside, reported in *E. giganteum* by Francescato *et al.*[15] and Alavarce *et al.*,[16] none of the compounds detected herein had been previously described in this species.

3.2. Evaluation of the antioxidant activity

The results of the antioxidant activity of both plant extracts are shown in Table 3. In general, *T. platyphyllos* evidenced a higher antioxidant potential than *E. giganteum*, both as a free radical scavenger, and also as a lipid peroxidation inhibitor, these results being clearly confirmed, respectively, by the lowest RSA, RP, CBI and LPI EC_{50} values. Not least interesting to highlight is that this biological activity seems to be directly correlated with the relative abundance in phenolic compounds: for the plant extract with higher antioxidant effects, *i.e. T. platyphyllos*, a high concentration of phenolic compounds was found (Tables 1 and 2). Thus, it is feasible to infer that the most prominent antioxidant potential evidenced to *T. platyphyllos*, through all the *in vitro* assays performed, was mainly attributed to their richness in phenolic compounds, particularly flavonoids (quercetin and kaempferol derivatives), as shown in Table 2.

Majer *et al.*[20] aiming to assess the *in vitro* antioxidant potential (singlet oxygen scavenging) of *T. platyphyllos* leaf flavonoids, concluded that sun leaves possess a higher myricetin content than shade leaves, and that quercetin and myricetin derivatives, mainly occurring as rhamnosides, exerted much higher singlet oxygen scavenger effects than kaempferol glycosides. In fact, a higher content in quercetin derivatives was observed in *T. platyphyllos*, to the detriment of kaempferol derivatives; furthermore, in *E. giganteum* only vestigial amounts of quercetin were determined, kaempferol being the most abundant. On the other hand, Yayalaci *et al.*[19] evaluating the *in vivo* antioxidant potential of *T. platyphyllos* flowers against ethanol-induced oxidative stress, concluded that linden flowers were able to not only prevent oxidative damage in the studied tissues, but also to inhibit the production of ethanol-induced free radicals in rats. Thus, and considering the obtained results, it is feasible to suppose that the antioxidant activity of *T. platyphyllos* is directly correlated with the relative abundance in phenolic compounds, mainly quercetin derivatives.

Table 3 Antioxidant, antitumor and anti-inflammatory activities of the studied plant extracts

	E. giganteum	*T. platyphyllos*	Student's *t*-test *p*-Value
Antioxidant activity (EC_{50} values, μg mL^{-1})			
DPPH scavenging activity (RSA)	123 ± 5	105 ± 1	<0.001
Reducing power (RP)	136 ± 1	123 ± 7	<0.001
β-Carotene bleaching inhibition (CBI)	202 ± 3	167 ± 2	<0.001
TBARS inhibition (LPI)	57 ± 1	56 ± 1	0.024
Antitumor activity (GI_{50} values, μg mL^{-1})			
MCF-7 (breast carcinoma)	250 ± 15	224 ± 19	0.058
NCI-H460 (non-small cell lung cancer)	258 ± 13	247 ± 22	0.380
HeLa (cervical carcinoma)	268 ± 16	195 ± 15	0.001
HepG2 (hepatocellular carcinoma)	239 ± 18	173 ± 13	0.002
Hepatotoxicity (GI_{50} values, μg mL^{-1})			
PLP2	>400	>400	—
Anti-inflammatory activity (EC_{50} values, μg mL^{-1})			
Nitric oxide (NO) production	239 ± 20	225 ± 22	0.314

The antioxidant activity was expressed as EC_{50} values, which means that higher values correspond to a lower reducing power or antioxidant potential. EC_{50}: extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox EC_{50} values: 41 μg mL^{-1} (reducing power), 42 μg mL^{-1} (DPPH scavenging activity), 18 μg mL^{-1} (β-carotene bleaching inhibition) and 23 μg mL^{-1} (TBARS inhibition). Results of the anti-inflammatory activity are expressed in EC_{50} values: sample concentration providing 50% of inhibition of nitric oxide (NO) production. Dexamethasone EC_{50} value: 16 ± 2 μg mL^{-1}. Cytotoxicity results are expressed in GI_{50} values corresponding to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. Ellipticine GI_{50} values: 1.2 μg mL^{-1} (MCF-7), 1.0 μg mL^{-1} (NCI-H460), 0.91 μg mL^{-1} (HeLa), 1.1 μg mL^{-1} (HepG2) and 2.3 (PLP2).

3.3. Evaluation of the anti-inflammatory activity

The anti-inflammatory potential of both plant extracts was evaluated through the measurement of nitric oxide (NO)

production, and the obtained results are presented in Table 3. Once again, the phenolic composition seems to exert a determinant role in the anti-inflammatory potential of plant extracts: *T. platyphyllos* appears to be more effective than *E. giganteum* because a lower concentration of the plant extract was necessary to achieve 50% of inhibition of NO production, than using *E. giganteum*. As previously mentioned, only Farinon *et al.*[17] assessed the *in vivo* anti-inflammatory potential of *E. giganteum*; otherwise, to the author's best knowledge, no studies have been previously reported on the anti-inflammatory potential of *T. platyphyllos*. However, the *in vitro* anti-inflammatory potential of other phenolic matrices has been also previously assessed by other authors.[35,36] Chen and Kang[35] by assessing the *in vitro* anti-inflammatory potential of a methanolic extract obtained from *Capsicum annuum* L. (red pepper) stalk extracts, observed that a concentration of 100 μg mL^{-1} was able to inhibit 53.5% of NO production. On the other hand, Silva *et al.*[36] aiming to assess the *in vitro* anti-inflammatory potential of three different phenolic extracts (two aqueous and one methanol extract) obtained from *Gomphrena globosa* L. inflorescences reported that aqueous extracts were able to inhibit by 50% the NO production at 472.3 ± 31.3 μg mL^{-1} (decoction) and 1260 ± 50.5 μg mL^{-1} (infusion), while for methanolic extracts, a concentration of 1166.0 ± 16.7 μg mL^{-1} was necessary.

Thus, considering the results obtained in the present study, despite *E. giganteum* also acting as an anti-inflammatory agent, the effect of *T. platyphyllos* was higher; therefore, it may be considered a rich source of phenolic compounds with anti-inflammatory potential. In fact, a concentration of 225 ± 22 μg mL^{-1} of *T. platyphyllos* hydroethanolic extract was effective at inhibiting 50% of NO production, while for *E. giganteum* it was necessary to use 239 ± 20 μg mL^{-1}.

3.4. Evaluation of the cytotoxic activity

The cytotoxicity of both plant extracts was evaluated in non-tumor porcine liver cells (PLP2) and also in tumor cell lines (breast carcinoma – MCF-7, non-small cell lung carcinoma – NCI-H460, cervical carcinoma – HeLa and hepatocellular carcinoma – HepG2), and the results are given in Table 3. Neither *E. giganteum* nor *T. platyphyllos* extracts showed toxicity in non-tumor liver cells (PLP2). On the other hand, a relatively low concentration of the extracts was necessary to cause 50% of the growth inhibition in human tumor cell lines. Once again, *T. platyphyllos* was revealed to be the more effective than *E. giganteum*, for all the tested tumor cell lines. Cytotoxic properties of the mentioned extracts were not previously mentioned in the literature.

Overall, the total phenolic compounds, phenolic acids and flavonoids were highly correlated with the antioxidant, anti-inflammatory and antitumor activities of both samples, presenting high correlation factors, especially for the DPPH scavenging activity (R^2 = 0.89), reducing power (R^2 = 0.66), β-carotene bleaching inhibition (R^2 = 0.98), TBARS (R^2 = 0.73), and for human tumor cell lines HeLa (R^2 = 0.94), HepG2 (R^2 = 0.93) and MCF-7 (R^2 = 0.62), confirming the statements mentioned above.

4. Conclusion

Hydroethanolic extracts rich in phenolic compounds obtained from *E. giganteum* and *T. platyphyllos* seem to be very interesting matrices for a wide variety of conditions. The *in vitro* antioxidant, anti-inflammatory and antitumor effects were further investigated in this experiment, with promissory results being achieved. *T. platyphyllos* showed a higher biological potential than *E. giganteum*, its phenolic compound content and composition being directly correlated with the observed potentialities. In fact, in *T. platyphyllos* a higher content of total flavonoids (50.4 ± 0.4 mg g^{-1}) and phenolic acids (11.65 ± 0.05 mg g^{-1}) was reached; however, despite the observed biochemical differences, *E. giganteum* also gave very positive results. Its content of total flavonoids and phenolic acids was significantly lower than that obtained for *T. platyphyllos*, 21.7 ± 0.4 mg g^{-1} and 4.98 ± 0.03 mg g^{-1}, respectively, but the observed biological potential was also very interesting. While in *T. platyphyllos* the most abundant phenolic acid and flavonoid were, respectively, protocatechuic acid and (−)-epicatechin, followed by quercetin 3-*O*-glucoside, in *E. giganteum* the most abundant compounds were caffeic acid and kaempferol-*O*-glucoside-*O*-rutinoside, respectively.

Thus, in conclusion, despite the obtained results for both phenolic matrices, further studies are necessary to assess the *in vivo* efficacy, the involved mechanism of action and the related therapeutic doses of both plant extracts.

Author contribution

L. Achour and I. C. F. R. Ferreira conceived the study. I. Jabeur and N. Martins carried out the experiments. L. Barros gave support in the analysis of phenolic compounds. R. C. Calhelha and J. Vaz gave support for antitumor assays. L. Barros, I. C. F. R. Ferreira and C. Santos-Buelga performed data organization and analysis of the results. I. Jabeur, N. Martins and L. Barros wrote the manuscript. I. C. F. R. Ferreira, L. Achour and C. Santos-Buelga revised the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

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