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Multi-target neuroprotective effects of herbal medicines for Alzheimer's disease

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ABSTRACT

Ethnopharmacological relevance: Alzheimer's disease is the most common form of dementia, but its treatment options remain few and ineffective. To find new therapeutic strategies, natural products have gained interest due to their neuroprotective potential, being able to target different pathological hallmarks associated with this disorder. Several plant species are traditionally used due to their empirical neuroprotective effects and it is worth to explore their mechanism of action.

Aim of the study: This study intended to explore the neuroprotective potential of seven traditional medicinal plants, namely *Scutellaria baicalensis*, *Ginkgo biloba*, *Hypericum perforatum*, *Curcuma longa*, *Lavandula angustifolia*, *Trigonella foenum-graecum* and *Rosmarinus officinalis*. The safety assessment with reference to pesticides residues was also aimed.

Materials and methods: Decoctions prepared from these species were chemically characterized by HPLC-DAD and screened for their ability to scavenge four different free radicals (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, $O_2^{\bullet-}$ and $^{\bullet}$NO) and to inhibit enzymes related to neurodegeneration (cholinesterases and glycogen synthase kinase-3β). Cell viability through MTT assay was also evaluated in two different brain cell lines, namely non-tumorigenic D3 human brain endothelial cells (hCMEC/D3) and NSC-34 motor neurons. Furthermore, and using GC, 21 pesticides residues were screened.

Results: Regarding chemical composition, chromatographic analysis revealed the presence of several flavonoids, phenolic acids, curcuminoids, phenolic diterpenoids, one alkaloid and one naphthodianthrone in the seven decoctions. All extracts were able to scavenge free radicals and were moderate glycogen synthase kinase-3β inhibitors; however, they displayed weak to moderate acetylcholinesterase and butyrylcholinesterase inhibition. *G. biloba* and *L. angustifolia* decoctions were the less cytotoxic to hCMEC/D3 and NSC-34 cell lines. No pesticides residues were detected.

Conclusions: The results extend the knowledge on the potential use of plant extracts to combat multifactorial disorders, giving new insights into therapeutic avenues for Alzheimer's disease.

Abbreviations: 2,2-diphenyl-1-picrylhydrazyl radical, (DPPH$^{\bullet}$); 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical, (ABTS$^{\bullet+}$); Acetylcholinesterase, (AChE); Alzheimer's disease, (AD); amyloid beta, (Aβ); beta-secretase 1, (BACE-1); butyrylcholinesterase, (BuChE); gallic acid equivalents, (GAE); glycogen synthase kinase-3β, (GSK-3β); multitarget-directed ligands, (MTDLs); neurofibrillary tangles, (NFTs); nitric oxide radical, ($^{\bullet}$NO); peroxynitrite, ($ONOO^-$); reactive nitrogen species, (RNS); reactive oxygen species, (ROS); radical scavenging activities, (RSA); superoxide anion radicals, ($O_2^{\bullet-}$); Trolox equivalents, (TE).

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1. Introduction

Alzheimer's disease (AD), the most common form of dementia, is clinically characterized by the loss of memory and cognitive functions, and changes in personality and behaviour, that occur due to progressive tissue degeneration, starting in the perirhinal region of the hippocampus complex and ultimately to the temporal lobes together with the basal forebrain (Bredesen, 2009). Globally, nearly 40 million people over the age of 60 suffer from AD, and the number of patients is rising, expecting to double every 20 years. Early-life risk factors for this pathology include genetic mutations, chromosomal abnormalities, head injury, insulin resistance, and inflammation (Borenstein et al., 2006; Zagórska and Jaromin, 2020; Gul et al., 2021).

The major pathological hallmarks of AD include the impairment of acetylcholine neurotransmission and abnormal function of cholinesterases, the enzymes responsible for this neurotransmitter breakdown; the extraneuronal deposits of amyloid beta (Aβ) fibrils and plaques and intracellular accumulations of neurotoxic Aβ oligomers; the intracellular hyperphosphorylated tau protein leading to the formation of neurofibrillary tangles (NFTs); and neuronal loss in key brain regions involved in memory and cognition. Despite incredible efforts, there is still a lack of effective drugs for the treatment of AD. Chemical inhibition of cholinesterase enzymes may be therapeutically promising. There are five drugs in clinic, including three cholinesterase inhibitors, that only provide palliative and supporting treatment (Butterfield and Boyd-Kimball, 2020; Zagórska and Jaromin, 2020; Gul et al., 2021). It is increasingly evident that other targets must be explored and that a complex and multifactorial disorder, such as AD, must be approached with multitarget-directed ligands (MTDLs) (Agis-Torres et al., 2014). Nowadays, other possible targets are being studied, such as beta-secretase 1 (BACE-1) and glycogen synthase kinase-3β (GSK-3β) inhibition (De Simone et al., 2021; Prati et al., 2015). While the first one is involved in Aβ agglomeration into fibrils and plaques, the abnormal activation of GSK-3β, a serine/threonine protein kinase, has been associated with hyperphosphorylation of tau proteins into neurofibrillary tangles (NFTs). Furthermore, increased GSK-3β activity also induces Aβ deposition extracellularly (Griebel et al., 2019; Toral-Rios et al., 2020).

It is also recognized that other factors may be involved in the progression of AD cognitive loss, such as neuroinflammation and oxidative and nitrosative stresses in the brain. The main source of reactive oxygen species (ROS) is the electron transport chain at the mitochondrial inner membrane. Naturally, some electrons escape from the inner membrane and react with oxygen to produce superoxide anion radicals ($O_2^{\bullet -}$), which further lead to the production of other ROS. If not neutralized by superoxide dismutase, $O_2^{\bullet -}$ can react with nitric oxide ($^{\bullet}NO$) to produce the highly reactive peroxynitrite ($ONOO^-$), starting nitrosative stress. ROS and reactive nitrogen species (RNS) are capable of damaging and modifying several types of macromolecules, such as DNA, RNA, lipids, and proteins, compromising the normal functions of the cells (Butterfield and Boyd-Kimball, 2020; Persson et al., 2014).

Mixtures of drugs or plant extracts might have advantages over single drugs as they can impact different targets simultaneously, which could be a novel and more effective treatment option for AD (Chen et al., 2021). The secondary metabolites of plants including alkaloids, flavonoids, and phenolic acids play a key role in improving regeneration and/or inhibiting neurodegeneration (Kamran et al., 2020). Several natural products, mainly plants extracts, have been reported to be used in traditional medicine for neuroprotective, memory enhancing, and antiageing functions, such as *Curcuma longa* L. (Zingiberaceae) (Chen and Chang, 2015; Long et al., 2014), *Hypericum perforatum* L. (Hypericaceae) (Doležal et al., 2019), *Lavandula angustifolia* Mill. (Lamiaceae) (Hawrył et al., 2019), *Rosmarinus officinalis* L. (Lamiaceae) (Yeddes et al., 2019), *Trigonella foenum-graecum* L. (Fabaceae) (Zameer et al., 2018), *Ginkgo biloba* L. (Ginkgoaceae Family) (Isah, 2015) and *Scutellaria baicalensis* Georgi (Lamiaceae Family) (Sowndhararajan et al., 2018).

Su et al. (2014) reviewed the Chinese medicinal plants used for the treatment of AD, which included the rhizome of *C. longa* and its active constituents curcumin and turmerone and the leaves of *Ginkgo biloba* L. characterized by the presence of ginkgolides and phenolic compounds. The extract from *G. biloba* leaves has become one of the most widely used herbal remedies for dementia (Yuan et al., 2017). *G. biloba* constituents, standardized extracts and leaf tablets showed protection in different *in vivo* models of AD (Su et al., 2014). The effects of *G. biloba* extracts on dementia have been assessed by several controlled clinical trials, but, the findings are inconsistent due to heterogeneity in the dosages and duration of the interventions, and the sample characteristics across different trials (Yuan et al., 2017).

In Traditional Chinese medicine, *S. baicalensis* is used to treat diseases such as diarrhoea, dysentery, high blood pressure, bleeding, insomnia, inflammation and respiratory infections (Zhao et al., 2019) and in traditional Korean medicine, to treat cerebral ischemia in addition to bacterial infection and inflammatory diseases (Heo et al., 2009). Heo et al. (2009) showed that *S. baicalensis* improved memory in the ibotenic acid-induced memory deficient model and could be possibly a good therapeutic candidate to treat degenerating neuronal diseases accompanied by memory loss.

In Europe, *H. perforatum* has been employed to treat several neurological conditions, including anxiety, insomnia due to restlessness, irritability, neuralgia, trigeminal neuralgia, neurosis, migraine headaches, fibrositis, dyspepsia and sciatica (Kumar et al., 2000). Concerning AD, *H. perforatum* treatment has also shown to significantly reduce soluble and aggregated Aβ levels in the brains of transgenic mice (C57BL/6J-APP/PS±mice) (Brenn et al., 2014).

R. officinalis and *L. angustifolia* are among the plants used in Danish folk medicine to treat memory dysfunction. Both species contain rosmarinic acid that is active against amyloid fibrillation (Adsersen et al., 2006; Lobbens et al., 2017). In Portugal, *L. angustifolia* decoction is also traditionally used against anxiety, insomnia, anorexia, bronchitis, cough, nerves, rheumatism and heart disturbance (Ferreira et al., 2006). Although *R. officinalis* and *L. angustifolia* extracts are low to moderate AChE inhibitors (Adsersen et al., 2006; Ferreira et al., 2006), *R. officinalis* extract inhibited Aβ fibrillation to some extent (Lobbens et al., 2017).

T. foenum-graecum is native to Eastern Europe but is now cultivated worldwide and traditionally used in Ayurveda, traditional Chinese medicine and other traditional medicine systems. Its traditional uses include, among others, to treat anorexia, nervous disorders, fever gastritis, gastric ulcers, and to boost breastfeeding. Concerning neuroprotection, it was shown to attenuate aluminum chloride-induced tau pathology, oxidative stress, and inflammation in $AlCl_3$-induced Alzheimer rats (Prema et al., 2017). Zameer et al. (2018) have recently reviewed the positive effect of fenugreek and its constituents on several *in vitro* and *in vivo* AD models.

Although there are already some information on the pharmacological effects of these plants and their constituents, a deeper investigation of their effects on the human brain is still needed to bring a more complete view of the neuroprotective potential of these plants. Therefore, in a previous study, we have demonstrated that the decoctions prepared from the leaves of *G. biloba* and from the roots of *S. baicalensis* displayed moderate acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibition as well as a strong scavenging activity against hydrogen peroxide, a ROS (Delerue et al., 2021). Besides cholinesterase inhibition, in this study, we extended not only the number of species to be evaluated, but also thoroughly investigated other targets related with neurodegeneration, namely the oxidative and nitrosative stresses and the GSK-3β inhibition. Moreover, the effect of all extracts on the viability of hCMEC/D3, a brain microvascular endothelial cell line, and NSC-34, a motoneuron-like cell line, was screened and the chemical composition of all extracts was fully characterized by HPLC-DAD. A particular challenge in safety data on herbal medicines is the quality of the products. The purity of the plants may be compromised

due to several factors, namely, by the presence of dirt, weeds, bacteria, moulds, and multiple contaminants (e.g., pesticides, toxic metals, polychlorinated biphenyls (PCB), etc) (Jordan et al., 2010; Luo et al., 2021). The WHO guidelides for assessing quality of herbal medicines describes the potentially hazardous contaminants and residues that may occur in herbal medicines and reports the national and regional limits set for the various types contaminants (World Health Organization, 2007). Regarding pesticides residues, several possible sources were described, such as, air, soil, water, during cultivation/growth and postharvest processing. Therefore, the presence of different families of pesticides in plant products is a topic of public concern due to the potential health risks.

2. Material and methods

2.1. Samples

Commercial samples of *S. baicalensis* root were acquired from SoriaNatural® (Espanha), while *G. biloba* leaves (Lot. ABCDE1236913), *L. angustifolia* inflorescences (Lot. ABCDE1236913), *R. officinalis* leaves (Lot. ALC21062017), *H. perforatum* leaves (Lot. HIKNEIP10032017), *C. longa* rhizome (Lot. ABCDE1236913), and *T. foenum-graecum* seeds (Lot. ABCDE1236913) were purchased from Ervanário Portuense (Portugal). Samples were grinded to a mean particle size <1000 μm and stored at room temperature and protected from light until further use.

2.2. Reagents, solvents, and materials

Tris(hydroxymethyl)aminemethane (Tris), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI), galantamine, acetylcholinesterase (AChE) from *Electrophorus electricus*, butyrylcholinesterase (BuChE) from equine serum, bovine serum albumin (BSA), glycogen Kinases 3β (GSK-3β), muscle glycogen synthase (GSM), 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetra acetic acid (EGTA), magnesium acetate, potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic trihydrate ($K_2HPO_4.3H_2O$), β-nicotinamide adenine dinucleotide (NADH) disodium salt hydrate, phenazine methosulphate (PMS), nitrotetrazolium blue chloride (NBT), sodium nitroprusside dihydrate (SNP), sulphanilamide, naphthylethylenediamine dihydrochloride, ortho-phosphoric acid 85%, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate ($K_2O_8S_2$), sodium carbonate, Folin–Ciocalteau reagent, gallic acid and sodium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA, and Steinheim, Germany). Magnesium chloride hexahydrate was obtained from VWR (Leuven, Belgium), and sodium chloride from Fisher Scientific (Fair Lawn, NJ, USA). Kinase-Glo Luminescent Kinase assays was purchased from Promega Italia S. r.l (Milan, Italy). Ultrapure water (resistivity of 18.2 MΩ cm at 25 °C) was produced using a Simplicity 185 system (Millipore, Molsheim, France). The eluents used in HPLC-DAD analysis (Methanol Chromasolv for HPLC from Riedel-de Haën (Seelze, Germany) and formic acid from Carlo Erba (Val de Reuil, France)) were filtered through a 0.22 μm nylon membrane filter (Fioroni Filters, Ingré, France) using a vacuum pump (Dinko D-95, Barcelona, Spain) and degassed for 15 min in an ultrasonic bath (Sonorex Digital 10P, Bandelin DK 255P, Germany). Standards for HPLC-DAD analysis were from Fluka (caffeic acid, ≥99%), Alfa Aesar (5-*O*-caffeoylquinic acid, ≥95%), Sigma-Aldrich (protocatechuic acid, ≥99%, ferulic acid, ≥99% and *p*-coumaric acid, ≥98%), and Extrasynthèse (Genay, France): trigonelline (≥95%), (+)-catechin (≥99%), (−)-epicatechin (≥99%), (−)-epicatechin-3-*O*-gallate (≥97.5%), luteolin-8-*C*-glucoside (≥99%), apigenin-8-*C*-glucoside (≥99%), luteolin-6-*C*-glucoside (≥99%), apigenin-6-*C*-glucoside (≥99%), apigenin-7-*O*-glucoside (≥99%), rosmarinic acid (≥99%), quercetin-3-*O*-galactoside (≥99%), quercetin-3-*O*-glucoside (≥99%), quercetin-3-*O*-rhamnoside (≥98.5%), carnosic acid (≥90%), carnosol (≥90%), hypericin (≥95%), and curcumin (≥97.5%). PTFE 0.45 μm filters were purchased from VWR international (PA, USA) and Nylon 0.22 μm from Specanalítica (Portugal).

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Hank's Balanced Salt Solution (HBSS), non-essential amino acids, penicillin, streptomycin and trypsin–EDTA were obtained from Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). Endothelial growth medium (EGM-2) was provided by Lonza. DMSO and Triton X-100 were purchased from Sigma-Aldrich (Steinheim, Germany), respectively.

The 21 pesticide standards (purity ≥95%) and the internal standards (4,4′-dichlorobenzophenone and triphenyl phosphate) were purchased from Sigma-Aldrich Co (Darmstadt, Germany). Standards solutions of 14 organochlorine pesticides (α-,β-, γ- and δ-hexachlorocyclohexanes (HCHs), hexachlorobenzene (HCB), o,p'-DDT ([1,1,1 trichloro-2, 2-bis-(p-chlorophenyl) ethane]), p,p'-DDE ([2,2bis(p-chlorophenyl)-1,1-dichloroethylene]), p,p'-DDD (dichlorodiphenyldichloro-ethane), aldrin, dieldrin, endrin, α, β-endosulfan, and methoxychlor) and 7 organophophorus pesticides (dimethoate, diazinon, chlorpyrifos-methyl, parathion-methyl, malathion, chlorpyrifos, and chlorfenvinphos)) were prepared in n-hexane (Chromatography grade) supplied by Merck (Steinheim, Germany). For the solid phase extraction (SPE), C18e (500mg/3 mL) solid phase extraction (SPE) cartridges were provided by Phenomenex (Spain) and methanol was supplied by SigmaAldrich (Steinheim, Germany). Ultrapure water (resistivity of 18.2 MΩ cm) was produced using a Simplicity 185 system (Millipore, Molsheim, France).

2.3. Decoction preparation

Decoctions ($n = 4$) were prepared as previously described (Delerue et al., 2021) by boiling grinded plant material (0.5 g, <1000 μm) in water (125 mL) for 10 min. After that, extracts were filtered and lyophilized. The extraction yields obtained were 26.88 ± 1.42% (*G. biloba*) (Delerue et al., 2021), 47.17 ± 1.82% (*S. baicalensis*) (Delerue et al., 2021), 24.84 ± 1.68% (*H. perforatum*), 18.25 ± 0.63% (*C. longa*), 28.43 ± 1.59% (*L. angustifolia*), 34.24 ± 2.35% (*T. foenum-graecum*) and 27.06 ± 1.07% (*R. officinalis*).

2.4. Total phenolic content (TPC)

TPC values were determined by a colorimetric assay based on Folin-Ciocalteu reagent following the procedure previously reported by Barroso et al. (2016). Calibration curves were performed using gallic acid and results were expressed as mg gallic acid equivalents (GAE) per g of dried sample. The experiment was performed in triplicate ($n = 3$).

2.5. HPLC-DAD analyses

The extracts were analysed ($n = 3$) on an analytical HPLC unit (Shimadzu) composed by a low-pressure quaternary pump (model LC-20AT), a degasser (model DGU-20A5R), an auto-sampler (model SIL-20AT), a column oven (model CTU-20AC) and a photodiode array detector (model SPD-M20A High-Performance Liquid Chromatography PDA detector). The gradient and column used were previously described (Delerue et al., 2021). Briefly, compounds' separation was achieved with a C18 Spherisorb ODS2 (25.0 × 0.46 cm; 5 μm particle size) column from Waters (Ireland). The solvent system consisted in formic acid 5% (A) and methanol (B), starting with 5% B, and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 45% B at 42 min, 55% B at 47 min, 75% B at 56 min, 100% B at 60 min, 100% B at 65 min, 5% B at 66 min and stop at 80 min. The solvent flow rate was 920 μL/min. Spectral data from all peaks were collected in the range of 200–600 nm, and chromatograms were recorded at 260, 280, 320, 340, 350, 425 and 590 nm. Data were

processed on LabSolutions software. Compounds were identified by comparing their retention times and UV–vis spectra with standards injected in the same conditions and/or by comparison with literature (Cesur Turgut et al., 2017; Chen and Chang, 2015; Delerue et al., 2021; Doležal et al., 2019; Janicsák et al., 1999; Lee and Choung, 2011; Long et al., 2014; Mena et al., 2016; Mohammadi et al., 2020; Raclariu et al., 2017; Rayyan et al., 2010; Shailajan et al., 2011; Troncoso et al., 2005; Yeddes et al., 2019; Yilmaz et al., 2018; Zameer et al., 2018; Zhang et al., 2012).

External calibration curves (Table 1) were prepared to quantify the identified compounds in the samples, using six concentrations ($n = 3$, each concentration). Peak areas were recording at 260 nm for trigonelline, at 280 nm for protocatechuic acid, catechin, epicatechin, epicatechin-3-*O*-gallate, carnosic acid and carnosol, at 320 nm for 5-*O*-caffeoylquinic, caffeic, *p*-coumaric, ferulic and rosmarinic acids, at 340 nm for apigenin-6-*C*-glucoside, apigenin-8-*C*-glucoside and apigenin-7-*O*-glucoside, at 350 nm for luteolin-6-*C*-glucoside, luteolin-8-*C*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rhamnoside, at 425 nm for curcumin and at 590 nm for hypericin.

The identified compounds were quantified with their corresponding standards, except for compounds **4** and **8** (quantified as 5-*O*-caffeoylquinic acid), **26** and **33** (as apigenin-7-*O*-glucoside), and **39** and **41** (as curcumin), according to the equations displayed in Table 1.

2.6. Safety assessment - pesticides screening analysis

All the extracts prepared in 2.3 section were treated for the pesticides screening analysis. A SPE C18e (500 mg/3 mL) cartridge was used for cleanup and preoconcentration of the 21 pesticides from the 7 medicinal plants extracts according to the procedure set out by (Silva et al., 2021).

Briefly, SPE cartridge was preconditionated and equilibrated with the elution solvent (n-hexane) followed by methanol and ultrapure water (2 × 2 mL of each). 15 mL extract was then subjected to SPE cartridge cleanup. After the concentration step, cartridges were rinsed with 5 mL of ultrapure water, dried for 10 min and eluted with 2 × 2 mL n-hexane. The elution was collected, dried using a gentle stream of nitrogen and reconstituted in 1 mL of n-hexane before injection in the chromatographic system. The gas chromatography analyses were performed according to Lobato et al. (2021) for organochlorine pesticides and to Fernandes et al. (2018) for organophophorus pesticides.

2.7. Bioassays

2.7.1. Radical scavenging activities (RSA)

The antiradical activity of the extracts was evaluated by several complementary *in vitro* assays, namely 2,2-diphenyl-1-picryl-hydrazyl free radical scavenging (DPPH$^{\bullet}$), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid assay (ABTS$^{\bullet+}$), superoxide anion radical scavenging ($O_2^{\bullet-}$) and nitric oxide radical scavenging ($^{\bullet}$NO), according to stablished procedures (Barroso et al., 2016; Cvetanović et al., 2019; Soares et al., 2021). For DPPH$^{\bullet}$-RSA assay, a calibration curve was prepared with Trolox, and the antioxidant activity was expressed as mg of Trolox equivalents per g of dw of extract (mg TE/g dw) and as IC_{50} values. In ABTS$^{\bullet+}$-RSA, the absorbance was taken at 734 nm, and TE was also used as standard. The obtained results were expressed as mg TE equivalents per g of dw of extract (mg TE/g dw) and as IC_{50} values. For all the assays, triplicate measurements were made for each extract. Concerning $O_2^{\bullet-}$ and $^{\bullet}$NO scavenging activities, results are expressed as IC_{50} values and decoctions were tested in triplicate and the experiments repeated three times.

2.7.2. Enzyme inhibition

AChE and BuChE inhibition assays were performed according to the procedure described by (Soares et al., 2021). Results are expressed as IC_{50} values and decoctions were tested in triplicate and the experiments repeated three times. To estimate K_m and V_{max}, four different concentrations of substrate (ATCI or BTCI) were tested. Slopes of the reaction (OD/min) were transformed in V (M/min) using the molar absorption coefficient of TNB (13.68×10^3 $M^{-1}cm^{-1}$). Competitive, non-competitive and uncompetitive models were fitted to the experimental results and the models with the highest R^2 and Adj R^2 and the lowest sum of the squared error were chosen.

For GSK-3β inhibition assay, 1 mg/mL solutions were prepared in DMSO for each herbal extract. The obtained solutions were mixed by a vortex for 5 min and then sonicated at room temperature. The samples were filtered by 0.45 μm PTFE filters. Then a 1:60 dilution in buffer assay (containing 50 mM 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetra acetic acid (EGTA), and 15 mM magnesium acetate) was obtained for each sample. The obtained samples were tested by performing Kinase Glo assay using white 96-well plates. The following procedure was carried

Table 1
Regression equations, R^2, limits of quantification (LOQ) and limits of detection (LOD) of the standards.

Compound[a]	RT	Calibration curve	R^2	[Range], mg/mL	LOD, mg/mL	LOQ, mg/mL
Trigonelline	5.54	$y = 3.01 \times 10^7x - 5.72 \times 10^4$	0.9996	$1.69 \times 10^{-3} - 3.37 \times 10^{-1}$	1.16×10^{-4}	3.52×10^{-4}
Protocatechuic acid	10.09	$y = 3.25 \times 10^7x + 1.50 \times 10^4$	0.9988	$1.89 \times 10^{-3} - 3.78 \times 10^{-1}$	5.89×10^{-4}	1.79×10^{-3}
Catechin	13.80	$y = 1.60 \times 10^7x - 9.22 \times 10^3$	0.9998	$7.80 \times 10^{-4} - 1.56 \times 10^{-1}$	1.14×10^{-5}	3.46×10^{-5}
5-*O*-Caffeoylquinic acid	17.70	$y = 8.11 \times 10^7x - 4.27 \times 10^4$	0.9998	$1.28 \times 10^{-3} - 7.90 \times 10^{-1}$	1.16×10^{-4}	3.53×10^{-4}
Caffeic acid	18.36	$y = 1.34 \times 10^8x - 9.92 \times 10^4$	0.9997	$1.25 \times 10^{-3} - 2.49 \times 10^{-1}$	6.45×10^{-6}	1.95×10^{-5}
Epicatechin	18.92	$y = 1.42 \times 10^7x - 1.02 \times 10^4$	0.9998	$8.10 \times 10^{-4} - 1.62 \times 10^{-1}$	3.10×10^{-5}	9.40×10^{-5}
***p*-Coumaric acid**	24.37	$y = 1.43 \times 10^8x + 3.01 \times 10^4$	0.9999	$5.85 \times 10^{-4} - 1.17 \times 10^{-1}$	5.22×10^{-6}	1.58×10^{-5}
Epicatechin-3-*O*-gallate	24.91	$y = 3.86 \times 10^7x - 4.40 \times 10^4$	0.9998	$9.25 \times 10^{-4} - 1.85 \times 10^{-1}$	3.48×10^{-5}	1.06×10^{-4}
Ferulic acid	29.63	$y = 1.27 \times 10^8x - 2.00 \times 10^5$	0.9997	$1.25 \times 10^{-3} - 2.49 \times 10^{-1}$	1.77×10^{-5}	5.38×10^{-5}
Luteolin-8-*C*-glucoside	31.41	$y = 6.23 \times 10^7x - 3.43 \times 10^4$	0.9987	$4.17 \times 10^{-4} - 8.33 \times 10^{-2}$	2.55×10^{-5}	7.72×10^{-5}
Apigenin-8-*C*-glucoside	33.74	$y = 4.60 \times 10^7x - 8.32 \times 10^3$	0.9998	$4.00 \times 10^{-4} - 8.00 \times 10^{-2}$	4.56×10^{-5}	1.38×10^{-4}
Luteolin-6-*C*-glucoside	35.56	$y = 6.28 \times 10^7x - 4.13 \times 10^4$	0.9998	$6.30 \times 10^{-4} - 1.26 \times 10^{-1}$	3.23×10^{-5}	9.80×10^{-5}
Apigenin-6-*C*-glucoside	42.04	$y = 6.69 \times 10^7x - 5.55 \times 10^4$	0.9998	$6.85 \times 10^{-4} - 1.37 \times 10^{-1}$	1.80×10^{-5}	5.44×10^{-5}
Rosmarinic acid	42.70	$y = 6.30 \times 10^7x - 2.72 \times 10^4$	0.9999	$1.29 \times 10^{-3} - 1.83 \times 10^{-1}$	3.43×10^{-5}	1.04×10^{-4}
Quercetin-3-*O*-galactoside	42.99	$y = 5.16 \times 10^7x - 1.84 \times 10^5$	0.9983	$9.20 \times 10^{-4} - 2.86 \times 10^{-1}$	2.19×10^{-5}	6.62×10^{-5}
Quercetin-3-*O*-glucoside	43.31	$y = 4.99 \times 10^7x + 1.14 \times 10^4$	0.9999	$6.70 \times 10^{-4} - 1.34 \times 10^{-1}$	2.92×10^{-5}	8.86×10^{-5}
Apigenin-7-*O*-glucoside	46.07	$y = 5.97 \times 10^7x - 4.90 \times 10^4$	0.9998	$7.30 \times 10^{-4} - 1.46 \times 10^{-1}$	1.39×10^{-5}	4.22×10^{-5}
Quercetin-3-*O*-rhamnoside	47.02	$y = 4.65 \times 10^7x + 1.59 \times 10^5$	0.9993	$2.22 \times 10^{-3} - 4.44 \times 10^{-1}$	8.03×10^{-5}	2.43×10^{-4}
Carnosol	61.70	$y = 8.20 \times 10^6x + 2.23 \times 10^4$	0.9998	$1.55 \times 10^{-4} - 1.55 \times 10^{-1}$	9.19×10^{-5}	1.50×10^{-4}
Curcumin	63.96	$y = 3.60 \times 10^8x - 1.43 \times 10^5$	0.9997	$1.50 \times 10^{-4} - 5.00 \times 10^{-2}$	6.36×10^{-6}	1.93×10^{-5}
Carnosic acid	65.50	$y = 6.98 \times 10^6x - 4.79 \times 10^3$	0.9948	$1.30 \times 10^{-3} - 2.60 \times 10^{-1}$	4.77×10^{-4}	1.30×10^{-3}
Hypericin	72.17	$y = 4.40 \times 10^7x + 8.81 \times 10^4$	0.9985	$1.30 \times 10^{-3} - 2.60 \times 10^{-1}$	5.46×10^{-4}	1.30×10^{-3}

[a] Calibration curves corresponding to the compounds identified in *S. baicalensis* and *G. biloba* can be found in Delerue et al. (2021).

out, 10 μL of 1:60 sample solution (final concentration was equal to 4.2 μg/mL) and 10 μL ATP (1 μM) were added to each well followed by 20 μL of assay buffer containing 25 μM substrate and 20 ng of GSK-3β. The final DMSO concentration in the reaction mixture did not exceed 1%. After 30 min of incubation at 37 °C, the enzymatic reaction was stopped with 40 μL of Kinase-Glo reagent. Glow-type luminescence was recorded after 10 min using a multiwells plate reader Victor X3 PerkinElmer. The activity is proportional to the difference of the total and consumed ATP. The inhibitory activities were calculated based on the maximal kinase (average positive) and luciferase (average negative) activities measured in the absence of inhibitor and in the presence of reference compound inhibitor (SB415286, IC_{50} = 70 nM) at a total inhibition concentration (5 μM), respectively (Baki et al., 2007). All decoctions were tested in duplicate, and the experiments were repeated three times.

2.7.3. Cell viability

Neuroblastoma x spinal cord cells (NSC-34) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal calf serum (FCS) and 0.5% penicillin/streptomycin solution as previously described (Maier et al., 2013). Cells were subcultured every 2–3 days. Passages 4–6 were used ($n = 5$).

Blood-Brain Barrier hCMEC/D3 cells were maintained in EGM-2 culture medium adding 5% (v/v) FBS, 1% (v/v) penicillin–streptomycin, hydrocortisone (0.5 μg/mL), ascorbic acid (5 μg/mL), 1% (v/v) lipid concentrate, 1% (v/v) HEPES and bFGF (1 ng/mL - added directly into the flasks when cells were cultured), as described as (Sánchez-Dengra et al., 2021). Passages 50–54 were employed ($n = 5$).

Cells were grown according to the methodology described by (Pinto et al., 2020). Briefly, both cell lines were maintained in an incubator at 37 °C, 5% CO_2 and 90% humidity in 75 cm^2 flasks at a cell density of 2.5 × 10^4 cells/cm^2. After, cells were seeded and incubated during 24 h with fresh medium in the absence or presence of extracts (250, 500 and 1000 μg/mL) dissolved in cell culture medium. Following the extracts removal from each well, cells were washed with HBSS. The number of viable cells was determined by adding MTT reagent and incubating for 3 h at 37 °C. DMSO was used to solubilize the crystals. The positive control used was DMEM and the negative control was 1% (w/v) Triton X-100. The absorbance was read at 590 nm with background subtraction at 630 nm. Results were expressed as percentages of cell viability.

2.8. Statistical analysis

All the experiments were repeated at least three times. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Tuckey's test and p values less than 0.05 were considered to be statistically significant.

Pearson correlation analyses were carried out to determine the possible relations between the contents of phenolic compounds and the observed bioactivities.

All statistical analyses, as well as the calculation of IC_{50} values and the kinetic parameters V_{max}, K_m and K_i were performed with the software GraphPad Prism 8.0.1.

3. Results and discussion

3.1. Chemical composition

3.1.1. Extracts bioactive composition

The values of TPC for the analysed samples varied between 29.00 ± 3.49 mg GAE/g extract dw (*T. foenum-graecum*) and 327.31 ± 26.88 mg GAE/g extract dw (*R. officinalis*) as displayed in Table 2. Values reported in literature for these species are quite variable. For instance, Li et al. (2013) have determined the TPC of 223 medicinal plant infusions, including *C. longa* (4.19 mg GAE/g extract), *G. biloba* (2.50 mg GAE/g extract) and *S. baicalensis* (46.31 mg GAE/g extract), reporting contents below to the ones achieved for our samples. However, in other studies, higher TPC values were reported for *S. baicalensis* (160.29 mg GAE/g extract) (Liau et al., 2019) and *C. longa* (39.38 mg GAE/g extract) (Alafiatayo Akinola et al., 2014). Concerning *H. perforatum* aqueous extracts, the amount of phenolics varies between c. a. 20 mg GAE/g extract (Altun et al., 2013) and 271.91 mg GAE/g extract (Öztürk et al., 2009) and for *R. officinalis* aqueous extracts and decoctions, values between 15.67 mg GAE/g extract (Sharma et al., 2020) and 127.87 mg GAE/g extract (Megateli and Krea, 2018) were also previously described. Other species of *Trigonella* genus displayed a value similar to the one displayed in Table 2 (18.59 mg GAE/g aqueous extract) (Aylanc et al., 2020), while for *L. angustifolia* decoctions the phenolics contents ranged from 50.6 mg GAE/g extract (Spiridon et al., 2011) to 124.8 mg GAE/g extract (Détár et al., 2020). Some of the factors that influence the different contents of phenolics obtained by the different studies are related with the cultivars, the extraction type and the maturation stage of the plant species used. Indeed, for *G. biloba*, it was found that leaf maturation (green *vs* yellow) and plant sex (male *vs* female) have influence on the amount of phenolics, being these factors responsible for the wide range of TPC values reported in literature (Kobus-Cisowska et al., 2020; Koczka et al., 2015).

Regarding the HPLC profile of the analysed decoctions (Fig. 1, Table 3), in a previous study, we have already shown that *S. baicalensis* root decoction was characterized by the presence of four flavones – baicalein-7-*O*-glucuronide (**30**), baicalein (**36**), wogonin-7-*O*-

Table 2
Total phenolic content and antiradical activity of plant decoctions.

Sample	TPC (mg GAE/g extract dw)	DPPH$^{\bullet}$ scavenging activity (mg GAE/g extract dw)	DPPH$^{\bullet}$ scavenging activity (IC_{50}, μg/mL)	ABTS$^{\bullet+}$ scavenging activity (mg TE/g extract dw)	ABTS$^{\bullet+}$ scavenging activity (IC_{50}, μg/mL)	$O_2^{\bullet-}$ scavenging activity (IC_{50}, μg/mL)	$^{\bullet}$NO scavenging activity (IC_{50}, μg/mL)
S. baicalensis	285.00 ± 14.40^{a} (Delerue et al., 2021)	29.96 ± 4.48^{a}	59.14^{a}	ND^{a}	[a]	116.81^{a}	106.37^{a}
G. biloba	123.40 ± 5.00^{b} (Delerue et al., 2021)	47.79 ± 2.27^{b}	137.67^{b}	58.15 ± 4.08^{b}	102.46^{a}	114.93^{a}	240.53^{a}
H. perforatum	207.89 ± 18.94^{c}	100.01 ± 7.43^{c}	66.05^{a}	350.45 ± 10.55^{c}	19.82^{b}	59.34^{a}	151.25^{a}
C. longa	43.23 ± 4.34^{d}	22.54 ± 3.34^{a}	[a]	30.38 ± 1.96^{d}	[a]	412.75^{b}	>1000^{b}
L. angustifolia	163.00 ± 5.66^{e}	47.25 ± 1.67^{b}	59.02^{a}	182.96 ± 17.00^{e}	32.00^{c}	76.94^{a}	717.29^{c}
T. foenum-graecum	29.00 ± 3.49^{d}	61.36 ± 1.32^{b}	106.82^{c}	23.68 ± 3.29^{d}	[a]	490.38^{b}	468.52^{d}
R. officinalis	327.31 ± 26.88^{f}	91.57 ± 9.16^{c}	71.84^{a}	282.94 ± 12.30^{e}	22.00^{b}	53.65^{a}	121.33^{a}

ND – Not detected (below LOD of the equation).
Different superscript letters correspond to statistically significant differences ($p < 0.05$).
[a] IC_{50} values above the maximum concentration tested (DPPH$^{\bullet}$: *C. longa* – IC_{50} > 120.45 μg/mL; ABTS$^{\bullet+}$ - *S. baicalensis* – IC_{50} > 327.27 μg/mL; *T. foenum-graecum* - IC_{50} > 188.00 μg/mL; *C. longa* - IC_{50} > 106.00 μg/mL).

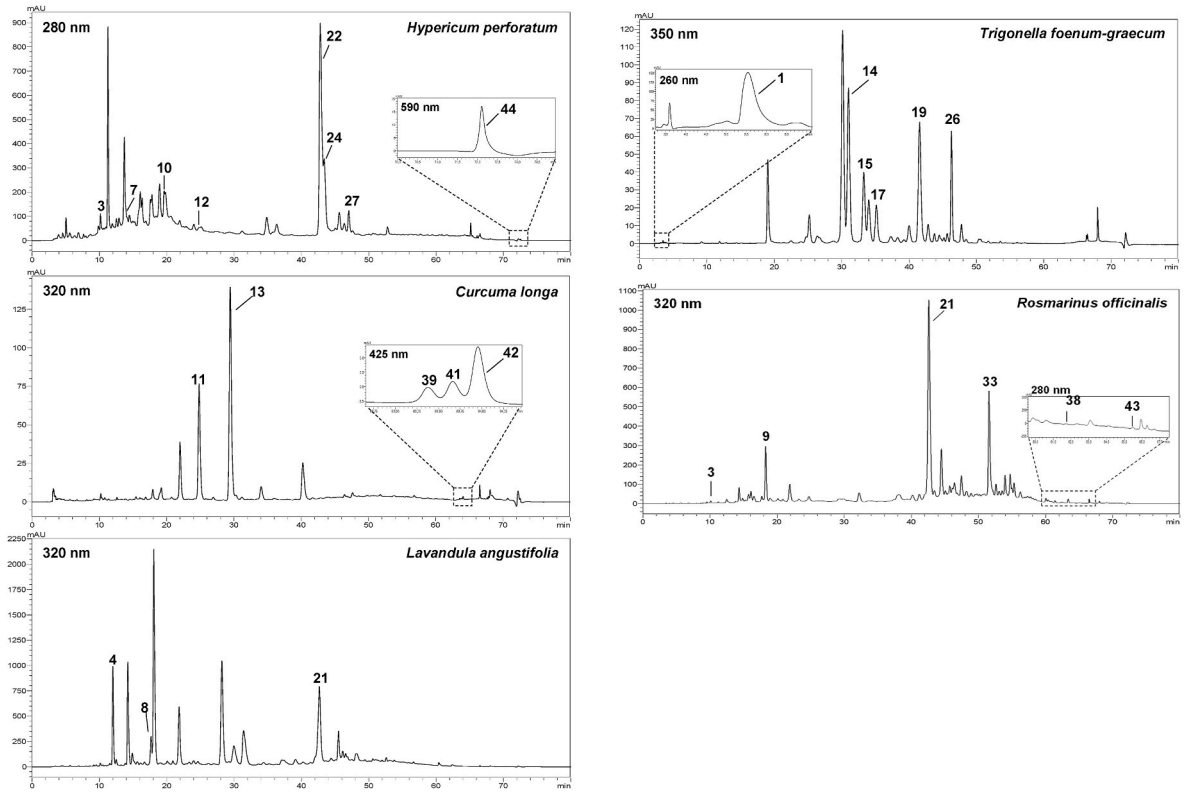

Fig. 1. HPLC-DAD chromatograms of *H. perforatum*, *C. longa*, *L. angustifolia*, *T. foenum-graecum* and *R. officinalis*. Chromatograms of *G. biloba* and *S. baicalensis* can be seen in Delerue et al. (2021). Peaks assignment according to Table 3.

glucuronide (**35**) and wogonin (**37**) – being baicalin the major compound (184.85 ± 6.64 mg/g extract dw) (Delerue et al., 2021). Sixteen compounds were also identified in *G. biloba* decoctions, comprising hydroxybenzoic acids (**2** and **3**), derivatives of catechin (**6** and **12**), of quercetin (**16**, **20**, **23**, **25**), of kaempferol (**18**, **28** and **29**) and of isorhamnetin (**31**), as well as acylated kaempferols (**32** and **34**) and trace amounts of amentoflavone (**40**). Protocatechuic acid (**3**; 2.27 ± <0.01 mg/g extract dw), quercetin-3-*O*-rutinoside (**25**; 1.47 ± 0.01 mg/g extract dw) and kaempferol-3-*O*-rutinoside (**29**; 1.48 ± <0.01 mg/g extract dw) were the dominant compounds in *G. biloba* decoction (Delerue et al., 2021).

The HPLC-DAD analysis of the other five decoctions showed very distinct chemical compositions (Fig. 1, Table 3). Besides *p*-coumaric (**11**) and ferulic (**13**) acids, three curcuminoids were identified in *C. longa* decoction, namely, bisdemethoxycurcumin (**39**), demethoxycurcumin (**41**) and curcumin (**42**) (Table 3). *T. foenum-graecum* contained an alkaloid, trigonelline (**1**), as its major compound (16.41 ± 0.24 mg/g extract dw), followed by four *C*-glycosyl flavones (luteolin-8-*C*-glucoside (**14**), apigenin-8-*C*-glucoside (**15**), luteolin-6-*C*-glucoside (**17**) and apigenin-6-*C*-glucoside (**19**)) and one apigenin derivative (**26**) (Table 3). Rosmarinic acid (**21**) was present in both species from Lamiaceae family, in higher amounts in *R. officinalis* than in *L. angustifolia* (21.06 ± 1.97 *vs* 13.28 ± 1.68 mg/g extract dw). Other two hydroxycinnamic acids (**4** and **8**) were also identified in *L. angustifolia*, while protocatechuic acid (**3**), caffeic acid (**9**), an apigenin derivative (**33**), carnosol (**38**) and carnosic acid (**43**) were also found in *R. officinalis* decoction (Table 3). *H. perforatum* contained small amounts of the naphthodianthrone hypericin (**44**; 0.18 ± 0.08 mg/g extract dw), and the major constituents were quercetin-3-*O*-galactoside (**22**; 27.00 ± 7.74 mg/g extract dw) and quercetin-3-*O*-glucoside (**24**; 11.74 ± 1.99 mg/g extract dw), followed by catechin derivatives (**7**, **10** and **12**), quercetin-3-*O*-rhamnoside (**27**) and protocatechuic acid (**3**) (Table 3).

The obtained results are similar to the findings published by other authors. The presence of the three identified curcuminoids, in which curcumin is in higher amount than the other two, was also verified in the extracts obtained by (Chen and Chang, 2015; Lee and Choung, 2011; Long et al., 2014). Trigonelline is an important chemotaxonomic marker of *Trigonella* species (Mohammadi et al., 2020; Shailajan et al., 2011; Zameer et al., 2018) and was found to be the major compound in the sample analysed herein. Moreover, a series of *C*-glycosyl flavones was already reported (Rayyan et al., 2010). Concerning *R. officinalis* and *L. angustifolia*, it is also worth to mention that rosmarinic acid is also the most important chemotaxonomic marker of species from Lamiaceae family (Janicsák et al., 1999) and, for *R. officinalis*, the phenolic diterpenes carnosic acid and carnosol are also considered important (Mena et al., 2016; Troncoso et al., 2005; Yeddes et al., 2019; Yilmaz et al., 2018; Zhang et al., 2012). Other phenolic acids and flavonoids previously reported for these two species were not found in the current samples (Cesur Turgut et al., 2017; Mena et al., 2016). Although most of the studies performed with *H. perforatum* focus on hypericin, pseudohypericin and hyperforin, other compounds are also considered as characteristic from this species and were also detected in the sample analysed, namely, quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside (Doležal et al., 2019; Raclariu et al., 2017).

3.1.2. Pesticides screening analysis

The quality of herbal medicines in terms of contaminants can have an impact on their safety and efficacy. However, it is a difficult task to guarantee total safety as it involves different areas, such as environmental and agricultural practices (World Health Organization, 2007). The analyses performed in this study demonstrated that among the 7 analysed samples, no presence of organochlorine and organophophorus pesticides was found (Fig. 2). Although the presence of organochlorine and organophosphorus pesticides in medicinal plants is commonly reported in the literature (Fu et al., 2019; Luo et al., 2021; Xu et al., 2011), in this work it has not been verified. It should be noted that the pesticide

Table 3
Quantification of the identified compounds in plant decoctions (mg/g of extract dw).

			S. baicalensis (Delerue et al., 2021)	*G. biloba* (Delerue et al., 2021)	*H. perforatum*	*C. longa*	*L. angustifolia*	*T. foenum-graecum*	*R. officinalis*
1	**Trigonelline**	5.54	–	–	–	–	–	16.41 ± 0.24	–
2	**Gallic acid**	6.75	–	0.47±<0.01	–	–	–	–	–
3	**Protocatechuic acid**	10.09	–	2.27±<0.01	0.93 ± 0.07	–	–	–	1.43 ± 0.04
4	**Hydroxycinnamic acid 1**	11.97	–	–	–	–	13.31 ± 0.02	–	–
5	**Procyanidin B1**	12.58	–	0.90 ± 0.02	–	–	–	–	–
6	**Catechin derivative**	13.46	–	0.78 ± 0.02	–	–	–	–	–
7	**Catechin**	13.80	–	–	2.19 ± 0.39	–	–	–	–
8	**Hydroxycinnamic acid 2**	17.62	–	–	–	–	5.48 ± 0.01	–	–
9	**Caffeic acid**	18.36	–	–	–	–	–	–	2.12 ± 0.11
10	**Epicatechin**	18.92	–	–	5.26 ± 0.70	–	–	–	–
11	***p*-Coumaric acid**	24.37	–	–	–	0.26±<0.01	–	–	–
12	**Epicatechin-3-*O*-gallate**	24.91	–	0.46 ± 0.02	0.44 ± 0.03	–	–	–	–
13	**Ferulic acid**	29.63	–	–	–	0.67±<0.01	–	–	–
14	**Luteolin-8-*C*-glucoside**	31.41	–	–	–	–	–	2.60 ± 0.33	–
15	**Apigenin-8-*C*-glucoside**	33.74	–	–	–	–	–	1.51 ± 0.17	–
16	**Quercetin/isorhamnetin derivative 1**	35.50	–	0.95±<0.01	–	–	–	–	–
17	**Luteolin-6-*C*-glucoside**	35.56	–	–	–	–	–	0.80 ± 0.20	–
18	**Kaempferol derivative 1**	40.75	–	0.72±<0.01	–	–	–	–	–
19	**Apigenin-6-*C*-glucoside**	42.04	–	–	–	–	–	2.15 ± 0.38	–
20	**Quercetin/isorhamnetin derivative 2**	42.21	–	0.44±<0.01	–	–	–	–	–
21	**Rosmarinic acid**	42.70	–	–	–	–	13.28 ± 1.68	–	21.06 ± 1.97
22	**Quercetin-3-*O*-galactoside**	42.99	–	–	27.00 ± 7.74	–	–	–	–
23	**Quercetin/isorhamnetin derivative 3**	43.26	–	0.36±<0.01	–	–	–	–	–
24	**Quercetin-3-*O*-glucoside**	43.31	–	–	11.74 ± 1.99	–	–	–	–
25	**Quercetin-3-*O*-rutinoside**	43.86	–	1.47 ± 0.01	–	–	–	–	–
26	**Apigenin derivative 1**	46.09	–	–	–	–	–	1.12 ± 0.12	–
27	**Quercetin-3-*O*-rhamnoside**	47.02	–	–	1.33 ± 0.11	–	–	–	–
28	**Kaempferol-3-*O*-glucoside**	47.36	–	0.56±<0.01	–	–	–	–	–
29	**Kaempferol-3-*O*-rutinoside**	48.06	–	1.48±<0.01	–	–	–	–	–
30	**Baicalein 7-*O*-glucuronide**	49.76	184.85 ± 6.64	–	–	–	–	–	–
31	**Isorhamnetin-3-*O*-rutinoside**	49.84	–	1.36 ± 0.01	–	–	–	–	–
32	**Acylated kaempferol 1**	50.55	–	0.83±<0.01	–	–	–	–	–
33	**Apigenin derivative 2**	51.60	–		–	–	–	–	9.74 ± 0.67
34	**Acylated kaempferol 2**	53.72	–	0.73±<0.01	–	–	–	–	–
35	**Wogonin-7-*O*-glucuronide**	54.00	24.26 ± 0.96	–	–	–	–	–	–
36	**Baicalein**	58.13	16.82 ± 0.07	–	–	–	–	–	–
37	**Wogonin**	60.87	4.04±<0.01	–	–	–	–	–	–
38	**Carnosol**	61.70	–	–	–	–	–	–	0.07 ± 0.02
39	**Bisdemethoxycurcumin**	63.38	–	–	–	0.01±<0.01	–	–	–
40	**Amentoflavone**	63.58	–	n.q.	–	–	–	–	–
41	**Demethoxycurcumin**	63.66	–	–	–	0.02±<0.01	–	–	–
42	**Curcumin**	63.96	–	–	–	0.02±<0.01	–	–	–
43	**Carnosic acid**	65.50	–	–	–	–	–	–	n.q.
44	**Hypericin**	72.17	–	–	0.18 ± 0.08	–	–	–	–
	Total (mg/g d.w.)		229.97	13.78	49.07	0.98	32.07	24.59	34.42

n.q. – Not quantified (<LOQ).

screening work was carried out on the liquid extracts of the plants. However, most of the works described in the literature were carried out on plants. Recently, Florea et al. (2020) reported the degree of pesticides transfer in three types of preparations (infusion, decoction and maceration), and demonstrated that some pesticides showed a significant decrease in transfer rate from plant to infusion. Therefore, the evaluation of pesticides in liquid extracts is still a topic that should be further explored, as they are the ones chosen for possible nutraceutical applications. We can say that looking at the 21 pesticides under study, the 7 decoctions are safe for their possible nutraceutical application.

3.2. Antioxidant activity

Four different methodologies were used to assess the antioxidant potential of the seven decoctions, namely, DPPH$^{\bullet}$-RSA, ABTS$^{\bullet+}$-RSA, $O_2^{\bullet-}$ and $^{\bullet}$NO scavenging activities (Table 2). From all samples, *H. perforatum* and *R. officinalis* decoctions were the most potent ones for DPPH$^{\bullet}$ (100.01 ± 7.43 and 91.57 ± 9.16 mg GAE/g extract dw, respectively) and ABTS$^{\bullet+}$ (350.45 ± 10.55 and 282.94 ± 12.30 mg TE/g extract dw, respectively) assays, while *C. longa* was the weakest one (22.54 ± 3.34 and 30.38 ± 1.96 TE/g extract dw). DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ RSA were well correlated ($r = 0.867$), however, a poor correlation was found between these two activities and TPC ($r = 0.410$ for DPPH$^{\bullet}$ and r = 0.503 for ABTS$^{\bullet+}$). In terms of IC_{50} values, results also demonstrated that the most active extracts against these two radicals were those of *H. perforatum*, *L. angustifolia* and *R. officinalis*.

The results obtained for these two antioxidant activities are in agreement with the strong activity already reported for *R. officinalis* (Megateli and Krea, 2018; Sharma et al., 2020; Skaperda et al., 2021), *H. perforatum* (Skaperda et al., 2021), *L. angustifolia* (Spiridon et al., 2011), and the weak activity already observed for *C. longa* (Li et al., 2013). The strong antioxidant activity of *R. officinalis* is attributed to some of its chemical constituents, which include carnosol, carnosic acid, rosmarinic acid, and caffeic acid (Rašković et al., 2014), and the antioxidant activity of the *Lavandula* extracts is mainly due to the presence of rosmarinic acid (Blažeković et al., 2010). According to Orčić et al.

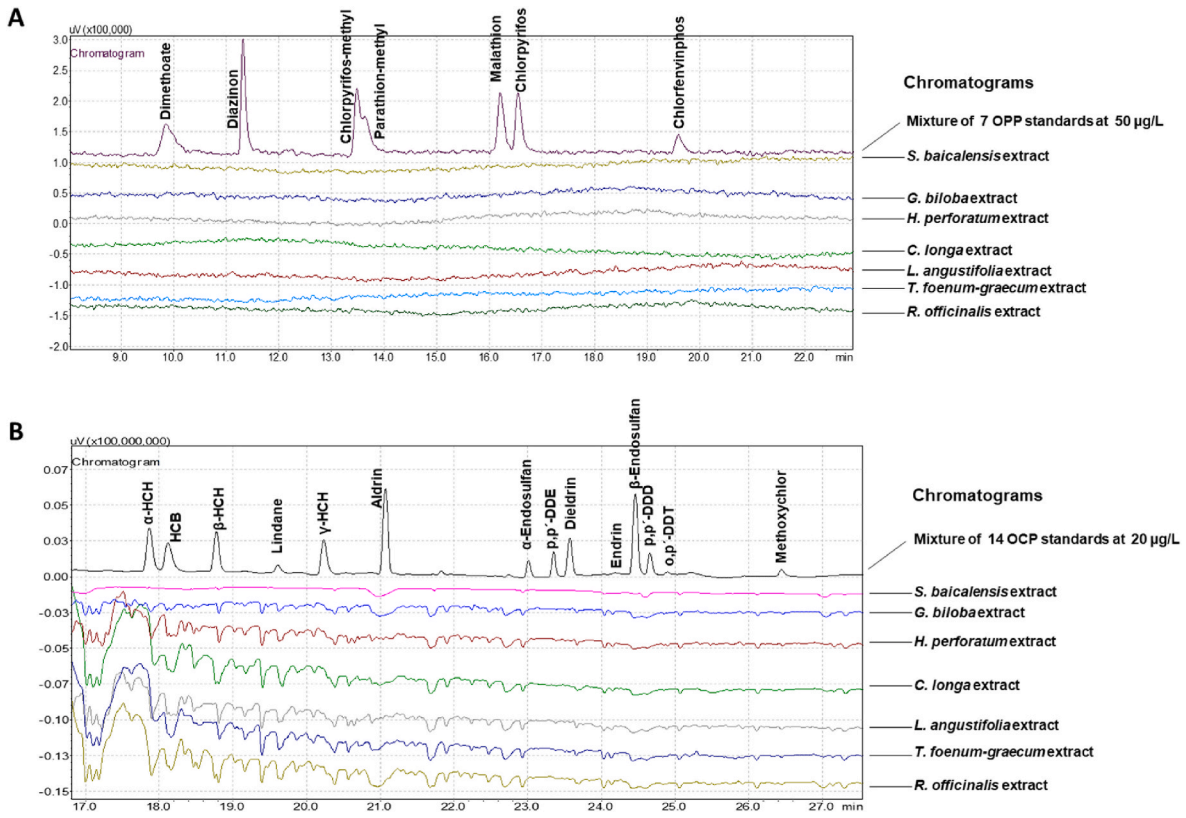

Fig. 2. GC-FPD (A) and GC-ECD (B) chromatograms of pesticides standards (7 OPP and 14 OCP) and analysed extracts.

(2011), the antioxidant activity of *H. perforatum* can be attributed to flavonoids and phenolic acids, while phloroglucinols and naphthodianthrones have no significant activity.

S. baicalensis decoction showed better DPPH$^{\bullet}$ than ABTS$^{\bullet+}$ RSA, which may reflect that it exerts antioxidant activity principally by single-electron transfer instead of hydrogen-atom transfer. Similar results were found by Liau et al. (2019). On the other hand, Li et al. (2013) observed that *S. baicalensis* was more active than *C. longa* and *G. biloba* against ABTS$^{\bullet+}$, which was not verified in the current study.

As far as we know, there are few previous studies on the capacity of these samples to scavenge $^{\bullet}$NO and $O_2^{\bullet-}$ radicals. These radicals have important roles as vascular signalling molecules, however, when overproduced, they react generating $ONOO^-$. An imbalance between protective $^{\bullet}$NO and highly cytotoxic $ONOO^-$ may be crucial in the initial step of the development of several vascular and neuronal diseases, including AD (Malinski, 2007).

As reported in Table 2, the most active decoctions against $O_2^{\bullet-}$ and $^{\bullet}$NO were those prepared with *R. officinalis* (IC_{50} = 53.65 µg/mL and 121.33 µg/mL, respectively) and *H. perforatum* (IC_{50} = 59.34 and 151.25 µg/mL, respectively). Moreover, no statistical differences were found between both decoctions and those of *S. baicalensis* and *G. biloba* (Table 2). Both $O_2^{\bullet-}$ and $^{\bullet}$NO scavenging activities were positively correlated, although with a *r* far from 1 ($r = 0.600$) and were negatively correlated with TPC ($r = -0.813$ and $r = -0.631$, respectively). Moreover, a poor correlation was found, in general, between the four antioxidant activities ($r = -0.403$ between $O_2^{\bullet-}$ and DPPH$^{\bullet}$ assay; $r = -0.618$ between $O_2^{\bullet-}$ and ABTS$^{\bullet+}$ assay; $r = -0.577$ between $^{\bullet}$NO and DPPH$^{\bullet}$ assay; and $r = -0.350$ between $^{\bullet}$NO and ABTS$^{\bullet+}$ assay).

An aqueous extract of *H. perforatum* displayed 69.32% inhibition against $^{\bullet}$NO and no activity against $O_2^{\bullet-}$ at 2000 µg/mL (Altun et al., 2013), revealing weaker activity than those displayed in Table 2.

The ethanolic extract from *S. scutellaria* displayed an IC_{50} = 40 µg/mL against $^{\bullet}$NO (Zhang et al., 2011), while an aqueous extract presented an IC_{50} = 81.78 µg/mL against $O_2^{\bullet-}$ (Liau et al., 2019). Huang et al. (2006) tested several flavones of *S. baicalensis* on a lipopolysaccharide (LPS)-induced inflammation model of RAW 264.7 macrophages and measured their capacity to scavenge $^{\bullet}$NO. It was found that wogonin (IC_{50} = 45.3 µM) was more active than baicalein (IC_{50} = 66.4 µM). EGb 761, a standardized extract of *G. biloba*, was shown to provoke 40% inhibition of $O_2^{\bullet-}$ at concentrations equal or above 10 mg/L (Liu et al., 2006).

Taking all the results together, *T. foenum graecum* and *C. longa* were the weakest antioxidant samples, while *R. officinalis* and *H. perforatum* are among the most active ones, showing their potential to counteract ROS and RNS.

3.3. CNS enzymes activity

According to the cholinergic hypothesis, the main cause of AD is the reduction in acetylcholine synthesis. Therefore, one of the potential therapeutic strategies is to increase the cholinergic levels in the brain by inhibiting the biological activity of cholinesterases. However, ChE inhibitors are not able to completely stop the progression of AD, and research should focus on the development of multi-target drugs able to counteract the decreased levels of ACh, protein misfolding and associated Aβ aggregation, hyperphosphorylation of tau protein, and oxidative stress (Butterfield and Boyd-Kimball, 2020; Zagórska and Jaromin, 2020). Therefore, the extracts were not only screened for their inhibitory activity against ChEs, but also against GSK-3β.

Concerning ChEs inhibition, it was only possible to achieve 50% of AChE inhibition for decoctions prepared with *S. baicalensis* (IC_{50} = 796.55 µg/mL) (Delerue et al., 2021), *G. biloba* (IC_{50} = 2076.07 µg/mL) (Delerue et al., 2021), and *R. officinalis* (IC_{50} = 1068.44 µg/mL), while only *L. angustifolia* and *R. officinalis* displayed considerable BuChE inhibition (IC_{50} = 2145.94 and 858.75 µg/mL, respectively) (Fig. 3A–E). These results demonstrated that the decoctions prepared from these four species are moderate to weak cholinesterases inhibitors, while those prepared from *C. longa* and *T. foenum graecum* (Fig. 3B and **D**) are not

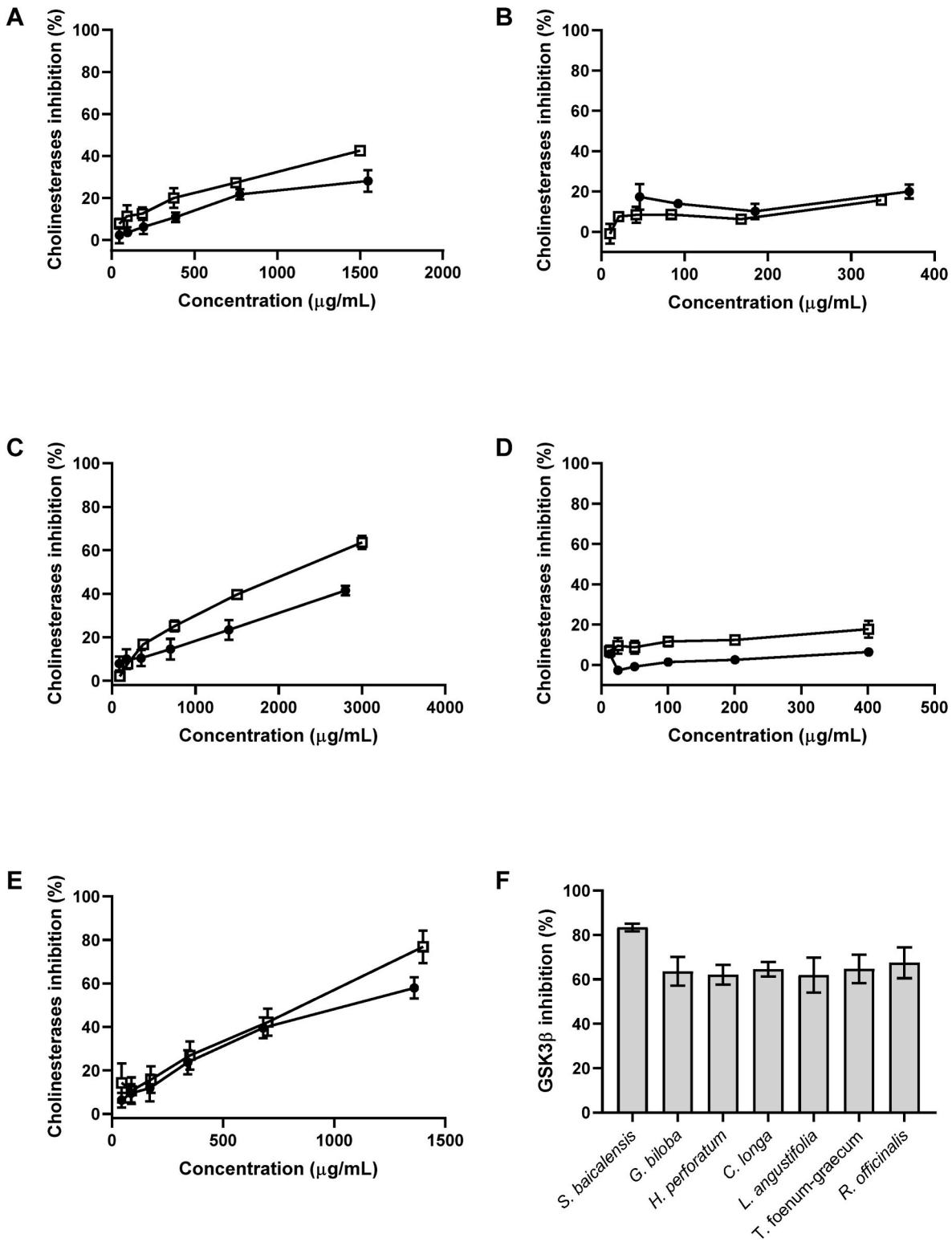

Fig. 3. Dose-response curves of AChE (●, A-E), BuChE (□, A-E) inhibition and GSK-3β inhibition (F) at 4.2 μg/mL. A – *H. perforatum*; B – *C. longa*; C – *L. angustifolia*; D – *T. foenum-graecum*; E – *R. officinalis*. Results are expressed by mean ± SEM.

active against these enzymes. Altun et al. (2013) tested concentrations up to 200 μg/mL of an aqueous extract of *H. perforatum*, observing no activity against AChE and weak activity (<30%) against BuChE. The low activity observed by these authors is consistent with the data obtained in the current study (Fig. 3A). Moderate to low activities were also reported previously for some of these species. A decoction prepared from *L. angustifolia* displayed no inhibition (<5%) even at 5 mg/mL (Ferreira et al., 2006). A hydro-alcoholic extract of *T. foenum graecum* displayed better activity (IC_{50} = 140.26 μg/ml against AChE) than our aqueous extract (SatheeshKumar et al., 2010), but no activity was observed for the aqueous extract of other species, *Trigonella spruneriana* (Aylanc et al., 2020). On the other hand, Sharma et al. (2020) obtained a stronger activity of *R. officinalis* decoction against AChE than that reported herein. Lastly, Kalaycıoğlu et al. (2017) tested three curcuminoids

isolated from *C. longa* and observed an order of potency against AChE and BuChE of bisdemethoxycurcumin > demethoxycurcumin > curcumin, with demethoxycurcumin and curcumin being not active against BuChE. These results may indicate that these compounds alone can display some degree of inhibition but not in mixture, as in the case of decoctions.

Since *R. officinalis* decoction was the only one displaying inhibitory potential over 50% for both cholinesterases, the type of enzyme inhibition was further studied for this extract (Fig. 4 A and B). Different models (uncompetitive, competitive, non-competitive) were fitted to the experimental results and, for both enzymes, non-competitive inhibition model resulted in the highest R^2 (0.8530 for AChE and 0.9275 for BuChE) and $AdjR^2$ (0.8412 for AChE and 0.9214 for BuChE) values and the lowest sum of the squared errors (1.11×10^{-10} for AChE and 2.15×10^{-11} for BuChE). The parameters V_{max}, K_m, and K_i were determined with this model and were found to be 2.22×10^{-5} M/min, 15.28×10^{-2} mM and 501.60 μg/mL for AChE inhibition, respectively, while for BuChE inhibition values of $V_{max} = 1.62 \times 10^{-5}$ M/min, $K_m = 18.98 \times 10^{-2}$ mM and $K_i = 747.50$ μg/mL were obtained.

On the other hand, at the tested concentration (4.2 μg/mL), all extracts displayed more than 60% of GSK-3β inhibition, being *S. baicalensis* the most potent extract (83.42 ± 2.99%) (Fig. 3F). However, no statistical differences were found between the results. To the best of our knowledge, this is the first study on the GSK-3β inhibition of these plant species. Only a study performed with an hydromethanolic extract from *L. officinalis* has been published, reporting an IC_{50} value of 40.35 μg/mL (Gürbüz et al., 2019). Other studies performed with some compounds present in the tested decoctions resulted in GSK-3β inhibition. For instance, an IC_{50} value of 17.95 μM was obtained for curcumin (di Martino et al., 2016), 135.35 μM for rosmarinic acid (Paudel et al., 2018) and 10.28 μM for quercetin-3-*O*-rutinoside (Johnson et al., 2011). Jung et al. (2017) demonstrated that 34 flavonoids, including kaempferol-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, baicalein and baicalein-7-*O*-glucuronide displayed GSK-3β inhibition. Moreover, it was also shown that the increased activity of GSK-3β in AD mice was reversed by baicalein (Gu et al., 2016).

3.4. Cell viability

Fig. 5 presents the cell viability results of non-tumorigenic D3 human brain endothelial cells (hCMEC/D3) and NSC-34 motor neurons after exposure to the seven decoctions under study. hCMEC/D3 represents a stable, easily grown blood brain barrier (BBB) model cell line and NSC-34 is a hybrid cell line produced by fusion of neuroblastoma with mouse motoneuron-enriched primary spinal cord cells.

Except for *G. biloba* (IC_{50} > 1000 μg/mL) and *L. angustifolia* (IC_{50} > 1000 μg/mL), which were not cytotoxic at the tested concentrations, all the other samples induced a concentration-dependent decrease in cell viability in both cell lines. Concerning NSC-34, *G. biloba*, *H. perforatum*, *C. longa* and *L. angustifolia* were the less cytotoxic (IC_{50} > 1000 μg/mL), followed by *S. baicalensis* (IC_{50} = 722.11 μg/mL) and *R. officinalis* (IC_{50} = 634.66 μg/mL) and the most toxic one was *T. foenum graecum* (IC_{50} < 250 μg/mL). hCMEC/D3 cells viability was moderately affected by *S. baicalensis* (IC_{50} = 633.52 μg/mL) and *Hypericum perforatum* (IC_{50} = 731.87 μg/mL) while *T. foenum graecum*, *R. officinalis* and *C. longa* induced a marked decreased in cells viability (IC_{50} < 250 μg/mL).

To the best of our knowledge, there are no previous studies about the

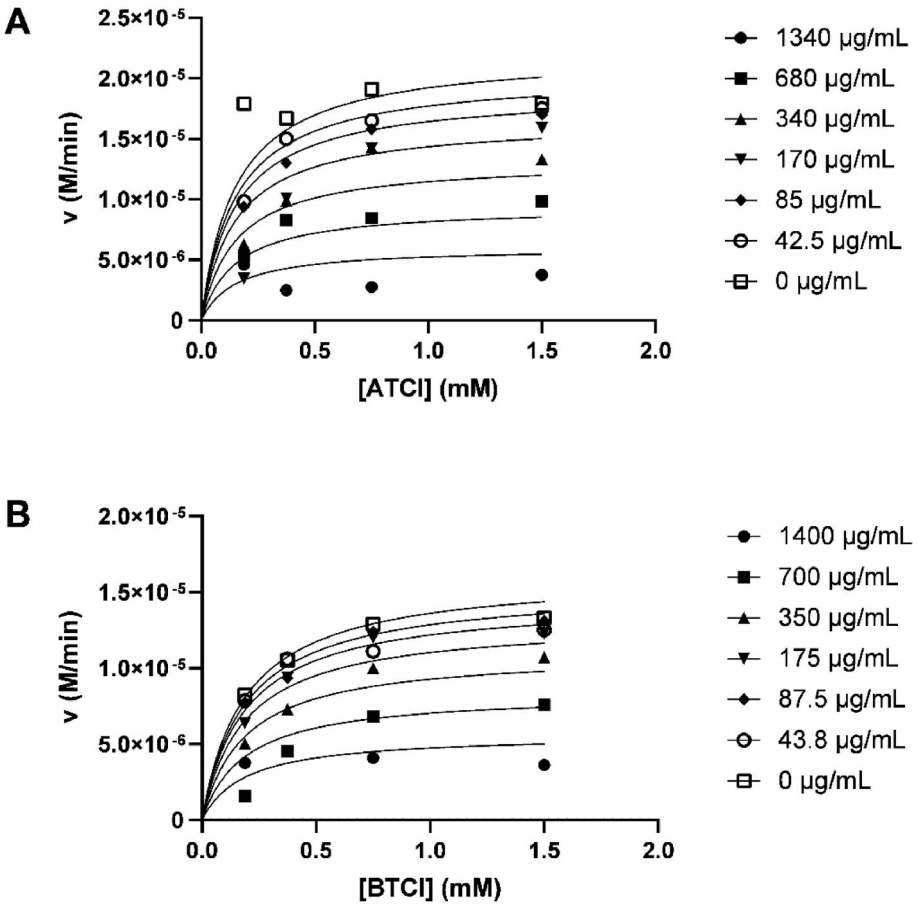

Fig. 4. Velocity *versus* substrate concentration plots for *R. officinalis* decoction. A non-competitive model was fitted to the experimental results obtained for AChE (A) and BuChE (B) inhibition.

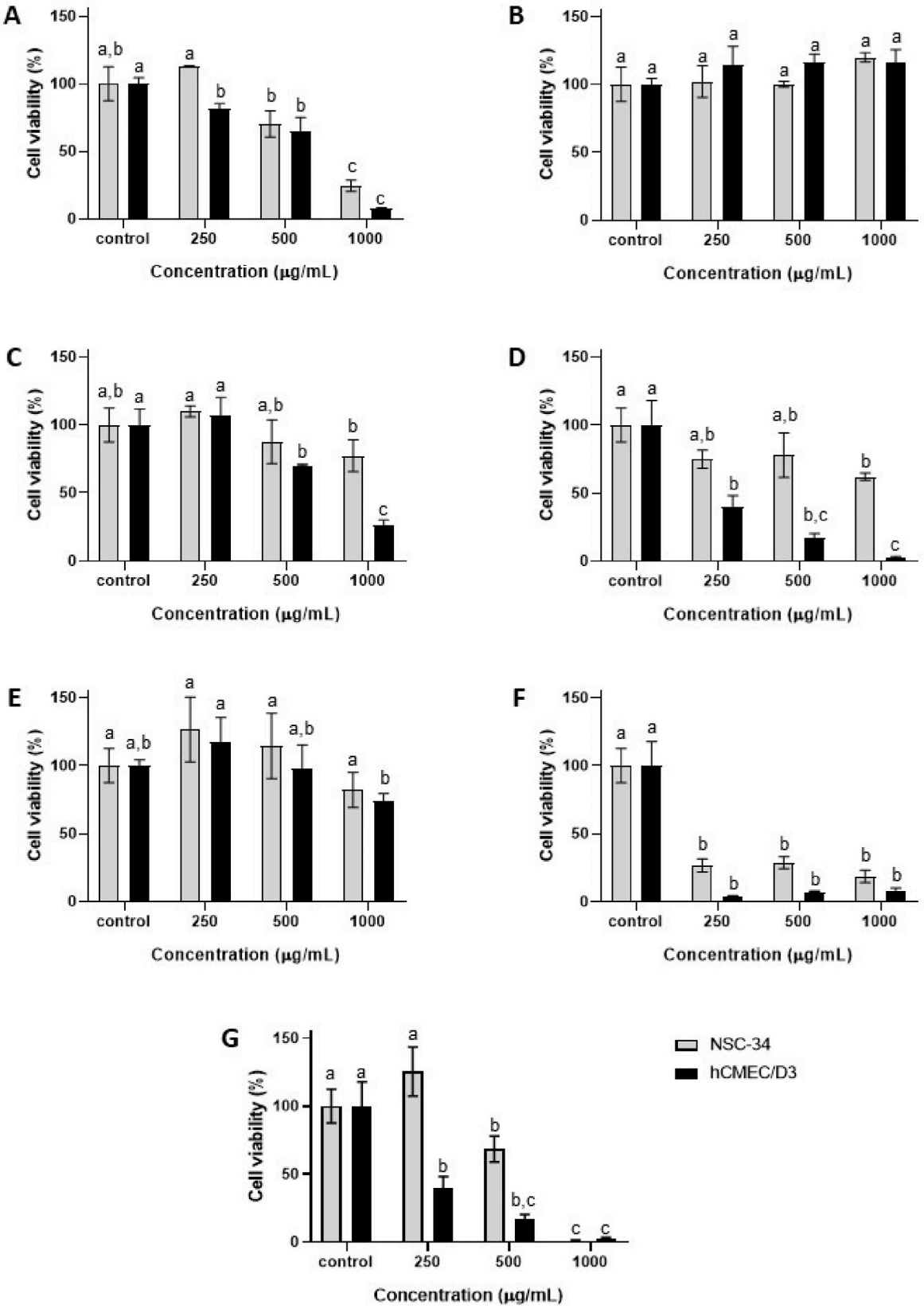

Fig. 5. The effect on cell viability (%) of the decoctions after 24h of incubation and evaluated by MTT assay. A – *S. baicalensis*; B – *G. biloba*; C – *H. perforatum*; D – *C. longa*; E – *L. angustifolia*; F – *T. foenum-graecum*; G – *R. officinalis*. Values are expressed as mean ± SD. For each cell line, different letters correspond to statistically significant differences ($p < 0.05$).

effect of these decoctions on the viability of these cell lines.

4. Conclusions

This study demonstrated the importance of plant extracts as multi-target agents, showing the benefits of seven different medicinal plants against different Alzheimer's hallmarks, including oxidative and nitrosative stresses and cholinesterases and GSK-3β inhibition. Except for the least active species (*C. longa* and *T. foenum-graecum*), these results can contribute to future studies of nanoencapsulation of these extracts to protect them for metabolization during digestion and be delivered to the targets of interest through the BBB.

Declaration of interest and authorship conformation form

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

CRediT authorship contribution statement

Filipe Fernandes: Investigation, Formal analysis, Writing, Writing – review & editing. **Fátima Barroso:** Investigation, Formal analysis, Writing, Writing – review & editing. **Angela De Simone:** Formal analysis, Writing, Writing – review & editing. **Eliška Emriková:** Investigation, Formal analysis, Writing, Writing – review & editing. **Mónica Teixeira:** Resources, Writing, Writing – review & editing. **José Paulo Pereira:** Resources, Writing, Writing – review & editing. **Jakub Chlebek:** Formal analysis, Writing, Writing – review & editing. **Virgínia Cruz Fernandes:** Investigation, Formal analysis, Writing, Writing – review & editing. **Francisca Rodrigues:** Investigation, Formal analysis, Writing, Writing – review & editing. **Vincenza Andrisano:** Resources, Funding acquisition, Writing, Writing – review & editing. **Cristina Delerue-Matos:** Resources, Funding acquisition, Writing, Writing – review & editing. **Clara Grosso:** Conceptualization, Methodology, Investigation, Formal analysis, Funding acquisition, Supervision, Writing, Writing – review & editing.

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