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Phytochemical diversity and biological activities of *Hypericum japonicum* and *Hypericum sampsonii*: potential for natural product-based food applications

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ABSTRACT

This study characterizes two species of the genus *Hypericum* to envisage their applicability as effective and versatile functional foods, dietary supplements, and food preservatives. A wide phenolic composition was found in both extracts, highlighting flanovoids for *H. japonicum* and xanthones for *H. sampsonii*. Moreover, anthocyanins were analyzed for the first time in the latter plant. Antioxidant capacity was highlighted by oxidative hemolysis inhibition assay (OxHLIA), where *H. japonicum* was more effective (lower EC_{50}) than antioxidant Trolox (16.3 < 21.8 μg/mL). *H. sampsonii* extract inhibited lipid peroxidation in the thiobarbituric acid reactive substances (TBARS) method (EC_{50} = 17.05 μg/mL) compared to Trolox (EC_{50} = 5.8 μg/mL). *H. japonicum* antibacterial activity showed a minimum inhibitory concentration (MIC) of 0.007 mg/mL, even lower than the control. These results indicate the bioactive potential of both extracts, as well as the importance of evaluating the food-related bioactive components of medicinal plants and the mechanisms involved in their bioactivities.

1. Introduction

The genus *Hypericum* Tourn. ex L., the largest within the Hypericaceae family, comprises about 500 flowering plant species classified under 36 taxonomic sections based on morphological and molecular characteristics (Bálintová, Bruňáková, Petijová, & Čellárová, 2019; Dresler, Kováčik, Strzemski, Sowa, & Wójciak-Kosior, 2018). It is found in grasslands, thickets, forest clearings, oak groves, and burned areas, ranging from temperate regions to high tropical mountains, and avoiding extreme habitats (Caldeira, Gouveia, Serrano, & Silva, 2022). The distinction among *Hypericum* (*HP*) species is made on the distribution of dark glands on bracts, leaves, petals, sepals and stems (R. Zhang et al., 2021). There are 64 species of *HP* in China, 33 of which are endemic, and 19 have been used in folk and traditional medicine due to their association with health benefits (Caldeira et al., 2022; Ji et al., 2021). As a globally distributed genus, the possible number of unexplored *HP* species is enormous. According to ethnopharmacological records, Chinese herbalists used *H. perforatum* and other shrubby species (*H. hookerianum, H. bellum, H. polyanthemum, H. androsaemum, and H. patulum*) in traditional medicine for treating heat or fever, hemostasis and detumescence, hepatitis, dysentery, colds, burns, wounds, and as a sedatives or antibacterial agents (Allegra, Tonacci, Spagnolo, Musolino, & Gangemi, 2021; Caldeira et al., 2022; Q. Chen, Di, Zhang, & Li, 2020; Jabeur et al., 2016; Ji et al., 2021; Liu, Liu, & He, 2014; Wu et al., 2018; Xue et al., 2023). *In vitro* and *in vivo* bioactivities attributed to natural extracts from the genus *HP* and its compounds include antimicrobial, antioxidant, anti-inflammatory, astringent, analgesic, antitumor, antidiabetic, antidepressant, antihyperglycemic, and hepatoprotective properties (Caldeira et al., 2022, 2023; Ion et al., 2022; Velingkar, Gupta, & Hegde, 2017).

Species of the genus *HP* have been reported to contain a wide range of phytochemicals of different classes that may display additive, synergistic and partly antagonistic effects among themselves, which may significantly modulate the biological activities that can be achieved from different *HP* extracts and formulations (Zhang, Ji, Zhang, Kennelly,

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Abbreviations

Generic

HP	*Hypericum*
SJW	St. John's wort
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
GALAEs	Galanthamine equivalents
AChE	Acetylcholinesterase inhibitor
TNF-α	Tumor necrosis factor-alph
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Rt	Room temperature
dw	Dry weight
OS	Oxidative stress
EMA	European Medicines Agency

Nutritional and chemical profile

CH	Carbohydrates
L	Lipids
P	Proteins
E	Energy
TFA	Trifluoroacetic acid
TLC	Total Lipid Content
SFA	Saturated fatty acids
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
UFA	Unsaturated fatty acids
TPC	Total phenolic compound content
TFC	Total flavonoid content

Compounds

PPAPs	Prenylated acylphloroglucinol
MTs	Meroterpenoids
mPPAP	Methylated polycyclic polyprenylated acylphloroglucinol derivatives
NaCL	Sodium chloride
Na_2SO_4	Anhydrous sodium sulphate
P36"g	Pelargonidin-3-O-(6″-malonylglucoside)
C3R	Cyanidin-3-O-rutinoside
PK	Polyketide
MeOH	Methanol

Equipment and techniques

UPLC	Ultra-high performance liquid chromatography
QTof	Quadrupole time-of-flight
NMR	Nuclear magnetic resonance spectroscopy
MS	Mass spectrometry
UV–Vis	UV–visible spectrophotometry
UFLC	Ultra-Fast liquid chromatography
HPLC	High-performance liquid chromatography
GC	Gas chromatography
DAD	Diode array detection
ESI	Electrospray ionization source
FID	Flame Ionization Detector
FAME	Fatty acid methyl esters
LC	Liquid chromatography

Antioxidant assays

EC_{50}	Effective concentration for 50 % antioxidant activity
IC_{50}	Half maximal(50 %) inhibitory concentration
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid
CAA	Cellular antioxidant activity
OxHLIA	Oxidative Hemolysis Inhibition Assay
TBARS	Thiobarbituric Acid Reactive Substances
OD	Optical density
MDA-TBA	Malondialdehyde-thiobarbituric acid
PBS	Phosphate buffered saline

Anti-inflammatory, cytotoxic and antitumoral assays

GI_{50}	Concentration responsible for 50 % of cell growth inhibition
RAW 264.7	Mouse macrophage-like leukemia cell line
PLP2	Porcine liver cell line
SW480	Cryopreserved human colon adenocarcinoma cell line
AGS	Gastric adenocarcinoma cell line
CaCo2	Colorectal adenocarcinoma cell line
MCF-7	Breast adenocarcinoma cell line
NCI-H460	Non-small cell lung cancer cell line
A375	Human melanoma cell line
MDA-MB-231	M.D. Anderson - Metastatic Breast 231 cell line
SiHa	Human cervical squamous carcinoma cell line
TPH-1	Human leukemia monocytic cell line
sw480	Large intestine of a Dukes C colorectal cancer cell line
SMMC-7721	Human hepatocellular carcinoma cell line
DNA	Dalton's ascites lymphoma cell line
SHSY-5Y	Human Neuroblastoma cell line
HepG2	Hepatoblastoma cell line
HeLa	Cervical cancer cell line
SH-SY5Y	Thrice-subcloned cell line
PC12	Catecholamine cell line
A549	Hypotriploid alveolar basal epithelia cell line
SRB	Sulforhodamine B assay
RPMI-1640	Roswell Park Memorial Institute Medium
FBS	Fetal bovine serum

Antimicrobial assays

MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
DCFH	2′,7′-dichlorohydrofluorescein
AAPH	2.2 2′-azobis(2-methylpropionamide) dihydrochloride
DMEM	Dulbecco's Modified Eagle medium
HBSS	Hanks' Balanced Salt Solution
DMSO	Dimethyl sulfoxide
UFC	Colony Forming Units
MEB	Malt extract broth
DMSO	Dimethyl sulfoxide
TSB	Tryptic soy broth
INT	*p*-iodonitrotetrazolium chloride

& Long, 2020). As a result, raise scientific interest on the identification of these compounds as well as public interest in the application of this medicinal herb in phytotherapy (Bridi, Meirelles, & Von Poser, 2018; Kimáková et al., 2018). Up to date, over 900 chemical compounds have been isolated from *HP* spp., which include prenylated acylphloroglucinols (PPAPs), meroterpenoids, phloroglucinol derivatives (e.g., hyperforin), xanthones (e.g., *cis*-kielcorin), naphthodianthrones (e.g., hypericin and pseudohypericin); phenolic compounds as flavonoids (e.g., rutin, quercetin, and myricetin glucosides), biflavones (e.g., I3, II8-biapigenin and amentoflavone), phenolic acids (e.g., coumaroylquinic, chlorogenic, caffeic, vanillic, *p*-hydroxybenzoic, and ferulic acids), proanthocyanidins and tannins, catechins, essential oils, and other phenolic and terpenoid compounds (Bridi et al., 2018; Eroğlu Özkan, Özsoy, Özhan, Özbek Çelik, & Mat, 2013; Ion et al., 2022). Hyperforin,

hypericin and pseudohypericin are the most well-known and significant secondary metabolites of *HP* plants for industry applications (Caldeira et al., 2022; Rizzo, Altschmied, Ravindran, Rutten, & D'auria, 2020; Sobhani Najafabadi et al., 2019). Although a variety of biological effects have been associated with these metabolites, it has been difficult to attribute the health-promoting properties of the extract to a single constituent, so the extract is considered to be the active principle (Zeliou et al., 2020).

Regarding the biological activities of *HP* plants, pharmacological research in the last decade highlights the antitumor and anti-inflammatory activity over others (Allegra et al., 2021; *M.* Huang, Geng, & Ding, 2022; N. Huang et al., 2011). Table 1 provides an overall review of several *HP* species, showing these effects against different cancer cell lines of the crude extracts (**Table 1**A) and pre-isolated and purified active compounds of *HP* species (**Table 1**B). Phenolic compounds predominate and their link to the biological activities of *HP* species has been studied. These bioactivities are thought to be due to inhibition of monoamine oxidase, inhibition of synaptosomal reuptake of amines (serotonin (5-hydroxytryptamine), norepinephrine, dopamine, γ-aminobutyric acid, and L-glutamate), as well as effects on monoamine transporters and effects on serotonin receptors. While these mechanisms are primarily associated with antidepressant effects, they

Table 1
Phytochemical compounds, antitumor and anti-inflammatory activity in *Hypericum* genus.

***Hypericum* species**	**Phytochemistry**						**Antitumor and anti-inflammatory**		**Ref.**
	Main metabolite	**Molecular formula**	**Compound type**	**Technique**	**[M-H]** (*m/z*)	**Quant.** (mg/g)	**Cell line**	**Result** (GI_{50}, μM)	
A. Crude extract									
H. japonicum	Taxifolin-7-*O*-α-L-rhamnoside	$C_{21}H_{22}O_{11}$	Flavononol	HPLC-DAD-ESI/MS^n	449	23.03	N/A	N/A	(Gao et al., 2009)
H. japonicum	Isoquercitrin	$C_{18}H_{14}O_6$	Flavonol glycosides	UPLC-QTof-MS	463.0874	N/A	SMMC-7721	59.80 (mg/mL)	(R. Zhang et al., 2021)
	Betulinic acid	$C_{38}H_{48}O3$	Triterpenoid		455.352				
	Taxifolin	$C_{21}H_{22}O_{11}$	Flavononol		449.107				
H. japonicum	Quercetin-3-*O*-*β*-*D*-glucuronide	$C_{21}H_{17}O_{13}$	Flavonol glycosides	LC–MS^n coupled with UPLC-QTOF	477.0647	5.39	THP-1	5.4 (mg/mL)	(Peron et al., 2019)
	Quercitrin	$C_{21}H_{19}O_{11}$			447.0911	3.41			
	Coumaroylquinic acid	$C_{16}H_{17}O_8$	Phenolic acid		337.0910	0.86			
H. himalaicum	Hyperoside	$C_{21}H_{20}O_{12}$	Flavonol glycosides	UPLC-QTof-MS	463.0874	N/A	SW480	N/A	(R. Zhang et al., 2021)
H. lanuginosum	Quinic acid	$C_{16}H_{17}O_9$	Phenolic acid	LC-HRMS-MS	191.055	N/A	N/A	N/A	(Mahomoodally et al., 2019)
H. androsaemum	5-*O*-Caffeoylquinic acid	$C_{16}H_{18}O_9$	Phenolic acid	HPLC-MS	353	40.1	HepG2	100	(Jabeur et al., 2016)
	Quercetin-3-*O*-glucoside	$C_{24}H_{22}O_{15}$	Flavonol glycoside		463	10.69	MCF-7	106	
H. pamphylicum	Chlorogenic acid	$C_{16}H_{18}O_9$	Phenolic acid	HPLC-DAD	N/A	5.58	HeLa	1.89	(Eroğlu Özkan et al., 2013)
H. androsaemum	5-*O*-Caffeoylquinic acid	$C_{16}H_{18}O_9$	Phenolic acid	HPLC-DAD	353	40.1	N/A	N/A	(Jabeur et al., 2016)
H. sampsonii	Rutin	$C_{27}H_{30}O_{16}$	Flavonol glycosides	HPLC	N/A	N/A	SH-SY5Y	49.57 (mg/mL)	(Q. Chen et al., 2020)
	Kaempferol	$C_{15}H_{10}O_6$							
	Gallic acid	$C_7H_6O_5$	Phenolic acid						
B. Purified or isolated compound									
H. wightianum	Hyperwightin	$C_{20}H_{16}O_6$	Flavone derivative	HR-ESI-MS	51.0875	N/A	PC12	N/A	(Yang et al., 2019)
H. stellatum	Hypxanthone	$C_{18}H_{15}O_7$	Xanthone	HR-ESI-MS	343.0823	N/A	HepG2	10.1	(Ji et al., 2019)
H. henryi	Hyperhenone	$C_{33}H_{40}O_6Na$	PPAPs	HR-ESI-MS	555.271	N/A	A549	40	(Duan et al., 2018)
H. beanii	Hyperxylone	$C_{33}H_{43}O_5$	PPAPs	HR-ESI-MS	519.3105	N/A	RAW 264.7	18.46	(X. Y. Li et al., 2023)
H. elodeoides	C-glycoside	$C_{19}H_{24}NaO_9$	Phenolic glycosides	HR-ESI-MS	419.1314	N/A	MCF-7	13.24	(Mu et al., 2023)
H. japonicum	Hyjapone	$C_{41}H_{57}O_8$	PK-MTs	HR-ESI-MS	677.4044	N/A	RAW 264.7	>20	(Deng et al., 2022)
H. japonicum	Wighteone	$C_{23}H_{25}O_6$	Xanthone	HR-ESI-MS	396.16180	N/A	RAW 264.7	6.34 (mg/mL)	(X. Li et al., 2023)
H. sampsonii	Hypericumone	$C_{32}H_{40}O_4Na$	BPS	HR-ESI-MS	511.2824	N/A	RAW 264.7	≤ 40.32	(C. Huang et al., 2020)
H. sampsonii	Sampbenzophenone	$C_{28}H_{34}O_5Na$	BPS	HR-ESI-MS	473.2304	N/A	SMMC-7721	13.95	(H. Zhu et al., 2016)
H. sampsonii	Norhypersampsone	$C_{20}H_{24}O_3$	PPAPs	HR-ESI-MS	335.1630	N/A	RAW 264.7	30.2	(J. S. Zhang et al., 2017)

Abbreviations: HR-ESI-MS: High-resolution mass spectrometry with electrospray ionization; NMR: Nuclear magnetic resonance spectroscopy; MS: Mass spectrometry; PK: Polyketide; MTs: Meroterpenoids; RAW 264.7: Mouse macrophage-like leukemia cell line; UPLC-QTof-MS: Ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry; SW480: Cryopreserved human colon adenocarcinoma cell line; SMMC-7721: Human hepatocellular carcinoma cell line; TPH-1: Human leukemia monocytic cell line; sw480: Large intestine of a Dukes C colorectal cancer cell line; HepG2: Hepatoblastoma cell line; MCF-7: Breast adenocarcinoma cell line; HeLa: Cervical cancer cell line; SH-SY5Y: Thrice-subcloned cell line; PC12: Catecholamine cell line; A549: Hypotriploid alveolar basal epithelia cell line; LC–MS^n: Liquid chromatography coupled with multistage accurate mass spectrometry; mPPAPs: Methylated polycyclic polyprenylated acylphloroglucinol derivatives; HPLC-MS: High performance liquid chromatography-mass spectrometry; GI_{50}: Concentration resulting in a 50 % inhibition of cell growth; PPAPs: Polycyclic polyprenylated acylphloroglucinol; LC-HRMS: Liquid chromatography-High resolution mass spectrometry.

may also contribute to anti-inflammatory and antitumor activities, possibly due to the presence of compounds such as xanthones, flavonoids and other phenolic compounds (Ion et al., 2022; Ji et al., 2021; Sobhani Najafabadi et al., 2019).

H. perforatum (St. John's wort (SJW)) is the main species among *HP* investigated chemically and pharmacologically (H. Chen et al., 2019; Kakouri et al., 2023). Its naphthodianthrones, flavonoids, PPAPs and tannins have been highlighted for their natural pharmacological activities (Sobhani Najafabadi et al., 2019; Süntar, Oyardi, Akkol, & Ozçelik, 2016). According to the European Medicines Agency (EMA), the aerial parts of SJW and its preparations can be an active ingredient of herbal medicinal products. The EMA has highlighted that dried aqueous-alcoholic extracts of SJW are well-supported for the treatment of mild depressive disorders. Other preparations, based on prolonged use, are prescribed for the treatment of gastrointestinal distress, sleep disturbances, temporary mental exhaustion, and certain dermatological conditions (European Medicines Agency, 2022). Nowadays, *HP*-based medicinal preparations are approved for use in both Europe and the United States, reflecting the recognized pharmacological potential of this plant (de Carvalho Meirelles, Bridi, von Poser, & Nemitz, 2019). Moreover, the generalized use of these species has led to pharmacoeconomic analyses to endorse the consumption of SJW preparations as a dietary supplement product (Ristevski, Ristevski, Jurukovska, Jurukovska, & Petrusevska-Tozi, 2022).

Hypericum japonicum Thunb. is an annual herbaceous plant with a height ranging between 40 and 70 cm that grows mainly in the temperate biome of the southern drainage area of the Yangtze River, China (Gao, Luo, & Kong, 2009; Liu et al., 2014; W. Zhu et al., 2019). Phytochemical characterization of whole plants of *H. japonicum* has shown more than 80 chemical compounds relevant to its composition (R. Zhang et al., 2021); some of them with potential bioactivities such as anticancer, antimalarial, antibacterial, antiviral and anti-OS agents (Deng et al., 2022; Liu et al., 2014; W. Zhu et al., 2019). However, despite the absence of consumption records, this knowledge could lead to its application by the food sector as a natural dietary supplement rich in antioxidants such as phenolic acids and flavonoids, among other possibilities (Samaga & Rai, 2013).

On the other hand, the available bibliography for *Hypericum sampsonii* Hance. is even scarcer. *H. sampsonii* is a perennial erect herb that grows mainly in Southern China, Japan, Burma, Myanmar, and Northern India (Q. Chen et al., 2020; J. S. Zhang et al., 2017). Prior phytochemical studies have highlighted the isolation of PPAPs, xanthones, benzophenones, and flavonoids from *H. sampsonii* (Hu et al., 2016; C. Huang, Chang, Wu, Chen, & Chen, 2020). Despite the composition of potentially bioactive compounds, no research studies still evaluate their biological effects.

Considering the properties previously described in other species of the genus and their use in traditional medicine. It is hypothesized that both *HP* species could be considered as promising new sources of bioactive compounds for product-based food applications with health benefits. For this reason, they were screened to evaluate their nutritional, chemical and biological properties to assess their potential and applicability.

2. Material and methods

2.1. Standards and reagents

Acetonitrile and formic acid HPLC grade were purchased from Fisher Scientific (Waltham, MA, USA). All the other reagents were of analytical grade and supplied from scientific retailers. Taxifolin (≥98 %), quercetin-3-*O*-glucoside (≥98 %), *p*-coumaric acid (≥98 %), chlorogenic acid (≥99 %), gallic acid (≥99 %), protocatechuic acid (≥90 %), (+)-epicatechin (≥99 %), rutin (≥99 %), and kaempferol-3-*O*-glucoside (≥99 %) were purchased from Extrasynthèse (Genay, France). Fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47,885-U) was from Supelco (Bellefonte, PA, USA) and purchased from Sigma (St. Louis, MO, USA).

2.2. Plant material and extract preparation

The species used were *Hypericum japonicum* and *Hypericum sampsonii*. Both species were sourced from the Botanical Garden of the National Museum of Natural Sciences located in Taichung City, Taiwan, China, and collected at their optimal maturity (between flower budding and full flowering). For extract preparation, the whole plant (both aerial and underground parts) was used. The dried samples were first crushed, sieved, and vacuum-packed for storage at −80 °C. These dried samples were used for nutritional and chemical characterization. To prepare the extracts for biological testing, 5 g of dried plant material from each species were placed in 250 mL amber glass vials. A total of 100 mL of a methanol-water mixture ($MeOH/H_2O$, 60:40, *v*/v) was added as the extraction solvent. The extraction process was conducted in a water bath at 45 °C with continuous magnetic stirring for 3 h to maximize the extraction of bioactive compounds. Post-extraction, the mixture was centrifuged at 8400 rpm for 8 min to separate the solid plant residues from the liquid phase. The supernatant was then concentrated using a rotary evaporator at a temperature below 40 °C to remove the solvent. Prior to lyophilization, the liquid was filtered through 0.22 μm PVDF membranes to ensure removal of any particulate matter. The final step was lyophilization of the filtered extract in a lyophilizer, resulting in a powdered form of the extract.

2.3. Characterization of nutritional and chemical composition

The inorganic material (ash) was determined by gravimetry after sample incineration (550 °C). Total protein content was estimated through Duma's method (6.25 factor) (Saha et al., 2017). Briefly, aliquots of 5 mg of each sample were subjected to flash combustion, leading to complete and instantaneous oxidation of the organics. Then, an elemental analysis unit (FISONS Carlo Erba EA1108) with a CHNS microanalyzer was used for nitrogen quantification (detection limit of 10 ppm). Lipids were extracted using a modified Bligh-Dyer method under non-thermal conditions (Bligh & Dyer, 1959). Total carbohydrates were calculated by difference to 100 (centesimal) after the sum of the other nutrients. Unless otherwise specified, the analyses were performed following AOAC Official Methods (Helrich, 1990). All assays described were performed in triplicate and the results obtained were presented as mean values ± standard deviation calculated using Microsoft Excel. All results were expressed in percentage (%, g of the nutrient per 100 g of sample in dry weight - dw).

2.3.1. Fatty acid composition

The lipid extracts were obtained by Bligh-Dyer method and the fatty acid composition was determined by gas-chromatography coupled to flame ionization detection (GC-FID, GC 1000, DANI instruments, Contone, Switzerland) according to (Barros et al., 2007), following the ISO 5509 trans-esterification method (2000). Briefly, fatty acids obtained from lipid extracts by the Bligh-Dyer method (1.2 g in one case and 1.4 g in the other), were methylated with 5 mL of methanol: sulfuric acid: toluene (2:1:1 *v*/v) overnight under constant stirring (160 rpm) at 50 °C. Then, 5 mL of distilled water was added to the tubes, followed by 5 mL of diethyl ether. After shaking, the upper phase was recovered, passed through sodium sulfate anhydrous to eliminate water, filtered with 0.22 μm nylon filters (Millipore, Burlington, MA, USA) and injected into the chromatographic system. Separation was carried out using a Zebron-Fame column (30 m × 0.25 mm ID × 0.20 μm df, Phenomenex, Lisbon, Portugal) following the chormatographic conditions detailed in (Carocho et al., 2020). Peaks identification was carried by comparing the retention times of the fatty acids methyl esters (FAME) peaks with the samples, and the results were expressed as a relative percentage (%) of each fatty acid.

2.3.2. Composition of organic acids

Organic acids were determined after extraction with 4.5 % metaphosphoric acid (1:25 sample to solvent) by stirring for 20 min at room temperature. Extracts were analyzed by HPLC-DAD at 215 nm in a Shimadzu liquid chromatograph (Shimadzu, Kyoto, Japan) according to procedures described in detail elsewhere (Barros, Pereira, & Ferreira, 2013). The results were expressed in g per 100 g of plant (dw).

2.3.3. Extraction of non-anthocyanin phenolic compounds from H. japonicum and H. sampsonii

Non-anthocyanin phenolic compounds were extracted from 1 g of each dried plant sample through vigorous shaking (150 rpm, 1 h, room temperature) with a hydromethanolic mixture (MeOH/H_2O, 60:40, *v*/v; 30 mL). The extracts were filtered through a 0.22 μm disposable liquid chromatography (LC) filter disc, and the residues reextracted under the same conditions. Extracts from the same specie were combined and concentrated under reduced pressure (T £ 40 °C, Büchi R-210 rotatory evaporator, Flawil, Switzerland). Finally, the remaining extract was frozen and subjected to lyophilization (Freeze Dryer Telstar LyoQuest-55—Milan, Italy) for 48 h at −55 ± 0.5 °C.

2.3.4. Extraction of anthocyanin phenolic compounds from H. sampsonii

Anthocyanins were extracted from dried plant sample (0.5 g) with 80 % MeOH (MeOH/H_2O, 80:20, v/v, 30 mL) containing 0.5 % trifluoroacetic acid (TFA) by agitation (magnetic stirring, 150 rpm, 1 h, 25 °C), based on the method described by (*D.* Silva et al., 2019). The methanol-water extract was filtered (0.22 μm) and the procedure was repeated twice on the remaining residue of the initial extraction. The combined extracts were concentrated (35 °C, BüchiR-210 rotary evaporator, Flawil, Switzerland) and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA). For purification, the extract solution was placed in a 3 cc C_{18} SepPak Vac cartridge (Phenomenex, 150 mm × 4.6 mm, 5 μm), previously activated and conditioned with MeOH followed by H_2O (20:80, *v*/v). Sugars and more polar substances were eliminated by passing 10 mL of water, and anthocyanin pigments were then eluted with 5 mL of 80 % MeOH containing 0.1 % TFA. The extract was concentrated under vacuum and lyophilized.

2.3.5. Analyses of anthocyanin and non-anthocyanin phenolic compounds by HPLC-DAD-ESI(−)/MS/MS

Phenolic compounds were analyzed using a Dionex Ultimate 3000 UPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a DAD detector and coupled in series to a Mass Spectrometer (MS, Orbitrap Exploris 120, Thermo Finnigan, San Jose, CA, USA). Separation was carried out on a C18 Waters Spherisorb S3 ODS2 (Milford, MA, USA), 3 μm (4.6150 mm) column kept at 35 °C. The chromatographic conditions described in detail by (Barros, Dueñas, Ferreira, Maria Carvalho, & Santos-Buelga, 2011) was used for compounds separation. For the MS conditions, heated electrospray (H-ESI) in negative mode (2500 V) was used. Sheath gas (N2) was applied at a flow rate of 50 (arbitrary unit), as well as auxiliary gas (10) and sweep gas (1). Ion transfer tube and vaporizer temperature operated at 325 °C and 350 °C, respectively. Data-dependent acquisition mode with a normalized collision energy of 30 % was also used. Mass spectra was recorded between *m/z* 110 and 1100.

Anthocyanins were analyzed in Dionex Ultimate 3000 and their separation was achieved on an Aqua® reversed-phase C18 column (150 mm × 4.6 mm, 5 μm,- Phenomenex, California-USA) kept at 35 °C. The equipment was connected with a MS detection (Linear Ion Trap LTQ XL mass spectrometer, Thermo) equipped with an ESI source operating in the positive mode. The previously described operation conditions by Gonçalves et al., 2017 (Gonçalves et al., 2017) were set in the compounds analyses.

Data processing was performed with the Xcalibur™ software (Thermo Scientific) and compound identification was based on the interpretation of chromatographic and spectral characteristics compared with commercial standards, literature and libraries such as MZCloud™ (Thermo Scientific). Quantification of phenolic compounds was carried out using external calibration curves of the respective standard or, if not available, with the most similar compound. Hence, taxifolin glycosides were quantified using an aglycone taxifolin standard curve. The content of mangiferin, quercetin, and myricetin glycosides and derivatives were determined using the calibration curve of quercetin-3-O-glucoside, while p-coumaric acid and chlorogenic acid curves were used to determine the concentrations of caffeoyl and coumaroylquinic acid isomers. Epicatechin was used to quantify procyanidin isomers, and gallic acid was applied to quantify galloyl derivatives. All anthocyanins were quantified using a calibration curve of cyanidin-3-O-gluoside. The results were expressed as mg of phenolic compounds/g of freeze-dried extract (dw).

2.4. Bioactivity assays of polyphenol extracts

All bioactivity assays described were performed in triplicate and the results obtained were presented as mean values ± standard deviation calculated using Microsoft Excel.

2.4.1. Antioxidant activity

2.4.1.1. Cellular Antioxidant Activity (CAA). Extracts capacity to neutralize reactive oxygen substances (ROS) formation was assessed using a murine macrophage cell line (RAW 246.7; 70,000 cells/mL) commercially acquired from DMSMZ – Leibniz – Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. The cells were routinely maintained in a CO_2 incubator at 37 °C (Heal Force) with DMEM culture medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), heat-inactivated fetal bovine serum (10 %), and nonessential amino acids (2 mM).

For the assay, the protocol descibred by (Wolfe & Rui, 2007), with the modifications of de la (De la Fuente et al., 2022) was applied. Polyphenol-rich extracts extracts of each sample were re-dissolved in H_2O (8 mg/mL), from which successive dilutions with 2′,7′-dichlorohydrofluorescein (DCFH; 50 μM prepared with ethanol and diluted with Hanks' Balanced Salt Solution (HBSS)), obtaining the concentrations to be tested (500–2000 μg/mL). The cells were treated with different extract concentrations, and after 1 h of incubation, ROS production was induced with 2,2′-azobis(2-methylpropionamide) dihydrochloride (AAPH; 600 μM), which decomposes and, in the presence of molecular oxygen, originates radicals. Quercetin was used as a positive control (0.3 μg/mL), and DCFH and DMEM culture medium were used as a negative control. The results were presented as the percentage of oxidation inhibition at a maximum concentration tested.

2.4.1.2. Oxidative Hemolysis Inhibition Assay (OxHLIA). Free radical-induced hemolysis was tested using sheep erythrocytes according to (Lockowandt et al., 2019; Takebayashi, Iwahashi, Ishimi, & Tai, 2012) with extracts diluted in phosphate-buffered saline (PBS). Peroxyl radicals were used as pro-oxidants to induce oxidative stress (OS), and erythrocytes were used as oxidizable targets. Concentrations ranged from 800 to 6.25 μg/mL, with distilled water as the baseline, Trolox as the positive control, and PBS as the negative control. The inhibition of free radical-induced hemolysis was determined by calculating the percentage of intact erythrocytes and the hemolysis time. The percentage of intact erythrocytes (P%) was calculated using the formula $(P\%) = \frac{St - CH_O}{So - CH_O} \times 100$, where *So* is the optical density of the sample at the initial time, *St* is the optical density at time t, and CH_O is the optical density corresponding to complete hemolysis at the initial time.

The hemolysis time (Δt) was calculated using the formula $\Delta t = Ht_{50}(sample) - Ht_{50}(control)$, where Ht_{50} represents the time required for 50 % of the erythrocytes to undergo hemolysis. The Δt values were then correlated with different concentrations of the extracts to determine the

concentration required to retard hemolysis at a fixed time. The concentration needed to delay hemolysis (IC_{50}) was calculated for both 60 and 120 min.

2.4.1.3. Thiobarbituric Acid Reactive Substances (TBARS) assay. The antioxidant capacity was estimated by the ability of the extract to inhibit lipid peroxidation in porcine (*Sus scrofa*) brain homogenates according to previously described procedure (Pinela, Barros, Carvalho, & Ferreira, 2011). For this purpose, the freeze-dried extracts were dissolved at a ratio of 5 mg/mL in EtOH/H_2O solution (80:20 *v*/v), and eight descending concentrations (serial dilutions) and two blanks were prepared to obtain a dose-response curve (0.009, 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25 mg/mL). The color intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at 532 nm. Using the dose-response values of the results obtained, a parameter was obtained that summarizes the potential antioxidant effect of each sample, that is, the concentration necessary to produce the effective concentration for 50 % of the antioxidant activity (EC_{50}).

2.4.2. NO-production inhibition

The anti-inflammatory activity was evaluated by determining the extract's capacity to inhibit the lipopolysaccharide (LPS)-induced nitric oxide (NO) release. The extracts were re-dissolved in H_2O (8 mg/mL) and successively diluted to obtain the different concentrations to be tested (0.125–8 mg/mL). The assay was performed using RAW 264.7 cells (cultured under the conditions described in section 2.4.1.) following the procedure previously described by de Medeiros et al., 2024. Cells (5×10^5 cells/mL) were treated with different extracts concentrations (15 μL, 6.25–400 μg /mL), and after 1 h incubation, cells with stimulated with a lipopolysaccharide solution (LPS; 1 mg/mL) for 24 h. Dexamethasone was used as positive control and the cells in the presence and absence of LPS as negative controls. The nitric oxide (NO) production was determined using a Griess reagent kit by reading absorbances at 540 nm (Biotek ELX800 microplate reader) and by comparison with the nitrite standard calibration curve ($y = 0.00068\times + 0.0951$, $R^2 = 0.9864$). The results were expressed as the extracts concentration responsible for 50 % inhibition of NO production ($IC_{50,}$ μg/mL).

2.4.3. Antiproliferative activity

The antiproliferative capacity of studied extracts was assessed using sulforhodamine B colorimetric assay following the procedure previously described by (de Medeiros et al., 2024). The extracts were tested against several tumor cell lines: gastric (AGS), colorectal (Caco-2), breast (MCF-7), and lung (NCI-H460) carcinoma. Additionally, a non-tumor porcine liver primary culture (PLP2) was also tested. Ellipticine (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control, and the cells in the absence of samples as a negative control. Results were expressed as the extract concentration responsible for 50 % of cell proliferation inhibition (GI_{50}, μg/mL).

2.4.4. Antimicrobial activity

Both food-borne and clinical bacteria were employed in the assays. For the former, the extracts were tested against five Gram-negative bacteria, namely, *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* subsp. (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610) and three Gram-positive bacteria, namely *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111) and *Staphylococcus aureus* (ATCC 25923). All these microorganisms were purchased from Frilabo, Porto, Portugal. Bacteria were incubated at 37 °C in a fresh medium for 24 h before analysis to maintain the exponential growth phase. Regarding clinical bacteria, the strains were clinical isolates obtained from patients hospitalized in various departments at the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Five Gram-negative bacteria (*Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Morganella morganii* and three Gram-positive bacteria (*Enterococcus faecalis, Listeria monocytogenes*, and *Staphylococcus aureus*, were tested. In addition, one more gram-positive clinical bacteria, (*Propionibacterium acnes*) was tested following the same procedure. The minimum inhibitory concentration (MIC) determinations on all bacteria were performed using a colorimetric assay described by (Pires et al., 2018). The lowest extract concentration required to kill a particular bacterium was expressed as the minimum bactericidal concentration (MBC, mg/mL).

Antifungal activity was performed according to the protocol described by Heleno et al., 2013 using *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404) (Heleno et al., 2013). The lowest concentrations with no visible fungal growth (under binocular microscopy) were defined as MIC. The lowest concentration with no visible fungal growth was defined as the minimum fungicidal concentration (MFC, mg/mL), indicating the death of 99.5 % of the original inoculum. A commercial fungicide (1 mg/mL Ketoconazole) was used as positive control.

3. Results and discussion

The nutritional composition was determined from the plant material to have a profile of the raw matrix. Both chemical characterization and bioassays were conducted with MeOH/H_2O (60:40) extract, which is widely recognized for achieving maximum extractability of phenolic compounds. The only exception was the analysis of anthocyanin compounds, performed with a MeOH/H_2O (80:20) extract optimized specifically for this type of compound, as indicated in the methodology. The choice of the 60 % MeOH extract for biological tests was also supported by preliminary optimization studies, which showed higher solubility and bioactivity at this ratio.

3.1. Nutritional composition

The nutritional composition of *HP* plants has been scarcely evaluated, and the literature is limited to a couple of studies. In the present study, the ash content of *H. sampsonii* (8.88 g/100 g dw) was twice that of *H. japonicum (*4.78 g/100 g dw) (Table 2). The two samples presented smaller differences in their protein content, 10.59 g/100 g dw for *H. japonicum* and 13.62 g/100 g dw for *H. sampsonii* (Table 2). The protein content of *HP* plants has not yet been documented in the literature. Nevertheless, considering the growing trend towards the consumption of plant proteins as alternatives to animal protein sources, the levels of this nutrient in both *H. japonicum* and *H. sampsonii* called attention as they are close to that reported for *Chenopodium quinoa* (~ 13 g/100 g dw) and higher than those of other cereals such as wheat or rice (Sá, Moreno, & Carciofi, 2020). The total lipid content was also similar between *H. japonicum* and *H. sampsonii* (1.24 and 1.39 g/100 g dw, respectively (Table 2). This low amount of lipids is in line to those results reported for species of the genus, which are generally in the range of 0.06–0.35 g/100 g dw (Hosni, Msaâda, Taârit, & Marzouk, 2017). Finally, carbohydrates were the major nutrient found in the dried plants studied, their contents being similar between the two species as well (83.39 g/100 g dw for *H. japonicum*, 76.11 g/100 g dw for *H. sampsonii*) (Table 2). As both species presented comparable nutritional composition, their energy values calculated from it were also similar, ranging from 370 to 390 kcal/100 g dw, with *H. japonicum* providing slightly higher energy intake.

3.2. Organic and fatty acid composition

Organic acids are essential to cellular metabolism, as they are versatile in their role in plant tolerance to different types of stress and exert mechanisms underlying the enhancement of biosynthesis, secretion and

Table 2
Nutritional and chemical composition of *Hypericum* plants.

	Hypericum japonicum	*Hypericum sampsonii*
NUTRICIONAL COMPOSITION (g/100 g dw)		
Ashes	4.78 ± 0.3	8.88 ± 0.3
Proteins	10.59 ± 0.22	13.62 ± 0.08
Lipids	1.24 ± 0.06	1.39 ± 0.17
Carbohydrates	83.39 ± 0.22	76.11 ± 0.08
Energy (kcal/100 g dw)	387.08	371.43
Energy (kJ/100 g dw)	1619.54	1554.06
CHEMICAL COMPOSITION		
Organic acids (g/100 g dw)		
Oxalic acid	1.48 ± 0.01	1.67 ± 0.22
Succinic acid	8.88 ± 0.17	5.06 ± 0.07
Fatty acids (%)		
Capric acid (10:0)	0.41 ± 0.24	N/A
Undecanoic acid (11:0)	0.27 ± 0.12	0.38 ± 0.15
Lauric acid (12:0)	0.68 ± 0.21	1.52 ± 0.34
Myristic acid (14:0)	1.17 ± 0.22	1.60 ± 0.25
Myristoleic acid (14:1)	0.74 ± 0.13	0.69 ± 0.07
Pentadecylic acid (15:0)	0.41 ± 0.05	0.82 ± 0.03
Palmitic acid (16:0)	37.04 ± 1.63	37.12 ± 3.92
Palmitoleic acid (16:1)	2.20 ± 0.56	1.20 ± 0.12
Margaric acid (17:0)	1.26 ± 0.11	1.11 ± 0.02
Stearic acid (18:0)	7.45 ± 3.27	9.86 ± 1.64
Oleic acid (18:1 n-9c)	10.68 ± 2.22	10.41 ± 0.90
Linoleic acid (18:2 n-6c)	22.06 ± 2.00	19.64 ± 2.33
α-linolenic acid (18:3 n-3)	13.63 ± 1.81	10.31 ± 0.51
Tricosylic acid (23:0)	N/A	0.92 ± 0.17
Lignoceric acid (24:0)	1.99 ± 0.30	2.98 ± 0.42
SFA (%)	50.69	56.31
MUFA (%)	13.62	12.30
PUFA (%)	35.69	31.39
ratio PUFA n6/n3 (%)	1.61	1.90

Abbreviations: SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. The results for nutritional and organic acid composition are expressed in g/100 g sample dry weight (dw) and the results for fatty acid composition are expressed as a percentage (%) of the total fatty acid content in the lipidic fraction. Results for energy value were expressed in kcal/100 g sample dw and kJ/100 g dw. N/A: no answer.

regulation (Panchal, Miller, & Giri, 2021). Oxalic acid and succinic acid were the only organic acids identified in both samples of *H. japonicum* and *H. sampsonii*, with values of 1.48 and 1.67 g/100 g for oxalic acid and, 8.80 and 5.06 g/100 g for succinic acid, respectively (Table 2). It is important to note that oxalic acid is an antinutrient that can be detrimental to health when consumed in large amounts, potentially inhibiting the absorption of essential minerals and contributing to the formation of kidney stones. Therefore, careful consideration of these factors is recommended when using these plants for dietary or medicinal purposes (Salgado, Silva, Figueira, Costa, & Albuquerque, 2023).

No earlier references to organic acid identification in *H. japonicum* and *H. sampsonii* have been found. A separate study quantified 0.042 g/100 g dw of succinic acid was quantified in *H. perforatum* (Bayram, Kutlu, Gerçek, Çelik, & Ecem Bayram, 2022). It has been reported that the content of organic acids in the plant could influence the growth control of several bacterial strains with variable levels of efficacy, which would directly contribute to the antimicrobial effect of the extract (Bushell, Tonner, Jabbari, Schmid, & Lund, 2019).

Plants utilize fatty acids to synthetize acyl-lipids that support physiological and protective cellular functions, such as essential membrane synthesis, storage, and surface lipids (Kalinger, Pulsifer, Hepworth, & Rowland, 2020). The fatty acid composition of the two *HP* species is listed in Table 2. Palmitic acid (37.04 % *H. japonicum*; 37.12 % *H. sampsonii*) and linoleic acid (22.06 % *H. japonicum,* 19.64 % *H. sampsonii)* were the most predominant fatty acids. Regarding their distribution, *H. japonicum* and *H. sampsonii* extracts are constituted mainly of saturated fatty acids (SFA = 50.69–56.31 %), followed by polyunsaturated fatty acids (PUFA = 35.69–31.39 %) and monounsaturated fatty acids (MUFA = 13.62–12.30 %), with a range of 1.61–1.90 for the value of the ratio PUFA n6/n3. To our knowledge, the fatty acid content of *H. japonicum* and *H. sampsonii* is being provided for the first time herein. Yet, although very sparingly, the fatty acid composition of some *HP* species has been described previously. In one study, palmitic acid was identified in *H. perforatum* (17.43 %), *H. perfoliatum* (11.2 %), *H. tomentosum* (20.67 %), and *H. ericoides* Ssp. Roberti (4.03 %). Linoleic acid was quantified in percentages of 11.21, 36.62, 12.4, and 36.47 %, respectively. In addition, oleic acid was reported to be present in all species in a range of 11.2 to 23.27 % (Hosni et al., 2017). Linoleic acid was also found in *H. perforatum* and *H. tetrapterum* at 44.1 % and 31.3 %, respectively (Heinrich, Lorenz, Daniels, Stintzing, & Kammerer, 2017). In another study, the SFA content in a fraction of *H. scabrum* extract was higher in stems (23.7 %) and lower in seeds (5.8 %). Meanwhile, the fraction with the highest unsaturated fatty acids (UFA) was the seeds (80.9 %), while the lowest was the flowers (48.9 %) (Shafaghat, 2011). In overall terms and as in the present study, the total amount of SFA, MUFA, and PUFA is very similar (Hosni et al., 2017).

3.3. Identification and quantification of phenolic compounds

3.3.1. Phenolic compounds in H. japonicum

HPLC-DAD-ESI(−)MS/MS analyses allowed the identification and quantification of 30 non-anthocyanin phenolic compounds in *H. japonicum* extract, being eight phenolic acids, five condensed tannins, and 17 flavonoids including 1 flavan-3-ol, 7 flavononols, and 9 flavonols (Table 3). The sum of total phenolic compounds (TPC, HPLC-DAD peaks) was 184.51 mg/g extract dw. In previous research, ten samples of *H. japonicum* from different habitats were assessed regarding their polyphenol content by HPLC-DAD, and TPC results ranged from 9.34 to 77.26 mg/g dw. In this study, TPC was roughly two times the highest value reported by those authors. Such variation might be due to several factors, such as environmental conditions, harvesting time, extraction and drying process or storage conditions (Gao et al., 2009).

Flavonoids accounted for up to 77.76 % of the total polyphenol composition (Fig. 1) in this species. The highest relative proportion of these compounds were flavononols (79.72 mg/g extract dw), a subgroup of flavonoids represented by taxifolin-*O*-rhamnoside (**P19**) and its isomers and derivatives (**P15, P16, P17, P18, P20, P21**). Taxifolin and derivative compounds have been extensively studied, for instance, in the therapy of tumors, microbial infections, OS, cardiovascular diseases, and liver disorders, as well as a multifunctional food additive (Mei et al., 2022; Thuan et al., 2022). Taxifolin derivatives were previously found in *H. japonicum* and reported to have antiproliferative effects on liver cancer cells (Gao et al., 2009; R. Zhang et al., 2021). Furthermore, taxifolin-rich extracts have exhibited a significant safety profile for food applications such as beverage fortification, food supplements, and chocolate confectionery (Hasibi et al., 2020; Taldaev et al., 2022; Turck et al., 2017). Taxifolin-*O*-rhamnoside was also found in other species, including *H. androsaemum*; however, in far lower quantities than those found in the present study (3.4 mg/g) dw (Jabeur et al., 2016). The flavonol group was the most numerous (63.08 mg/g extract dw), highlighting the content of isoquercitrin (**P26**, 15.56 mg/g) and quercitrin (**P29**, 23.18 mg/g). Both were previously reported in *H. japonicum* in ranges from 1.19 to 20.77 mg/g (isoquercitrin) and from 4.71 to 36.34 mg/g dw (quercitrin) (Gao et al., 2009). Isoquercitrin has been identified as one of the most abundant phenolic compounds in *HP* species. For example, it was quantified in the extract obtained from the aerial part of *H. empetrifolium* at 307.677 mg/g (Boga et al., 2021), in *H. humifusum* ethanolic extract at 106.82 mg/g (Toiu, Vlase, Drăgoi, Vodnar, & Oniga, 2016), in the crude extracts of *H. cardonae* (9.60 mg/g), *H. cuatrecasii* (75.01 mg/g), *H. myricariifolium* (109.61 mg/g), *H. humboldtianum* (17.33 mg/g), *H. carinosum* (34.39 mg/g), *H. laricifoliium* (115.27 mg/g) and, *H. garciae* (7.03 mg/g). In addition, another flavonoid glycosides were identified in significant amounts, such as quercetin dirhamnoside

Table 3
Identification, total and individual content and classification of phenolic compounds detected in *H. japonicum* extract.

1Id	Rt (min)	λ_{max} (nm)	[M-H]$^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Chemical formula	Quant. (mg/g E dw)	Ref.
Phenolic acids								
P1	3.36	321, 286	353	191, 135, 179	*cis*-3-Caffeoylquinic acid (chlorogenic acid isomer)[a]	$C_{16}H_{17}O_9$	1.80 ± 0.16	(Gao et al., 2009)
P2	3.36	321, 286	315	152, 151, 169, 125	Galloyl rhamnose[b]	$C_{13}H_{16}O_9$	1.97 ± 0.14	(Abou-Zaid & Nozzolillo, 1999)
P3	4.02	324	391	217, 205, 179, 135	Caffeoyl derivative [a]	–	9.19 ± 0.56	–
P4	4.02	324	353	191, 135, 179	*trans*-3-Caffeoylquinic acid [a] (chlorogenic acid isomer)	$C_{16}H_{18}O_9$	9.19 ± 0.56	(Bender et al., 2018)
P5	4.78	293, 260	153	–	Protocatechuic acid	$C_7H_6O_4$	2.89 ± 0.25	(Mekam, Martini, Nguefack, Tagliazucchi, & Stefani, 2019)
P6	5.04	310	337	119, 163, 191	3-*p*-Coumaroylquinic acid [c]	$C_{16}H_{17}O_9$	2.45 ± 0.17	(Gao et al., 2009)
P7	7.45	–	337	173, 163, 137, 119	4-*p*-Coumaroylquinic acid[c]	$C_{16}H_{18}O_8$	0.19 ± 0.02	(Bender et al., 2018)
P8	5.14	–	353	135, 173, 179, 191	4-Caffeoylquinic acid[a] (chlorogenic acid isomer)	$C_{16}H_{17}O_9$	4.01 ± 0.26	(Gao et al., 2009)
Condensed tannins								
P10	5.45		865	695, 713, 739, 577, 407, 425, 451, 287, 289	Procyanidin B-type trimer[d]		0.54 ± 0.03	(Dall'Acqua et al., 2021)
P11	5.6		577	289, 407, 245	Procyanidin B-type dimer[d]		3.59 ± 0.22	(Mekam et al., 2019)
P12	7.11	280	865	287, 289, 695, 577, 425, 407, 245	Procyanidin B-type trimer[d]	$C_{30}H_{26}O_{12}$	1.31 ± 0.06	(Dall'Acqua et al., 2021)
P13	7.77		1153	1135, 1027, 1001, 983, 865, 739, 695, 575, 425, 413, 289	Proanthocyanidin tetramer B-type[d]		2.60 ± 0.16	(Dall'Acqua et al., 2021)
P14	8.72		1441	1151, 863, 575	Proanthocyanidin pentamer B-type[d]		1.11 ± 0.09	(Lin, Sun, Chen, Monagas, & Harnly, 2014)
Flavan-3-ol								
P9	6.91	278	289	–	Epicatechin	$C_{15}H_{14}O_6$	0.87 ± 0.05	(Mekam et al., 2019)
Flavanonols								
P15	5.04	–	449	125, 285, 275, 181, 153	Taxifolin-3-*O*-rhamnoside (neoastilbin)[e]	$C_{21}H_{22}O_{11}$	3.95 ± 0.20	(R. Zhang et al., 2021)
P16	8.23	–	595	285, 303, 325, 431, 449, 179, 151	Taxifolin-*O*-deoxyhexoside-deoxyhexoside [e]	$C_{21}H_{22}O_{12}$	0.36 ± 0.01	–
P17	8.23	–	693	449, 303, 431, 325, 285, 151, 595	Taxifolin-*O*-deoxyhexoside-derivative [e]	$C_{21}H_{22}O_{12}$	0.36 ± 0.01	–
P18	8.23	–	449	285, 303, 151, 125	Taxifolin-*O*-rhamnoside isomer [e]		0.36 ± 0.01	–
P19	9.94	287	449	125, 275, 285, 151, 181	Taxifolin-*O*-rhamnoside [e]	$C_{21}H_{22}O_{11}$	69.60 ± 4.23	–
P20	12.01	286	449	125, 285, 275, 181, 151	Taxifolin-*O*-rhamnoside iso [e] mer		4.39 ± 0.33	(Mekam et al., 2019)
P21	14.74	286	303	125	Taxifolin	$C_{15}H_{12}O_7$	0.70 ± 0.07	(Goufo, Singh, & Cortez, 2020)
Flavonols								
P22	7.77	–	609	463, 446, 301	Quercetin-*O*-deoxyhexoside-hexoside[f]	$C_{27}H_{30}O_{15}$	0.57 ± 0.02	(Bender et al., 2018)
P23	12.01	–	593	446, 447, 301	Quercetin dirhamnoside[f]	$C_{21}H_{20}O_{11}$	4.39 ± 0.33	–
P24	13.8		477	301, 151, 179	Quercetin-*O*-glucoronide (miquelianin) [f]	$C_{21}H_{17}O_{13}$	1.99 ± 0.14	(Gao et al., 2009)
P25	14.3	353, 256	479	316, 317	Myricetin-*O*-hexoside[f]	$C_{22}H_{22}O_{13}$	15.56 ± 1.20	(Mekam et al., 2019)
P26	14.3	–	463	300, 301, 255, 271, 151, 179	Quercetin-3-*O*-glucoside (isoquercitrin)	$C_{21}H_{20}O_{12}$	15.56 ± 1.20	(Mekam et al., 2019)
P27	15.69	–	505	300, 301	Quercetin-acetyl-hexoside[f]	$C_{23}H_{22}O_{13}$	0.61 ± 0.04	(Goufo et al., 2020)
P28	17.25	345, 266	447	285, 284, 22, 255	Kaempferol-3-*O*-glucoside	$C_{21}H_{20}O_{11}$	1.22 ± 0.07	(Goufo et al., 2020)
P29	17.44	348, 256	447	300, 301, 271, 255, 179, 151	Quercetin-*O*-rhamnoside (quercitrin) [f]	$C_{21}H_{20}O_{11}$	23.18 ± 1.79	(Mekam et al., 2019)
P30	23.33	282, 231	385	153, 223	Roseoside	$C_{19}H_{29}O_8$	NQ	(Bender et al., 2018)
					TOTAL PHENOLIC COMPOUND CONTENT			184.51 ± 0.2

Notes: The contents of detected phenolic compounds are expressed as milligrams per gramme dry weight and are presented as mean ± sd ($n = 3$). Compounds to which no wavelength is informed were detected in low concentrations or coeluted with other compounds so that their UV–Vis spectra were not clearly seen or detected. In either situation, the peak assignment was possible as the mass spectra was detected, making the identification possible given the superior sensitivity of the MS over the DAD detector. Standard curves used for each compound: a – chlorogenic acid; b – gallic acid; c - *p*-coumaric acid; d – epicatechin; e – taxifolin; f – quercetin-3-*O*-glucoside.

(**P23**), kaempferol-3-*O*-glucoside (**P25**) and myricetin-*O*-hexoside (**P28**), recognized to be an effective antagonist of cell-cell signaling and to inhibit bacterial biofilm formation in a concentration-dependent mode (Mickymaray, 2019; Tocci et al., 2018). The presence of myricetin-*O*-hexoside was also verified in the methanolic extracts of *H. salsugineum* and *H. scabrum* (12.5 and 6.9 mg/g, respectively) (Bender et al., 2018; Llorent-Martínez et al., 2018). Epicatechin (**P9**, 0.87 mg/g) was the only flavan-3-ol found in this species. This compound was also detected in other *HP* species in variable amounts, including *H. androsaemum* (6.9 mg/g) (Jabeur et al., 2016), *H. triquetrifolium* (51.19 mg/g), *H. neurocalycinum* (9.39 mg/g), *H. perforatum* (19.09 mg/g) and *H. rochelii* (3.82 mg/g) (Babotă et al., 2022; Dall'Acqua et al., 2021). Plant epicatechin has been shown to present anticarcinogenic and antimicrobial properties and therapeutic benefits in preventing heart disease. Furthermore, it was recognized as an effective antioxidant in an *in vivo* study due to its capacity to regulate muscle development in human health (Seo et al., 2021). Catechins are used in food as natural antioxidants in oils and fats to prevent lipid oxidation, feed additives, food antimicrobials, and functional health ingredients in various foods and supplements (Yilmaz, 2006).

The analysis showed the presence of eight phenolic acids (**P1** to **P8**) in a total quantity of 31.69 mg/g in the extract of *H. japonicum*. The chlorogenic acid and its isomers, such as *trans*-3-caffeoylquinic (**P4**) and caffeoyl derivative (**P5**), were the major compounds in the extract (9.19 mg/g dw). Chlorogenic acid is the most widely identified and plentiful phenolic acid in *HP* species (Alahmad et al., 2022; Bayram et al., 2022; Caldeira et al., 2023; Kimáková et al., 2018; Kladar et al., 2017; Mathioudaki, Berzesta, Kypriotakis, Skaltsa, & Heilmann, 2018; Nogueira et al., 2013; Peron, Hošek, Rajbhandary, Pant, & Dall'Acqua, S., 2019; Samaga & Rai, 2013; A. R. Silva, Taofiq, Ferreira, & Barros, 2021). In *H. androsaemum,* it has been named as the most predominant phenolic acid, representing nearly 60 % of the total phenolic content (Jabeur et al., 2016). It has been previously suggested that the cytotoxicity of *HP* may correlate with the antiproliferative activity of quercetin and chlorogenic acid (Kladar et al., 2017). Further phenolic acids, such as 3-*p*-coumaroylquinic acid (**P6**) and 4-caffeoylquinic acid (**P8**), found at 2.45 and 4.01 mg/g, were also quantified in other *HP* species in varying amounts. 3-*p*-coumaroylquinic acid (2.13 mg/g) was quantified in the floral shoot of *H. perforatum* and the hydroalcoholic extracts of *H. hirsutum* (6.8 mg/g), *H. maculatum* (7.1 mg/g) and *H. acutum* (10.3 mg/g). On the other hand, 4-caffeoylquinic acid was identified in the hydroalcoholic extract of *H. barbatum* (0.5 mg/g) (Tusevski, Krstikj, Stanoeva, Stefova, & Gadzovska Simic, 2018; Zdunic, Godjevac, Savikin, & Petrovic, 2017). Gallic acid (**P2**) was found in *H. japonicum* but not in *H. samsponii* (Table 3). Protocatechuic acid (**P5**, 2.89 mg/g) was identified in the present study and in *H. olympicum* extract (0.82 mg/g) in another study (Llorent-Martínez et al., 2018). In recent years, natural antioxidant compounds have been incorporated into the design of functional foods to prevent many diseases related to OS, such as cardiovascular and neurodegenerative diseases. Owing to the antioxidant capacity of chlorogenic acids and the high presence of this compound and its derivatives in the plants studied, the incorporation of *HP* in functional foods is shown to be an interesting alternative (Wang et al., 2022). Additionally, chlorogenic acid and its derivatives have been used as food packaging materials with excellent results in maintaining the freshness of shrimp due to the high antioxidant capacity of these compounds (Hu et al., 2020). Hence, the application of *HP* in the food industry is promising with a wide spectrum ranging from functional foods to preservatives to extend the shelf life of food. Finally, a group of condensed tannins (9.15 mg/g) consisting of procyanidins and proanthocyanidins (**P10-P14**) was found. Procyanidin B was previously identified by other authors in *H. japonicum* (Gao et al., 2009).

3.3.2. Phenolic compounds in H. sampsonii

In *H. sampsonii*, 17 phenolic compounds belonging to six groups (phenolic acids, xanthones, tannins, flavan3-ols, flavononols and flavonols) were identified and quantified (Table 4). The TPC was 59.57 mg/g, significantly lower ($p < 0.05$) than that of *H. japonicum*. Polyphenols contributed up to 72.24 % to the global composition (Fig. 1). Among them, xanthones were the most abundant group (36.12 mg/g extract dw) and were mostly composed of mangiferin (**F7**, 34.90 mg/g extract dw). Although the literature on the phenolic composition of *H. sampsonii* is limited, xanthones have been reported as one of the major constituents, and in particular, mangiferin displays antioxidant and anti-inflammatory potential (Nguyen Viet et al., 2021). Mangiferin was found in the flower extract of other *HP* species, such as *H. retusum* (0.09 mg/g), *H. spectabile* (2.18 mg/g), and *H. elongatum* (0.05 mg/g); however, the quantities were considerably lower (Cirak et al., 2016). In *H. sampsonii*, procyanidin A-type trimer (**F12**, 0.91 mg/g), not found in *H. japonicum*, was identified. Based on the available literature, this could be the first time this compound has been described in *HP* species. As in the *H. japonicum* extract, taxifolin-*O*-rhamnoside (**F13**) was also observed, but in substantially less quantity.

The total flavonol content was 3.98 mg/g, and rutin (**F14**), a flavonol associated with the antidepressant activity in *HP* extracts, was found in *H. sampsonii* but not in *H. japonicum.* Rutin has been reported in several other *HP* species in significant quantities (Alahmad et al., 2022; Boga et al., 2021; Cirak et al., 2016; El-Hawary, Ahmed, Sheashea, & Ezzat, 2022; Ergin, Karakaya, Göger, Sytar, & Demirci, 2022; Kimáková et al., 2018; Mahomoodally et al., 2019; Napoli et al., 2018; Nogueira et al., 2013; Peron et al., 2019). This compound was shown to inhibit osteoclast growth by reducing reactive oxygen species (ROS) and tumor necrosis factor-alpha (TNF-α) by inhibiting the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation (Ganeshpurkar & Saluja, 2017). Rutin is widely recognized as an effective natural antioxidant, besides other anti-inflammatory, antimicrobial, anticarcinogenic or neuroprotective bioactivities reported (Choi, Park, & Lee, 2021). For instance, in the extract of the aerial part of *H. empetrifolium*, rutin was present in a similar amount (3.94 mg/g) (Boga et al., 2021). Chlorogenic acid, rutin, hyperoside, isoquercitrin and quercitrin from *HP* extracts have also been demonstrated to be potential acetylcholinesterase inhibitor (AChE) compounds (Tusevski et al., 2018). Hyperoside (**F15**) was identified and quantified in 2.04 mg/g. Hyperoside was the most abundant flavonol glycoside in previous screenings of *H. sampsonii* (Q. Chen et al., 2020). Hyperoside was found in the hydroalcoholic extracts of *H. richeri* (15.7 mg/g) and *H. perforatum* (15.5 mg/g) spp. (Zdunic et al., 2017). Isoquercitin (**F16**) and quercitrin (**F17**) were both quantified at 1.02 mg/g and 0.37 mg/g. These phytochemicals have been extensively reported in numerous *HP* spp. and variable concentrations (Cirak et al., 2016; Eroğlu Özkan et al., 2013; Liu et al., 2014; Llorent-Martínez et al., 2018; Toiu et al., 2016). Quercitrin and isoquercitrin were quantified in low amounts in eleven *HP* species over a range of 0.0004 to 1.01 mg/g dw (Nogueira et al., 2013).

The total content of phenolic acids was 16.54 mg/g, all classified as hydroxycinnamic acids. *Trans*-3-Caffeoylquinic acid (**F3**, 5.19 mg/g) was the most abundant. In a separate study, 3-caffeoylquinic acid was reported in six distinct *HP* species, ranging from 6.8 to 28.2 mg/g (Zdunic et al., 2017). Regarding other phenolic acids, such as 3-*p*-coumaroylquinic acid (**F4**, 1.71 mg/g), it was also identified in other species,

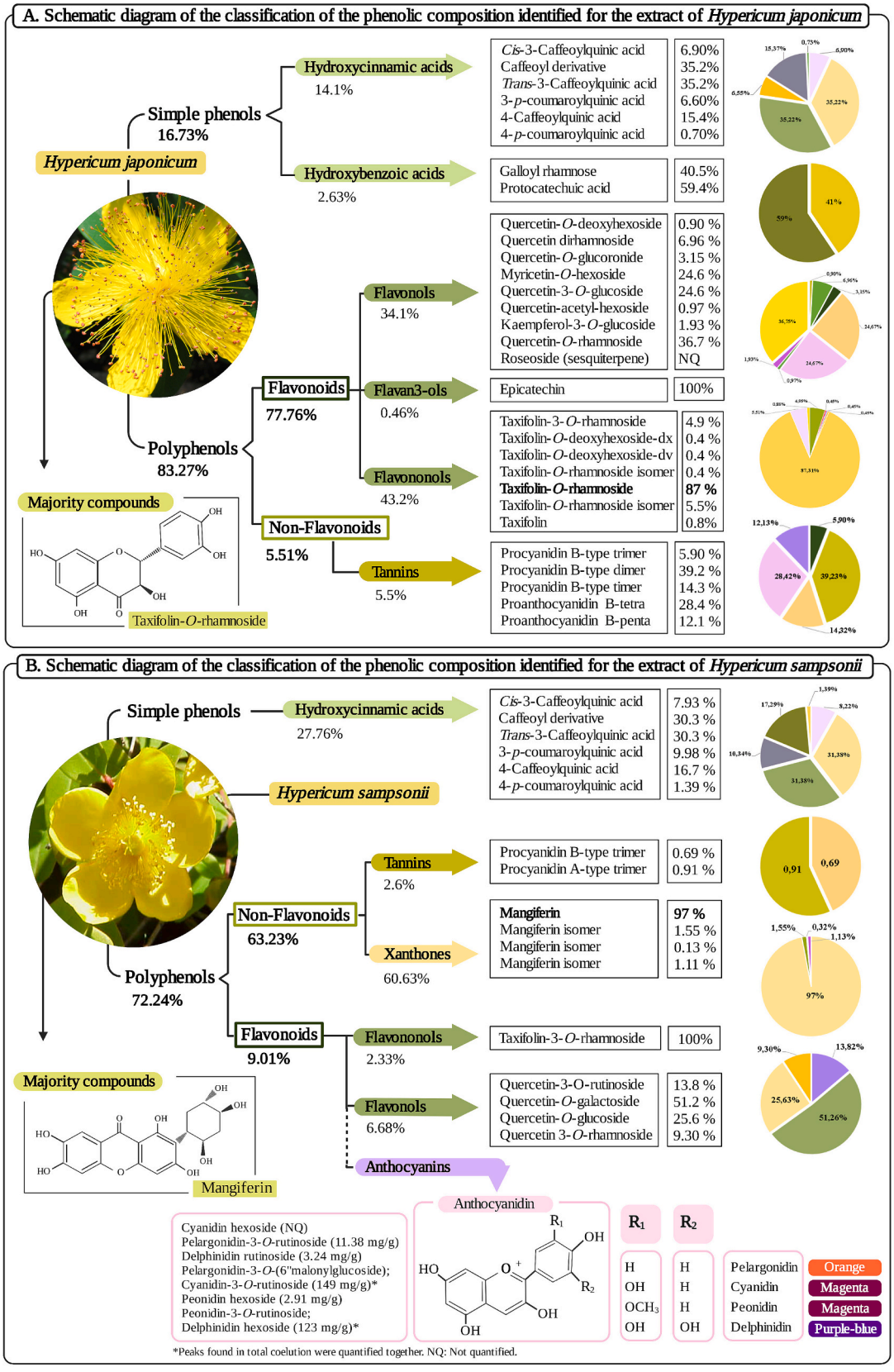

Fig. 1. Overview of non-anthocyanin and anthocyanin phenolic compounds found in *H. japonicum* and *H. sampsonii*. Results are expressed as percentage (%) within each group of compounds and their contribution to the total quantity. Created with BioRender.com.

Table 4
Identification, total and individual content and classification of phenolic compounds and anthocyanins in *H. sampsonii* extracts.

Id	Rt (min)	λ max (nm)	[M-H]$^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Chemical formula	Quant. (mg/g E dw)	Ref.
Phenolic compounds (non-anthocyanin compounds)								
Phenolic acids								
F1	3.36	323	353	191, 173, 359, 533, 135	*cis*-3-Caffeoylquinic acid[a] (chlorogenic acid isomer)	$C_{16}H_{17}O_9$	1.36 ± 0.06	(Babotă et al., 2022)
F2	4.01		391	217, 205, 179, 135	Caffeoyl derivative[a]		5.19 ± 0.14	(Mahomoodally et al., 2019)
F3			353	191, 179, 135	*trans*-3-Caffeoylquinic acid (chlorogenic acid isomer) [a]		5.19 ± 0.14	(Jabeur et al., 2016)
F4	5.15		337	119, 163, 191	3-*p*-Coumaroylquinic acid[b]		1.71 ± 0.06	(Gao et al., 2009)
F5			353	135, 191, 173, 179	4-Caffeoylquinic acid [a] (chlorogenic acid isomer)		2.86 ± 0.09	(Gao et al., 2009)
F6	7.45		337	173, 163, 137, 119	4-*p*-Coumaroylquinic acid[b]		0.23 ± 0.01	(Bender et al., 2018)
Xanthones								
F7	5.64	367, 318, 257,240	421	331, 301, 271, 273, 259, 258	Mangiferin[c]	$C_{19}H_{18}O_{11}$	34 90 ± 0.97	(Luo et al., 2012)
F8	6.38		421	331, 301, 313, 271, 285, 258	Mangiferin isomer[c]		0.63 ± 0.05	
F9	6.92	363, 316, 255	421	331, 301, 313, 271, 285, 258	Mangiferin isomer[c]		0.13 ± 0.01	
F10	11.26		421	301, 273, 331, 271	Mangiferin isomer[c]		0.46 ± 0.02	
Condensed tannins								
F11	7.14	279	865	847, 749, 713, 695, 577, 407, 287, 289	Procyanidin B-type trimer[d]	$C_{30}H_{26}O_{12}$	0.69 ± 0.02	(Jabeur et al., 2016)
F12	7.46		863	411, 289, 711, 693, 573, 451, 559	Procyanidin A-type trimer[d]	$C_{45}H_{36}O_{18}$	0.91 ± 0.02	(Rosero, Cruz, Osorio, & Hurtado, 2019)
Flavanonols								
F13	9.99	285	449	303, 285, 275, 125, 153, 181	Taxifolin-*O*-rhaminoside[e]	$C_{21}H_{22}O_{11}$	1.33 ± 0.19	(Goufo et al., 2020)
Flavonols								
F14	13.11	354	609	300, 301	Quercetin-3-*O*-rutinoside[f] (rutin)	$C_{27}H_{30}O_{16}$	0.55 ± 0.01	(Boga et al., 2021)
F15	13.90		463	300, 301, 255, 271, 151, 179	Quercetin-*O*-galactoside [f] (hyperoside)	$C_{21}H_{20}O_{12}$	2.04 ± 0.05	(Goufo et al., 2020)
F16	14.34		463	300, 301, 255, 271, 151, 179	Quercetin-*O*-glucoside[f] (isoquercitrin)	$C_{21}H_{20}O_{12}^-$	1.02 ± 0.09	(Jabeur et al., 2016)
F17	17.44		447	300, 301, 271, 151, 179	Quercetin 3-*O*-rhamnoside (quercitrin) [f]	$C_{21}H_{20}O_{11}$	0.37 ± 005.	(Heinrich, Daniels, Stintzing, & Kammerer, 2017)
				TOTAL PHENOLIC COMPOUND CONTENT			**59.57 ± 0.8**	
Anthocyanins								
F17	14.77	258, 318, 366, 512	423	405, 387, 357, 327	Manginferin**	$C_{19}H_{18}O_{11}$	14.34* ± 0.02	(Nitarska et al., 2018)
A1			449	287	Cyanidin hexoside	$C_{21}H_{21}O_{11}^+$		
A2	16.61	258, 317, 364, 515	519	453, 399, 421, 271	Pelargonidin-3-*O*-(6″-malonylglucoside)[g]	$C_{24}H_{23}O_{13}^+$	149.06* ± 0.01	
A3			595	449, 433, 287	Cyanidin-3-*O*-rutinoside [g]	$C_{27}H_{31}O_{15}^+$		
A4	19.72	234, 262, 314, 370, 507	579	433, 271	Pelargonidin-3-*O*-rutinoside [g]	$C_{27}H_{31}O_{14}^+$	11.38 ± 0.80	
A5	20.34	261, 314, 370, 515	463	301	Peonidin hexoside [g]	$C_{22}H_{23}O_{11}^+$	2.91 ± 0.21	–
A6	22.05	260sh, 280, 320sh, 517	609	463, 301	Peonidin-3-*O*-rutinoside [g]	$C_{28}H_{33}O_{15}^+$	123.01* ± 0.51	–
A7			465	303	Delphinidin hexoside [g]	$C_{21}H_{21}O_{12}^+$		
A8	26.23	220,257sh, 310sh, 360sh, 519	611	593, 465, 303	Delphinidin rutinoside [g]	$C_{27}H_{31}ClO$	3.24 ± 0.05	–
				TOTAL ANTHOCYANIN CONTENT			**303.94 ± 0.3**	

Notes: The contents of detected phenolic compounds and anthocyanins are expressed as milligrams per gramme dry weight (mg/g dw ± sd). More than one row per peak indicates coelution. *Peaks found in total coelution were quantified together: 1a + 1b; 2a + 2b; 5a + 5b. ** Mangiferin had to be considered even though it is not an anthocyanin due to its co-elution with cyanidin hexoside. Standard curves used for each compound: a – chlorogenic acid; b – *p*-coumaric acid; c – quercetin-3-*O*-glucoside; d – epicatechin; e – taxifolin; f – quercetin-3-*O*-glucoside; g – cyaniding-3-glucoside.

such as *H. androsaemum*, but in lower quantities (0.88 mg/g) (Jabeur et al., 2016). The extract of *H. lanuginosum* contained 4-caffeoylquinic acid (**F5**) (Mahomoodally et al., 2019). Flavonoids and hydroxycinnamic acids have been recognized as the phenolic compounds most involved in antioxidant responses. Furthermore, they may enhance plant resistance to elicitors of biotic origin, including attacks by pests and pathogens (Bruňáková, Bálintová, Petijová, & Čellárová, 2022).

3.4. Anthocyanin composition in H. sampsonii

From both species, only *H. sampsonii* showed the presence of anthocyanins. Anthocyanins are the glycosylated forms of anthocyanidins (aglycones), mainly based on the flavillium cation or 2-phenylbenzopyrilium, and consist of hydroxyl and methoxyl groups in different positions (Fig. 2) (D. Li, Wang, Luo, Zhao, & Chen, 2017; Mattioli, Francioso, Mosca, & Silva, 2020). Many studies have pointed out that anthocyanins exhibit a variety of biological activities, most notably strong antioxidant activity, by scavenging free radicals (H. Xue et al., 2023). To our knowledge, this is the first study reporting the anthocyanin identification in *H. sampsonii*. Eight anthocyanins were identified and quantified by HPLC-DAD-ESI(+)/MS/MS. Anthocyanins were eluted as follows: cyanidin hexoside > pelargonidin-3-*O*-(6″-malonylglucoside) (P36"g) > cyanidin-3-*O*-rutinoside (C3R) > pelargonidin-3-*O*-rutinoside > peonidin hexoside > peonidin-3-*O*-rutinoside > delphinidin hexoside and delphinidin rutinoside.

As can be seen in chromatogram (Fig. 2), compounds found in total coelution were quantified together. In addition, mangiferin was considered even though it is not an anthocyanin due to its co-elution with cyanidin hexoside. Based on the data presented in Table 4, the highest proportion of anthocyanins corresponds to the amount of P36"g and C3R (149.06 mg/g). Recently published studies have shown that P36"g may have a high antioxidant effect by reducing free fatty acid-induced OS, including increased ROS and superoxide anion levels, among other functional roles (Hao et al., 2023). C3R has been characterized as a natural anthocyanin with broad antioxidant, anti-hyperglycemic, antiglycation and cardioprotective properties (Thilavech & Adisakwattana, 2019).

3.5. Evaluation of in vitro biological properties of phenolic-rich extracts of H. japonicum and H. sampsonii

3.5.1. Antioxidant activity

The cellular antioxidant activity (CAA) assay showed, for both samples, unremarkable results (EC_{50} = 77.5 μg/mL) whereas the positive control (quercetin) displayed an antioxidant capacity 6000 times more potent (Table 5). Despite the results observed for CAA, the antioxidant activity of the extracts was supported by the OxHLIA and TBARS assays. In the OxHLIA, as show in Table 5, for a Δt of 60 min, the EC_{50} value of the *H. japonicum* extract was lower than the positive control made with Trolox (16.2 < 21.8 μg/mL). The EC_{50} value for *H. sampsonii* was also positive (30 μg/mL), demonstrating the protective effect of the extracts to prevent erythrocytes from hemolysis. A separate study tested nine different *HP* species using the OxHLIA assay. Their results agreed with ours, with a range of EC_{50} values between 7.77 and 21.40 μg/mL (Babotă et al., 2022). Previous results agree that the *HP* bioactive compounds modulate essential cellular processes via multiple mechanisms of action and interaction points in many intracellular signaling cascades (A. R. Silva et al., 2021). Besides, several studies have attributed the antioxidant potential to flavonoid glycosides and phenolic acids (Napoli et al., 2018). In another study on four *HP* species, a correlation was found between the composition of phenols and flavonoids and the antioxidant activity (Saddiqe, Naeem, Hellio, Patel, & Abbas, 2020). For example, taxifolin and its derivatives (flavononols), were the major compounds found in *H. japonicum* and are directly connected with antioxidant activity in the defense against OS-induced apoptosis through the activation of Nrf2, pro-capillary action and neuroprotective effects (Das, Baidya, Chakraborty, Samanta, & Roy, 2021). Mangiferin, the major xanthone in the MeOH extract of *H. sampsonii*, has substantiated its safety and effectiveness in clinical trials. The positive effects has been attributed to the inhibition of inflammation caused by the regulation of NF-kB, the redox control of soluble factor epoxide hydrolase (sEH) and in maintaining a reduced production and accumulation of low levels of ROS (Lum et al., 2022).

In the TBARS assay, the performance of both extracts was similar to that obtained for the commercial antioxidant Trolox (5.8 μg/mL) used as positive control. In this case, the MeOH extract of *H. sampsonii* obtained a better EC_{50} value (17.05 μg/mL) than *H. japonicum* (28.09 μg/mL). In another study, the EtOH extract of *H. androsaemum* showed an inhibition of TBARS of 36 μg/mL (EC_{50}) (Jabeur et al., 2016). Although the results reflect the antioxidant potential of the two *HP* species and their chemical components, the influence of the solvent fraction used should not be overlooked. In a study in which four different solvents were tested on *H. japonicum*, the results of antioxidant activity for the TBARS assay were completely different. The hexane extract obtained an IC_{50} value of 267.13 μg/mL while the ethyl acetate extract achieved a lipid peroxidation inhibition value as high as 7.68 μg/mL (IC_{50}) (Roy & Swargiary, 2023). These properties indicate a potential application of *HP* in the development of functional foods. For example, *H. japonicum* extract has been incorporated into biscuits, showing high antioxidant effects and positively influencing the antimetabolomic properties of the products (Jakubczyk et al., 2021), emphasizing the benefits of using these plants in food-related applications.

The different results between TBARS, OxHLIA and CAA are explained because techniques measure different mechanisms of action. Cell culture and erythrocyte-based assays are more complex than chemical ones. However, antioxidant effects are evaluated from a global perspective. Despite the scarce literature available for the two species, research on the antioxidant properties of the genus *HP* has provided evidence to highlight the antioxidant activity based on the phytochemical composition of the species (Boga et al., 2021; Kakouri et al., 2023; Napoli et al., 2018; Zdunic et al., 2017).

3.5.2. Anti-inflammatory activity

The samples from *H. japonicum* and *H. sampsonii* did not achieve a significant suppression of LPS-induced NO generation in the RAW 264.7 cell line (Table 5). These results contrast with the literature. In one study, an IC_{50} of 32.44 μg/mL was obtained with the EtOH extract of *H. sampsonii* in the same cell line (Chen et al., 2020). In a further study, sampsonione and otogirinin isolated from the aerial parts of *H. sampsonii* were effective as anti-inflammatory compounds with IC_{50} values of 35.25 and 32.87 μM, respectively (Huang et al., 2020). In a separate study, seven *HP* extracts displayed anti-inflammatory activity, with inhibitions ranging from 59.4 % to 81.9 %. These included *H. japonicum* extract, for which, of the 52 major constituents identified in the species, 11 exhibited anti-inflammatory properties (R. Zhang et al., 2021). Hyperjapone, isolated from *H. japonicum* extract displayed anti-inflammatory effects (IC_{50} value of 11.32 μM), based on decreasing the protein expression of iNOS and COX-2 in a dose-dependent manner. Nevertheless, the aforementioned compounds were not identified in the present study in either extract (Deng et al., 2022). Considering the common phytochemical differences between *HP* species, their biological properties vary influenced by plant's genetic characteristics, since many bioactive compounds are species-specific and, within species, lower-order taxa (Carrubba, Lazzara, Giovino, Ruberto, & Napoli, 2021).

3.5.3. Antitumoral activity and cytotoxic potential

HP species have been traditionally used as medicines to deal with a variety of illnesses. For this reason, the determination of cytotoxic effects could be of help for the human health (Eroğlu Özkan et al., 2013). Toxicity was observed to non-tumorous porcine liver cells (PLP2) of 154 μg/mL for *H. japonicum* and 266 μg/mL for *H. sampsonii*. These values did not imply hepatotoxicity as high as that achieved by the Ellipticine

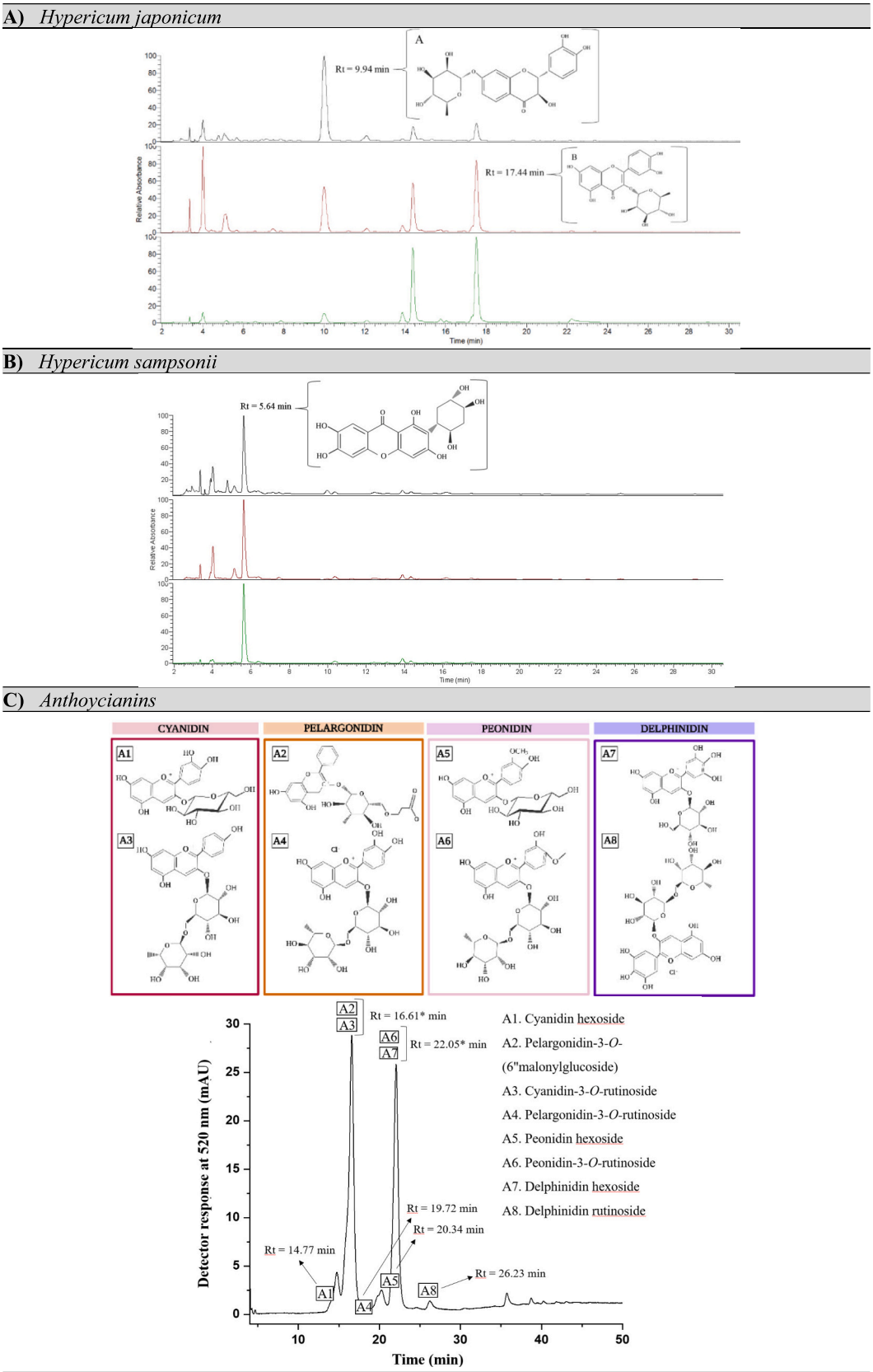

Fig. 2. A) and **B)** Chromatograms, obtained by HPLC-DAD, of the phenolic compound *H. japonicum* and *H. sampsonii* extracts. Chromatograms were processed at 280, 330 and 370 nm and are presented in this order. Peak assignments can be found in Table 3 and Table 4. **C)** Chromatogram and chemical structures of the anthocyanins obtained by HPLC-DAD in the extract of *H. sampsonii.* The chromatogram was processed at 520 nm. The color assigned to each group corresponds to the anthocyanin pigmentation. Peak assignments can be found in Table 4.

Table 5
Antioxidant, cytotoxic and anti-inflammatory activities of *Hypericum* species.

	Hypericum japonicum	*Hypericum sampsonii*	Positive control
Antioxidant activity[1] EC_{50} values (μg/mL)			
CAA (%)	77 ± 5	77 ± 5	95 ± 5
OxHLIA, Δt = 60 min	16.2 ± 0.4	30 ± 2	21.8 ± 0.28
TBARS	28.09 ± 0.2	17.05 ± 0.4	5.8 ± 0.6
Cytotoxic potential and antitumoral activity[2] GI_{50} values (μg/mL)			
AGS (gastric adenocarcinoma)	69 ± 2	201 ± 11	1.23 ± 0.03
CaCo2 (colorectal adenocarcinoma)	210 ± 19	237 ± 11	1.21 ± 0.02
MCF-7 (breast carcinoma)	131 ± 13	222 ± 10	1.02 ± 0.004
NCI-H460 (non-small cell lung cancer)	186 ± 18	232 ± 4	1.01 ± 0.01
PLP2 (porcine liver primary culture)	154 ± 6	266 ± 23	1.4 ± 0.1
Anti-inflammatory activity[3] IC_{50} (μg/mL)			
Raw 264.7 (murine macrophage cells)	>400	>400	6.3 ± 0.4

Abbreviations: CAA: Cellular Antioxidant Activity, OxHLIA: Oxidative Hemolysis Inhibition Assay; TBARS: Thiobarbituric Acid Reactive Substances; EC_{50}: Effective concentration required to obtain a 50 % of antioxidant effect; GI_{50}: Concentration achieving 50 % of growth inhibition in human tumor cell lines; IC_{50}: Half-maximal inhibitory concentration (50 %) for the inhibition of nitric oxide (NO) production. Extract concentrations: 2 mg/mL (CAA), 5 mg/mL (OxHLIA; TBARS), 8 mg/mL (cytotoxic potential, antitumoral and anti-inflammatory activities). Positive controls: [1]Quercetin 0.3 μg/mL (CAA), Trolox 5 mg/mL (OxHLIA) and Trolox 5 mg/mL (TBARS). [2]Ellipticine. [3]Dexamethasone 50 μM.

control (1.4 μg/mL) but still, they must be further assessed to draw a conclusion on the safeness of the use of the *HP* species. Other *HP* species as *H. androsaemum* exhibited non-toxicity to PLP2 cells at concentrations up to 400 μg/mL (Jabeur et al., 2016). However, the MeOH extracts were capable of inhibiting different human tumor cell lines ranging from 69 to 237 μg/mL (Table 5). AGS cells (GI_{50} = 69 μg/mL) were the most susceptible, followed by MCF-7 (GI_{50} = 131 μg/mL) and NCI-H460 (GI_{50} = 186 μg/mL) all three to *H. japonicum* extract. The most sensitive cell line to *H. sampsonii* extract was AGS (GI_{50} = 201 μg/mL) and the least sensitive were CaCo2 cells (GI_{50} = 237 μg/mL). These results could be explained by the diversity of the composition of phytochemicals compounds in the extracts and the synergistic activity that results in protection against toxicity (Vaou et al., 2022). As an example, myricetin and isoquercitrin are reported to exhibit *in vitro* antitumor activity, demonstrating potential for cancer therapy (Carrubba et al., 2021). In other studies, the EtOH extract of *H. androsaemum*, GI_{50} values were close to those achieved in our study with the MCF-7 (106 μg/mL) and NCI-H460 (215 μg/mL) tumor cell lines. In contrast, they had no toxic potential (IC_{50} > 400 μg/mL) with the non-tumor cell line PLP2 (Jabeur et al., 2016). Additionally, the cytotoxic effects of *H. scabrum* on the MDF-7 cell line were lower than those obtained in our study with an IC_{50} level of 333.9 μg/mL (Dastan, 2023). In a further study, the EtOH extract of *H. sampsonii* showed moderate cytotoxic activity against cancer cells including human melanoma (A375), M.D. Anderson-Metastatic Breast 231 (MDA-MB-231), human cervical squamous carcinoma (SiHa) cell line and human Neuroblastoma (SHSY-5Y) lines, with IC_{50} values of 52.35, 59.33, 30.24 and 49.57 μg/mL, respectively, while it displayed no cytotoxic activity (IC_{50} > 200 μg/mL) against SGC-7901 (Chen et al., 2020). Finally, in a screening of *in vitro* cytotoxicity against Dalton's lymphoma ascites (DLA) cell line with *H. japonicum* extract obtained with solvents of different polarity, the highest short-term cytotoxicity was displayed by the acetonic extract (GI_{50} = 29.30 μg/mL) and the lowest by the MeOH extract (GI_{50} = 60.20 μg/mL). This evidence suggests that antitumor potential may be influenced by the extraction solvent chosen (Puthur, Anoopkumar, Rebello, & Aneesh, 2018).

3.5.4. Antimicrobial activity

Phytochemical compounds isolated from medicinal plants could perform as potent bioactive pharmacological agents against pathogenic bacteria through reversing their resistance to antibiotics, boosting synergistic action with modern antibiotic agents, inhibiting virulence factors, targeting microbial cells as well as providing applications in the food industry as natural preservatives or functional ingredients (Mickymaray, 2019; Rasheed et al., 2024). The results obtained for *H. japonicum* and *H. sampsonii* in terms of MIC and MBC for clinical and food bacteria are compiled in Table 6.

In general, the values obtained for *H. japonicum* were more satisfactory compared to those obtained for *H. sampsonii.* On the one hand, regarding clinical bacteria, *H. japonicum* was able to successfully inhibit the growth of gram-positive *E. faecalis* (MIC = 0.07 mg/mL), far lower than that required by the antibiotic ampicillin (<15 mg/mL). Very few studies have evaluated the antimicrobial potential of *H. japonicum* and there are even fewer for *H. sampsonii.* Yet, for other *HP* species, similar results for the EtOH extract of *H. perforatum* (MIC = 0.016 mg/mL) against *E. feacalis* are available (Süntar et al., 2016). In a separate study, nine *HP* species were assayed against *E. feacalis*. The lowest MIC and MBC value was obtained by *H. perforatum* (0.13 and 0.51 mg/mL, respectively), followed by *H. delphicum* (0.31 and 0.63 mg/L, respectively) (Kakouri et al., 2023). Although the MIC obtained in our study is a very low value, the MBC obtained is quite significant. Antibacterial agents are generally accepted as bactericidal if the MBC is not more than four times the MIC. Therefore, more exhaustive studies of other antimicrobial parameters, proportionality studies and the degree of susceptibility may be necessary to advance the appropriate criteria for the interpretation of the results and to increase the probability of therapeutic success (Kowalska-Krochmal & Dudek-Wicher, 2021; Mogana, Adhikari, Tzar, Ramliza, & Wiart, 2020). Regarding *H. sampsonii*, the most outstanding MIC values are against L. *monocytogenes* and *MRSA* (0.6 mg/mL). These values are considerably superior to those achieved by the control antibiotic vancomycin (MIC = 0.25 mg/mL) using a 10-fold lower concentration of drug. The supercritical CO_2-prepared extract of. *H. rochelii* reached an MIC of 9.8 mg/mL and a MBC of 78 mg/mL against *MRSA,* which are far higher concentrations compared to our findings (Ilieva et al., 2023).

On the other hand, the results were better for foodborne bacterial strains than for clinical isolates. Again, *H. japonicum* showed greater inhibition of *H. sampsonii* bacterial growth. Of note was its effectiveness against *B. cereus*, *L. monocytogenes* and *S. aureus* (MIC = 0.07 mg/mL), as they are among the top three food-borne pathogens causing the greatest uncertainty and disquiet (Wei et al., 2019). Obtaining also better performance than ampicillin. Furthermore, the antimicrobial activity of *H. japonicum* and *H. sampsonii* was also higher than the achieved for the MeOH extract of *H. humifusum* against *B. cereus, L. monocytogenes* and *S. aureus* (MIC = 0.62, 0.15 and 0.15 mg/mL, respectively) (Toiu et al., 2016). Other species, such as *H. scabrum* reported MIC values for *B. cereus* (1.7 mg/mL) and *S. aureus* (2.6 mg/mL) approximately 30 times higher than in our study (Dastan, 2023). The MIC values against clinical and foodborne strains of *E. coli* of the MeOH extracts of *H. japonicum* (1.25 and 2.5 mg/mL) and *H. sampsonii* (5 and 2.5 mg/mL) were not the most remarkable results. However, they are comparable with a study in which 11 *HP* spp. were evaluated against *E. coli* and a constant MIC value of 2 mg/mL was observed. Thus, this gram-negative bacterium may show less susceptibility to *HP* spp. (Nogueira et al., 2013). Moreover, a study of the MeOH extract from *H. scabrum* showed a MIC value for *E. coli* of 0.6 mg/mL (root) and 2.5 mg/mL (aerial part); therefore, the plant part is an influential factor that must be considered (Ergin et al., 2022). Additionally, antibacterial studies were also carried out for *P. acnes*. *H. japonicum* and *H. sampsonii* had MIC of 0.6 and 1.25 mg/mL, respectively (Sinha, Srivastava, Mishra, & Yadav, 2014). These findings support the use of *H. japonicum and H. sampsonii* in potential food packaging applications, such as edible coatings to improve the preservation of fresh foods, as well as in *HP* species that have already

Table 6
Antibacterial and antifungal activities of the extracts of *H. japonicum* and *H. sampsonii*.

MICROBIAL STRAINS TESTED		*HYPERICUM* SPECIES		POSITIVE CONTROLS		
CLINICAL (BACTERIA)		***H. japonicum***	***H. sampsonii***	Ampicillin	Imipenem	Vancomycin
Escherichia coli	*MIC*	1.25	5	<0,15	<0.007	N/A
	MBC	>10	>10	<0,15	<0.007	N/A
Klebsiella penumoniae	*MIC*	5	5	10	<0.007	N/A
	MBC	>10	>10	>10	<0.007	N/A
Pseudomonas aeruginosa	*MIC*	10	10	>10	0.5	N/A
	MBC	>10	>10	>10	1	N/A
Morganella morganii	*MIC*	0.6	1.25	>10	<0.007	N/A
	MBC	>10	>10	>10	<0.007	N/A
Proteus mirabilis	*MIC*	1.25	5	<0.15	<0.007	N/A
	MBC	>10	>10	<0.15	<0.007	N/A
Enterococcus faecalis	*MIC*	0.07	2.5	<0.15	N/A	<0.007
	MBC	>10	>10	<0.15	N/A	<0.007
Listeria monocytogenes	*MIC*	2.5	0.6	<0.15	<0.007	N/A
	MBC	>10	>10	<0.15	<0.007	N/A
MRSA	*MIC*	1.25	0.6	<0.15	N/A	0.25
	MBC	>10	>10	<0.15	N/A	0.5
Propionibacterium acnes	*MIC*	0.6	1.25	0.07	N/A	N/A
	MBC	>10	>10	5	N/A	N/A
FOOD-BORNE (BACTERIA)		***H. japonicum***	***H. sampsonii***	Ampicillin	Streptomycin	Methicillin
Enterobacter cloacae (ATCC 49741)	*MIC*	5	5	0.15	0.007	N/A
	MBC	>10	>10	0.15	0.007	N/A
Escherichia coli (ATCC 25922)	*MIC*	2.5	2.5	0.15	0.01	N/A
	MBC	>10	>10	0.15	0.01	N/A
Yersinia enterocolitica (ATCC 8610)	*MIC*	2.5	5	0.15	0.007	N/A
	MBC	>10	>10	0.15	0.007	N/A
Salmonella enterocolitica (ATCC 13076)	*MIC*	10	10	0.06	N/A	N/A
	MBC	>10	>10	0.06	N/A	N/A
Pseudomonas aeruginosa (ATCC 9027)	*MIC*	10	10	0.6	0.06	N/A
	MBC	>10	>10	0.6	0.06	N/A
Bacillus cereus (ATCC 11778)	*MIC*	0.07	2.5	N/A	0.007	N/A
	MBC	>10	>10	N/A	0.007	N/A
Listeria monocytogenes (ATCC 19111)	*MIC*	0.07	0.3	0.15	0.007	N/A
	MBC	>10	>10	0.15	0.007	N/A
Staphylococcus aureus (ATCC 25923)	*MIC*	0.07	0.6	0.15	0.007	0.07
	MBC	>10	>10	0.15	0.007	0.07
ANTIFUNGAL ACTIVITY		***H. japonicum***	***H. sampsonii***	Ketoconazole		
Aspergillus brasiliensis	*MIC*	>10	>10	0.6		
	MFC	>10	>10	0.125		
Aspergillus fumigatus	*MIC*	>10	>10	0.5		
	MFC	>10	>10	1		

Abbreviations: MIC: minimum inhibitory concentration (mg/mL); MBC: minimum bactericidal concentration (mg/mL), MFC: minimum fungicidal concentration (mg/mL); *MRSA*: methicillin-resistant *Staphylococcus aureus*. Tested concentrations: extracts and Ampicillin at 10 mg/mL; Imipenem, Vancomycin, Streptomycin, Methicillin and Ketoconazole at 1 mg/mL. Clinical bacterial strains were isolated from hospitalized patients of the Hospital Center of Trás-os-Montes and Alto Douro (Portugal) and all food-borne bacteria were purchased from Frilabo, Porto, Portugal. N/A: no answer.

been described (Zamani Faradonbeh, Barzegar, Hojjati, Alizadeh Behbahani, & Taki, 2024).

Finally, limited data are available on the antifungal potential of this genus and only a few species have been assessed for this property (Tocci et al., 2018). The results evidenced the absence of antifungal activity of the two species (Table 6). Furthermore, no previous research was found regarding the antifungal action of *H. japonicum* and *H. sampsonii*.

4. Conclusions

This study is the first to explore the phytochemical characterization of *H. japonicum* and *H. sampsonii* and their connection to biological properties. TPC (184.51 mg/g dw) was three times that of *H. sampsonii* (59.57 mg/g dw), indicating superior biological characteristics. The dominant phenolic compounds in *H. japonicum* were flavonoids (77 % of the total), whereas xanthones dominated *H. sampsonii* (60 %). Anthocyanins (303.94 mg/g dw) were identified in *H. sampsonii* for the first time.

Antioxidant activity, assessed by OxHLIA, showed results equal to or superior to the positive control of Trolox (*H. japonicum*, $EC_{50} = 16.3 < 21.8$ μg/mL). While *H. sampsonii* extract revealed results with considerable potential, indicating that it similarly inhibited lipid peroxidation in the TBARS method ($EC_{50} = 17.05$ μg/mL) compared to Trolox ($EC_{50} = 5.8$ μg/mL). Although more data are needed to draw general conclusions about their cytotoxic activity against PLP2, both extracts showed significant antitumor activity, with *H. japonicum* extract showing a GI_{50} of 69 μg/mL against AGS. Additionally, the *H. japonicum* extract exhibited strong antimicrobial activity, achieving MIC values as low as 0.007 mg/mL for several bacterial strains (*E. faecalis, B. cereus, L. monocytogenes,* and *S. aureus*), outperforming the control.

The phytochemical characterization and bioactivity screening results suggest that *H. japonicum* and *H. sampsonii* may be suitable for product-based food applications.

CRediT authorship contribution statement

Paula Barciela: Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Daniele B. Rodrigues:** Writing – review & editing, Visualization, Supervision, Methodology. **Ana Perez-Vazquez:** Writing – original draft. **Tayse F.F. da Silveira:** Writing – review & editing, Supervision, Methodology. **Tânia C.S.P. Pires:** Writing – review & editing, Supervision, Methodology. **Filipa Mandim:** Supervision, Methodology. **Maria Carpena:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology. **Carla Pereira:** Supervision. **Isabel C.F.R. Ferreira:** Visualization, Validation, Project administration.

Lillian Barros: Supervision, Project administration, Funding acquisition. **Miguel A. Prieto:** Supervision, Project administration, Funding acquisition.

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.

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Data availability

No data was used for the research described in the article.

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