https://doi.org/10.31925/farmacia.2026.5.7 **ORIGINAL ARTICLE**

PHYTOCHEMICAL CHARACTERIZATION, ANTIOXIDANT POTENTIAL AND MULTIFUNCTIONAL ENZYME INHIBITORY ACTIVITY OF *HYSSOPUS OFFICINALIS* SUBSP. *ARISTATUS*: AN *IN VITRO* AND *IN SILICO* STUDY

TIJANA MIĆOVIĆ [1,2]*, STEVAN SAMARDŽIĆ [3], IVONA VELIČKOVIĆ [4], KSENIJA MILESKI [4], JELENA ĐOROVIĆ JOVANOVIĆ [5]

[1]*Institute for Medicines and Medical Devices of Montenegro, Podgorica, Montenegro*
[2]*Faculty of Medicine, University of Montenegro, Podgorica, Montenegro*
[3]*Department of Pharmacognosy, Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia*
[4] *Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Belgrade, Serbia*
[5]*Institute for Information Technologies, University of Kragujevac, Kragujevac, Serbia*

**corresponding author: tijana.micovic@cinmed.me*

Manuscript received: April 2026

Abstract

LC-DAD-ESI-MS analysis of the methanol extract from the flowering aerial parts of *Hyssopus officinalis* subsp. *aristatus* (Montenegro) revealed the predominance of hydroxycinnamic acid derivatives, flavonoid glycosides, and phenylethanoid glycosides. The extract exhibited moderate antioxidant activity in ABTS, TRC, and BCB assays, with the strongest effect in ABTS (IC_{50} = 73.41 µg/mL), compared to *L*-ascorbic acid and BHT (31.59 and 40.37 µg/mL, respectively). Major phenolics, chlorogenic and rosmarinic acids, showed potent antioxidant activity (IC_{50} = 24.00 and 12.23 µg/mL in the ABTS assay, respectively). The extract showed stronger inhibition of *α*-glucosidase (IC_{50} = 19.02 mg/mL) than *α*-amylase (26.41 mg/mL). The dominant phenolic acids showed potent *α*-glucosidase inhibition, comparable to acarbose. This study is the first to report inhibitory activity of this species against arginase (IC_{50} = 7.47 mg/mL) and elastase (IC_{50} = 26.24 mg/mL). Molecular docking indicated favorable binding of rosmarinic acid to *α*-glucosidase (ΔG_{bind} = -7.56 kcal/mol) and arginase (ΔG_{bind} = -7.77 kcal/mol), providing a theoretical rationale for the extract's activity. These findings highlight this plant as a promising source of multifunctional phenolics relevant to oxidative stress and metabolic disorders.

Rezumat

Analiza fizico-chimică a extractului metanolic obținut din părțile aeriene înflorite ale speciei *Hyssopus officinalis* subsp. *aristatus* (Muntenegru) a evidențiat prezența derivaților acidului hidroxicinamic, a flavonoidelor și a feniletanoide glicozilate. Extractul a prezentat o activitate antioxidantă moderată, cu cel mai puternic efect în testul ABTS (IC_{50} = 73,41 µg/mL), comparativ cu acidul *L*-ascorbic și BHT (31,59 și, respectiv, 40,37 µg/mL). Principalii compuși fenolici, acizii clorogenici și rozmarinici, au prezentat o activitate antioxidantă puternică (IC_{50} = 24,00 și, respectiv, 12,23 µg/mL în testul ABTS). Extractul a inhibat mai puternic *α*-glucozidaza (IC_{50} = 19,02 mg/mL) decât *α*-amilaza (26,41 mg/mL). Acizii fenolici dominanți au prezentat o inhibare puternică a *α*-glucozidazei, comparabilă cu cea a acarbosei. Simularea de andocare moleculară a indicat o legare favorabilă a acidului rozmarinic de *α*-glucozidază (ΔG_{bind} = -7,56 kcal/mol) și de arginază (ΔG_{bind} = -7,77 kcal/mol), oferind o justificare teoretică pentru activitatea extractului. În concluzie, această plantă este o sursă promițătoare de compuși fenolici multifuncționali relevanți pentru stresul oxidativ și tulburările metabolice.

Keywords: *Hyssopus officinalis* subsp. *aristatus*, antioxidant activity, *α*-glucosidase inhibition, arginase inhibition, molecular docking

Introduction

Plant secondary metabolites represent a highly diverse yet still underexplored source of bioactive molecules with significant pharmacological potential. In the context of the rising global prevalence of chronic non-communicable diseases, particularly type 2 diabetes, cardiovascular, inflammatory disorders and degenerative conditions, research focus has increasingly shifted from synthetic drugs toward natural multifunctional agents capable of modulating multiple pathophysiological mechanisms, often with a more favourable safety profile (1, 2).

This therapeutic concept, based on the application of plant secondary metabolites, primarily relies on two key mechanisms: neutralization of oxidative stress and modulation of metabolic enzymes. Phenolic acids and flavonoids, as major representatives of plant antioxidants, contribute to the mitigation of oxidative stress, which plays a central role in the pathogenesis of cardiovascular diseases, neurodegenerative disorders, and aging processes (3).

In addition to antioxidant activity, targeted inhibition of key metabolic and proinflammatory enzymes represents another important pharmacological approach. The α-amylase and α-glucosidase are essential targets in the regulation of postprandial glycemia and the management of type 2 diabetes. Furthermore, arginase and elastase are involved in inflammatory processes, vascular dysfunction, and extracellular matrix remodelling, thus being considered relevant targets for the development of anti-inflammatory and pro-endothelial agents (4-6).

Plant polyphenols, with their ability to modulate key enzymes involved in metabolic and inflammatory disorders, have attracted growing scientific interest over the last few decades. Thus, aromatic and medicinal species of the *Lamiaceae* family have been increasingly investigated as rich sources of bioactive phenolic compounds. Recent studies have further confirmed that phenolic-rich extracts of medicinal plants from the *Lamiaceae* family exhibit considerable antioxidant potential and represent valuable sources of bioactive compounds with multifunctional biological activities (7, 8).

Among these, the Mediterranean medicinal and culinary plant *Hyssopus officinalis* L., *Lamiaceae* (hyssop), has demonstrated multiple pharmacological properties, including antimicrobial, antioxidant, cytotoxic, antigenotoxic, and anti-inflammatory effects (9-15). Traditionally, the aerial parts of hyssop have been used in various cultures to treat digestive and respiratory ailments, rheumatism, and skin disorders (9, 16-18).

The pharmacological potential and ethnomedicinal applications of hyssop are closely associated with the rich and complex chemical composition of its aerial parts. In addition to essential oils, the plant contains significant groups of secondary metabolites, primarily flavonoids (*e.g.*, isoquercitrin and diosmin) and phenolic acids (including ferulic, caffeic, chlorogenic and rosmarinic acids) (9, 12-14, 16).

In our previous study (14), methanol extracts of flowering aerial parts of *H. officinalis* subsp. *aristatus* (Godr.) Nyman, collected from five different natural habitats in Montenegro, were examined. Among them, the extract from the Piva locality exhibited the highest total polyphenol content (112 mg GAE/g) and the strongest antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. Phytochemical analysis revealed chlorogenic and rosmarinic acids as the dominant constituents, quantified at 33.46 mg/g and 17.98 mg/g, respectively. However, the overall phytochemical characterization was limited to a small number of identified compounds, highlighting the need for more comprehensive profiling.

Based on these findings, the Piva extract was selected for further investigation. The aim of this study was to provide a detailed characterization of its polyphenolic profile, to evaluate its antioxidant activity using complementary *in vitro* assays, to assess its inhibitory effects on key metabolic and proinflammatory enzymes (including α-amylase, α-glucosidase, arginase and elastase), and to investigate potential molecular interactions through molecular docking simulations of its major phenolic constituents.

To the best of our knowledge, such an integrated phytochemical, *in vitro*, and *in silico* approach has not previously been applied to this subspecies, highlighting the novelty and scientific relevance of the present study.

Materials and Methods

Tested material

The methanol extract of the flowering aerial parts of *Hyssopus officinalis* subsp. *aristatus* was used for the bioactivity tests. The plant material was collected at the Piva locality, Montenegro (43°09′25″ N, 18°50′46″ E; 750 m a.s.l.), during the flowering stage in September 2018. The species was taxonomically identified by Dr. Danijela Stešević, Faculty of Natural Sciences and Mathematics, University of Montenegro, Podgorica, Montenegro. A voucher specimen (No. 1420162) was deposited in the Herbarium of the same institution.

The extract was prepared as previously described by Mićović *et al*. (14). Briefly, the dried and ground plant material was subjected to bimaceration. The obtained extract was filtered and evaporated to dryness using a rotary evaporator, affording a dark green semisolid mass. Its yield was 9.05%, calculated relative to the dry plant material (14). Prior to analysis, the extracts were stored at 4ºC in tightly closed glass jars.

Chemical analysis

Liquid chromatography coupled with diode-array detection and electrospray ionization mass spectrometry (LC-DAD-ESI-MS) analysis of the methanol extract of hyssop flowering aerial parts was conducted on an Agilent LC/MS System 1260/6130 (Agilent Technologies, Waldbronn, Germany) following the procedure previously described by Mićović *et al*. (14). The dry extract was dissolved in a suitable volume of methanol to obtain a final concentration of 5 mg/mL. After filtration through a 0.45 µm membrane filter (Captive Syringe Filters, Agilent, Germany), 3 µL of the sample was injected. Chromatographic separation was performed on a reversed-phase column (Zorbax SB-Aq, 150 × 3.0 mm, particle diameter 3.5 µm, Agilent Technologies) thermostated at 25°C. The binary mobile phase consisted of aqueous formic acid (solvent A) and acetonitrile (solvent B). The elution program was: 0 - 20 min (10 - 35% B), 20 - 24 min (35 - 90% B), 24 - 25 min (90% B), and 25 - 30 min (90 - 10% B). The

flow rate was 0.35 mL/min. UV detection was set at 210, 270, 320, and 350 nm, while UV spectra were recorded in the range 190 - 640 nm. Mass chromatograms were acquired in negative ion mode using fragmentation voltages of 100 and 250 V. Mass spectra were obtained in the range *m/z* 100 - 1000. The compounds were tentatively identified by comparing experimental UV and MS data with literature data (19-30).

Antioxidant activity assays

The antioxidant capacity of the methanol extract of *H. officinalis* subsp. *aristatus* and its two quantitatively dominant compounds, rosmarinic acid and chlorogenic acid, was assessed through a combination of complementary *in vitro* spectrophotometric methods, encompassing both radical scavenging and ferric reducing ability assays. Specifically, the 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay and β-carotene bleaching (BCB) assay were employed to evaluate the free radical scavenging potential of the tested samples (extract, rosmarinic acid, and chlorogenic acid), while the total reducing capacity (TRC) procedure served to estimate the ability to reduce iron from Fe^{3+} to Fe^{2+} under the applied conditions.

The ABTS assay was performed according to Xiao *et al*. (31) with procedural adjustments to accommodate the extract and optimise sensitivity, thus enabling a reliable estimation of antioxidant activity toward both hydrophilic and lipophilic radical species. The BCB assay was carried out as outlined by Lekouaghet *et al*. (32), based on monitoring the inhibition of *β*-carotene discoloration in a linoleic acid emulsion system. This method reflects the capacity of antioxidants to protect unsaturated lipids from oxidative degradation.

The TRC assay, performed in line with the procedure of Mokrani *et al*. (33), provided a comparative estimation of the samples' electron-donating capacity.

Results obtained from the ABTS and BCB assays were expressed as IC_{50} values (µg/mL and mg/mL, respectively), representing the concentration required to scavenge 50% of the respective radicals, whereas the IC_{50} (mg/mL) obtained for the TRC test indicated the effective concentration required to reach an absorbance of 0.5. To determine these values, a linear regression analysis was applied. For each spectrophotometric and enzyme inhibitory assay, the percentages of inhibition or absorbance values were plotted against the corresponding concentrations of the tested samples. The linear regression equation ($y = ax + b$) was generated from the resulting dose-response curves using Microsoft Excel software. The IC_{50} values were mathematically derived from these equations by calculating the exact concentration that corresponds to a 50% inhibitory effect or the required absorbance threshold, respectively.

To ensure consistency and comparative validity, the antioxidant performance of the tested samples was evaluated relative to the reference antioxidants – *L*-ascorbic acid and butylated hydroxytoluene (BHT) – analysed under identical conditions.

Enzyme inhibitory activity

The enzyme inhibitory potential of the methanol extract was evaluated toward four physiologically relevant enzymes involved in carbohydrate metabolism and inflammatory processes: *α*-amylase, *α*-glucosidase, arginase and elastase. The two dominant compounds, rosmarinic acid and chlorogenic acid, were tested specifically against *α*-amylase and *α*-glucosidase to determine their contribution to the carbohydrate-metabolism inhibitory activity, as the extract showed the most promising enzyme inhibitory activity towards these enzymes compared to the appropriate reference compound. All assays were performed in 96-well microplates using spectrophotometric detection. Each test was conducted in triplicate, and results were expressed as IC_{50} values (mg/mL) and compared with those of reference standards. Enzyme controls corresponding to 0% and 100% activity were prepared, where the complete reaction mixture without inhibitor represented full enzymatic activity, and the blank without enzyme was used as the zero-activity control. Colour controls were also prepared for all tested concentrations of the samples.

α-Amylase inhibition assay

The inhibition of *α*-amylase activity was determined following the modified Caraway–Somogyi iodine–potassium iodide (I/KI) colorimetric method described by Zengin *et al*. (34), optimised for microplate application. Briefly, 25 µL of the sample solution (*i.e.*, extract, rosmarinic acid, or chlorogenic acid) at various concentrations was mixed with *α*-amylase enzyme solution (0.5 mg/mL) prepared in phosphate buffer, pH 6.8, containing 6 mM NaCl. The mixture was pre-incubated for 10 min at 37°C, followed by the addition of a starch solution to initiate the reaction. After 15 minutes of incubation, the reaction was terminated by the addition of 25 µL of 1 M HCl, and the colour was developed with I/KI reagent. The absorbance was recorded at 630 nm using a microplate reader. Percentage inhibition was calculated relative to a control without inhibitor. Acarbose was used as a positive control under identical conditions.

α-Glucosidase inhibition assay

The inhibition of *α*-glucosidase was assessed according to the spectrophotometric method proposed by Wan *et al*. (35), based on the hydrolysis of *p*-nitrophenyl-*α*-D-glucopyranoside (PNPG). Tested samples (*i.e.*, extract, rosmarinic acid, or chlorogenic acid) were pre-incubated with the enzyme solution in phosphate buffer (pH 6.8) at 37°C in the final volume of 145 µL to allow potential inhibitor–enzyme

interaction, after which the 3.5 mM pNPG substrate was added to initiate the reaction. The release of *p*-nitrophenol, indicative of enzymatic activity, was quantified by measuring absorbance at 405 nm following reaction termination with 0.2 M sodium carbonate. Results were expressed as IC_{50} values and compared with the standard inhibitor acarbose analysed in parallel.

Arginase inhibitory activity assay

Inhibitory activity of the *H. officinalis* methanol extract toward arginase enzyme was determined using a microplate-based colorimetric assay adapted from literature reports: Zedet *et al*. (36) and Marchisio *et al*. (37) with slight modifications. Briefly, the assay involved pre-incubation of varying concentrations of the extract with arginase enzyme (0.025 U/µL) in a suitable buffer (50 mM Tris-HCl, pH 7.5 with addition of NaCl, BSA, and glycerol), *L*-arginine as substrate (50 mM), and $MnCl_2$ as cofactor in Tris-HCl buffer at 37°C for 60 minutes. After equilibration, the reaction was stopped by adding a colour-forming reagent (a 1:1 mixture of a solution of *o*-phthalaldehyde 10 mM in 15% sulphuric acid with a solution of primaquine diphosphate 4 mM with boric acid 130 mM and sulphuric acid 15%), and incubated for another 60 minutes at the same temperature. The amount of urea produced was quantified spectrophotometrically at 450 nm. A known arginase inhibitor, *S*-(2-boronoethyl)-*L*-cysteine (BEC), was included as a positive control.

Elastase inhibitory activity assay

The inhibitory effect of the *H. officinalis* methanol extract on elastase activity was evaluated using a protocol adapted from the study of Lee *et al*. (38). The varying concentrations of the extract were mixed with Tris-HCl buffer, pH 8, in a final volume of 160 µL and pre-incubated with elastase enzyme solution (10 µg/mL) for 20 minutes at 25°C. The reaction was initiated by adding 8 mM N-succinyl-Ala-Ala-Ala-p-nitroanilide (SANA) as substrate, and the mixture was incubated for an additional 20 minutes at room temperature and measured at 410 nm. The degree of inhibition was calculated by comparing enzyme activity in the presence and absence of the extract. Results were expressed as IC_{50} values, and quercetin hydrate was used as a positive control.

Molecular docking

The molecular docking simulations were used for *in silico* prediction of the inhibitory potency of rosmarinic acid and chlorogenic acid, the two quantitatively dominant compounds identified in the methanol extract, toward three selected enzymes: *α*-amylase, *α*-glucosidase, and arginase. These simulations aimed to further elucidate the binding mechanisms of the two dominant compounds. While docking was extended to include arginase to explore its interaction with these phenolics, elastase was excluded from the study as the methanol extract exhibited the lowest *in vitro* activity toward this enzyme. All simulations were performed using AutoDock 4.2 software (39). In the molecular docking simulations presented in this research, rosmarinic acid and chlorogenic acid were used as ligands. The Protein Data Bank provided the three-dimensional (3D) crystal structures of *α*-amylase, *α*-glucosidase, and arginase enzymes (PDB IDs: 1OSE, 4J5T, and 3KV2, respectively) (40, 41, 42). The chosen enzymes were prepared in BIOVIA Discovery Studio 4.0 to be employed as receptors in molecular docking simulations (43). Preparation of the targeted receptors was performed by removing the co-crystallized ligands, water molecules, and cofactors. The detection of pockets and cavities in the 3D structures of enzymes was performed using AutoGridFR (AGFR) software, and the binding sites of the target proteins were defined (44). The binding pocket analyses were achieved after the native bound ligands were removed from the structures of *α*-amylase, *α*-glucosidase, and arginase enzymes. In addition, re-docking was completed with the ligand compounds, and the same docking modes were created as were found in co-crystallized forms of target enzymes. AutoDockTools (ADT) graphical user interface was used for the addition of polar hydrogen atoms and the calculation of Kollman charges. The structures of target enzymes were kept rigid during the simulations, while the ligands were set as flexible and the bonds in the ligands were set to be rotatable. The Lamarckian Genetic Algorithm (LGA) was used for flexible enzyme–ligand molecular docking simulations. The grid boxes, centred with dimensions 32.363Å x 41.127Å x -0.901Å, -1.276Å x -17.236Å x -7.529Å, and 13.873Å x -11.394Å x -17.634Å in the -x, -y, and -z directions of *α*-amylase, *α*-glucosidase and arginase were applied to cover the enzyme's binding sites and allow ligands to move freely. The auto grid runs were performed with a grid point spacing of 0.375 Å. All the molecular docking simulations were accomplished at a temperature of 298.15 K. BIOVIA Discovery Studio was used for the analysis of the results of the molecular docking simulation and for their visualization.

The evaluation of the inhibitory potency was commonly based on two important parameters, free energy of binding (ΔG_{bind}) and the inhibitory constant (K_i). AutoDock calculated the values of both of these parameters, but the value of K_i was calculated by AutoDock after the estimation of the value of ΔG_{bind}. The energy that is released during the formation of contacts between a ligand and a target protein is presented by the value of ΔG_{bind}. The value of ΔG_{bind} is a summary of the values of Final Intermolecular Energy (FIE), Final Total Internal Energy (FTIE), Torsional Free Energy (TFE), and Unbound System's Energy (USE) (Equation 1). The value of FIE is de-

pendent on the Van der Waals energy, energy of hydrogen bonds, desolvation energy of the system, and electrostatic energy.

ΔG_{bind} = [(FIE) + (FTIE) + (TFE) - (USE)] (1)

As regards the value of the inhibitory constant, it is calculated by the following equation:

$K_i = \exp(\Delta G_{bind} /RT)$ (2)

where R is the gas constant (R = 1.99 cal/molK), and T is the value of the room temperature (298.15 K).

Statistical analysis

All results from antioxidant and enzyme inhibitory assays were calculated using MS Office Excel and presented as mean ± standard deviation (SD) from three independent experiments (n = 3), each performed in triplicate. Prior to evaluation of statistical differences among the obtained data their normal distribution and homogeneity of variance were confirmed by Shapiro-Wilk and Levene's tests. Differences between the group means and their significance were verified by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for multiple comparisons, and differences were considered statistically significant at $p < 0.05$. Data were acquired using PAST software (version 3.18).

Results and Discussion

Chemical composition

The LC-DAD-ESI-MS analysis expanded the current knowledge on the chemical profile of the methanol extract of hyssop collected at the Piva locality in Montenegro. In total, including previously reported phenolics by Mićović *et al*. (14), 14 constituents were identified (Table I), two of them (chlorogenic acid and rosmarinic acid) by comparison with commercially available reference standards, while the remaining ones were tentatively identified. In addition to the previously documented compounds, the presence of danshensu (21, 25, 28), two esters of quinic acid with hydroxycinnamic acids (caffeoylquinic acid and *p*-coumaroylquinic acid) (27), one flavone glycoside (apigenin di-*C*-glycoside) (20), one hydroxycinnamic acid dimer (methyl derivative of rosmarinic acid) (24, 30) and one tetramer (salvianolic acid B) (19, 26, 29), as well as the phenylethanoid glycoside martynoside and its acetylated derivative (22, 23), was revealed. Of the eight newly reported compounds, six (danshensu, caffeoylquinic acid, *p*-coumaroylquinic acid, the methyl derivative of rosmarinic acid, salvianolic acid B, and martynoside) have previously been reported in *H. officinalis* or other species of the same genus (13, 19, 45), while the remaining two (apigenin di-*C*-glycoside and acetylmartynoside) have been described as constituents of *Lamiaceae* species other than those of the genus *Hyssopus* (*Origanum majorana*, *Salvia bulleyana*, *Thymbra capitata*) (20, 46, 47). Overall, the chemical composition of the tested sample was in good agreement with the available literature.

Table I

Compounds identified in the methanol extract of the flowering aerial parts of *Hyssopus officinalis* subsp. *aristatus*, with their retention times (t_R), UV and MS spectral data

t_R (min)	UV λ_{max} (nm)	ESI-MS data (*m/z*)	Assignment
4.2	280	395 [2M-H]$^-$, 197 [M-H]$^-$, 179, 153, 135, 123	danshensu[bd]
5.9	298sh, 324	707 [2M-H]$^-$, 353 [M-H]$^-$, 191, 179, 135	caffeoylquinic acid[bd]
9.1	298sh, 326	707 [2M-H]$^-$, 353 [M-H]$^-$, 191	chlorogenic acid (5-*O*-caffeoylquinic acid)[ac]
11.0	298sh, 328	623 [2M-H]$^-$, 311 [M-H]$^-$, 134	caffeoyl pentoside[ad]
11.1	270, 338	593 [M-H]$^-$, 473, 353	apigenin di-*C*-hexoside[bd]
11.3	312	337 [M-H]$^-$, 173	*p*-coumaroylquinic acid[bd]
12.1	296sh, 326	735 [2M-H]$^-$, 367 [M-H]$^-$, 173	feruloylquinic acid[ad]
15.3	256, 266sh, 356	463 [M-H]$^-$, 300	quercetin *O*-hexoside[ad]
17.2	252, 266, 348	607 [M-H]–, 299, 284	diosmetin *O*-deoxyhexosyl-hexoside[ad]
18.5	286sh, 328	719 [2M-H]$^-$, 359 [M-H]$^-$, 197, 161	rosmarinic acid[ac]
19.7	286sh, 330	651 [M-H]$^-$, 475, 193, 175, 160	martynoside[bd]
20.4	254, 286, 308	717 [M-H]$^-$, 537, 519, 493, 339, 321, 295	salvianolic acid B[bd]
21.6	288sh, 328	747 [M-H]$^-$, 373, 197, 193, 179, 175, 160, 135, 134	methyl derivative of rosmarinic acid[bd]
22.7	286sh, 330	693 [M-H]$^-$, 651, 475, 193, 175, 160	acetylmartynoside[bd]

[a] Compound previously identified by Mićović *et al.* (14); [b] compound identified in the current study; [c] compound identified using a reference standard; [d] compound tentatively identified through comparison of experimental and literature data.

Antioxidant Activity

The antioxidant activity of the methanol extract of *H. officinalis* subsp. *aristatus* and its quantitatively dominant compounds, evaluated by three assays (ABTS, TRC, and BCB), is presented in Table II.

Table II

Antioxidant activity of the methanol extract of *Hyssopus officinalis* subsp. *aristatus* and chlorogenic and rosmarinic acids

Tested sample/ Reference compound	ABTS IC_{50} (µg/mL)	TRC IC_{50} (mg/mL)	BCB IC_{50} (mg/mL)
H. officinalis extract	$73.41 \pm 1.00^{a**}$	1.54 ± 0.13^{a}	1.89 ± 0.18^{a}
Chlorogenic acid	24.00 ± 0.22^{b}	0.22 ± 0.01^{b}	9.03 ± 0.59^{b}
Rosmarinic acid	12.23 ± 0.15^{c}	0.09 ± 0.00^{b}	1.16 ± 0.07^{c}
L-ascorbic acid	31.59 ± 0.29^{d}	0.08 ± 0.00^{b}	0.60 ± 0.01^{d}
BHT	40.37 ± 2.28^{e}	0.22 ± 0.02^{b}	0.25 ± 0.03^{d}

*The results were obtained from three experiments. **Different letters in each column mean significant differences ($p < 0.05$).

In the ABTS assay, the extract exhibited moderate radical-scavenging capacity with an IC_{50} value that was significantly higher than those of the used reference compounds (73.41 µg/mL *vs*. 40.37 and 31.59 µg/mL for BHT and *L*-ascorbic acid, respectively). Statistical analysis confirmed that all tested samples and standards differed significantly from one another within this assay ($p < 0.05$). Literature on hyssop extracts mostly reports DPPH-based antioxidant activity, with IC_{50} values varying widely due to extraction methods, plant origin, and plant part used (9, 12, 18, 48, 49). In the study by Hamoud and Maammeri (50), fractionated hyssop extracts (methanol/water followed by ethyl acetate) exhibited lower ABTS IC_{50} values (7.47 µg/mL for leaves and 15.65 µg/mL for stems), which can be attributed to the selective enrichment of phenolic components in the ethyl acetate fraction, whereas the results obtained in the present study reflected the antioxidant potential of the crude methanol extract.

The reducing power of the extract, determined by the TRC assay (IC_{50} = 1.54 mg/mL), further confirmed its ability to donate electrons. This finding is consistent with previously reported FRAP results for the same extract (0.959 mmol Fe^{2+}/g) (14), indicating the presence of compounds in the extract capable of participating in redox-based protective mechanisms. The methanol extract was also active in the BCB assay, with an IC_{50} value of 1.89 mg/mL. Although this value was closest to that of pure rosmarinic acid (IC_{50} = 1.16 mg/mL), post-hoc analysis indicated that the difference between the extract and rosmarinic acid remained statistically significant ($p < 0.05$).

Analysis of the dominant phenolic components, chlorogenic acid and rosmarinic acid, showed that rosmarinic acid exhibited stronger antioxidant activity in the ABTS and BCB assays compared to chlorogenic acid, as evidenced by significantly lower IC_{50} values ($p < 0.05$). However, in the TRC assay, no statistically significant difference was observed between the reducing power of rosmarinic acid (IC_{50} = 0.09 mg/mL) and chlorogenic acid (IC_{50} = 0.22 mg/mL), as well as in comparison to the reference standards (Table II). Compared to rosmarinic acid, the methanol extract displayed moderate activity, which is expected given its complex composition and the dilution of active components within the matrix. The superior activity of rosmarinic acid in the ABTS (IC_{50} = 12.23 µg/mL) assay can be attributed to its structural features, particularly the presence of two hydroxyl groups in the *ortho* position on both phenolic rings, which contribute to radical stabilization and efficient electron donation (51). Previous studies indicate that rosmarinic acid can exhibit activity comparable to or even higher than standard antioxidants such as BHT and *L*-ascorbic acid (51, 52), which is consistent with the results obtained in this study in the ABTS and TRC assays (Table II).

Although the literature reports that nonpolar extracts tend to exhibit higher efficacy in lipophilic systems (53), the activity of the methanol extract in the BCB assay can be explained by the amphiphilic nature of certain phenolic antioxidants, such as rosmarinic acid. This property allows rosmarinic acid to position itself at the interface between lipid and aqueous phases, where it efficiently intercepts lipid radicals during the initial stages of oxidation (54, 55).

Overall, the methanol extract of *H. officinalis* exhibited moderate antioxidant activity. The role of rosmarinic acid, together with possible synergistic effects of other phenolic components, suggests the potential of this plant species as a source of natural antioxidants. Future studies should include validation of these findings in cellular models of oxidative stress, as well as optimization of the extract's phenolic profile to enhance antioxidant efficacy.

Enzyme Inhibitory Activity

The inhibitory activity of the methanol extract of *H. officinalis* subsp. *aristatus* and its quantitatively dominant compounds against the tested enzymes is presented in Table III.

The methanol extract of hyssop, compared to the reference inhibitors, showed the most favorable inhibitory potential against digestive enzymes, primarily *α*-glucosidase, followed by *α*-amylase. Inhibition of these enzymes represents an established pharmacological approach for the regulation of postprandial hyperglycaemia and is therefore considered a relevant target in the management of type 2 diabetes (4, 56). In the present study, the methanol extract of *H. officinalis* exhibited moderate inhibitory activity toward both enzymes, with relatively stronger activity

observed against α-glucosidase (IC_{50} = 19.02 mg/mL) compared to α-amylase (IC_{50} = 26.41 mg/mL). Within each respective assay, the inhibitory activity of the extract was found to be significantly weaker ($p < 0.05$) than that of the reference compounds and isolated phenolics.

Table III

Enzyme inhibitory activity of the methanol extract of *Hyssopus officinalis* subsp. *aristatus*, and chlorogenic and rosmarinic acid

Tested sample/ Reference compound	Amylase IC_{50} (mg/mL)	Glucosidase IC_{50} (mg/mL)	Arginase IC_{50} (mg/mL)	Elastase IC_{50} (mg/mL)
H. officinalis extract	26.41 ± 1.03[a]	19.02 ± 1.73[a]	7.47 ± 0.90[a]	26.24 ± 0.86[a]
Chlorogenic acid	> 5	0.265 ± 0.002[b]	/	/
Rosmarinic acid	> 5	0.268 ± 0.003[b]	/	/
Acarbose	0.35 ± 0.00[b]	0.37 ± 0.01[b]	/	/
Quercetin hydrate	/	/	/	0.08 ± 0.01[b]
BEC	/	/	0.053 ± 0.005[b]	/

*The results were obtained from three experiments. **Different letters in each column mean significant differences ($p < 0.05$).

Literature data support the ability of *H. officinalis* to inhibit digestive enzymes involved in carbohydrate metabolism. Matsuura *et al*. demonstrated that water-methanol extracts of dried hyssop leaves inhibit α-glucosidase in an *in vivo* mouse model, which subsequently led to the isolation and identification of specific bioactive compounds responsible for this effect (57). Similarly, Miyazaki *et al*. found that hyssop extracts can inhibit the breakdown of complex carbohydrates in the intestines and significantly reduce postprandial blood glucose levels, indicating their potential in the regulation of hyperglycaemia (58). In the study by Loizzo *et al.*, chloroform, methanol, and n-hexane extracts of *H. officinalis* exhibited inhibitory activity against α-glucosidase, whereas α-amylase inhibition was not detected; the most pronounced activity was observed for the chloroform extract (IC_{50} = 127.3 µg/mL) (59). In contrast, Srivastava *et al*. reported that the methanol extract of *H. officinalis* can inhibit α-amylase (IC_{50} = 0.8366 mg/mL), although this activity was considerably weaker compared to the reference inhibitor acarbose (IC_{50} < 0.025 mg/mL) (18). These data indicate that the inhibitory potential of hyssop toward digestive enzymes largely depends on the type of extract, the composition of bioactive components, and the experimental methodology applied.

Chlorogenic acid and rosmarinic acid exhibited pronounced inhibitory activity against α-glucosidase, with IC_{50} values of approximately 0.27 mg/mL. Crucially, statistical analysis revealed that the IC_{50} values of chlorogenic acid (0.265 mg/mL) and rosmarinic acid (0.268 mg/mL) showed no statistically significant difference ($p > 0.05$) compared to each other, nor did they differ significantly from the reference inhibitor acarbose (0.37 mg/mL) (Table III). These results suggested that chlorogenic acid and rosmarinic acid may contribute to the α-glucosidase inhibitory activity observed *in vitro*. The obtained findings were consistent with previous *in vitro* studies reporting the inhibitory potential of chlorogenic and rosmarinic acids toward α-glucosidase. Specifically, comparable levels of activity to reference inhibitors have been observed, although the reported IC_{50} values vary depending on experimental conditions and the methodologies employed (60, 61).

In the present study, both chlorogenic acid and rosmarinic acid were evaluated for their potential inhibitory effects on α-amylase. No detectable activity was observed for either compound at concentrations up to 5 mg/mL, which is considered a relatively high concentration for pure phenolic acids. This finding suggests that, under the applied *in vitro* conditions, these compounds do not exert relevant α-amylase inhibition. Our observations are consistent with previous reports describing weak or negligible α-amylase inhibition by these phenolic acids (62, 63).

Interestingly, the methanol extract of *H. officinalis* exhibited measurable inhibitory activity against α-amylase (IC_{50} = 26.41 mg/mL), despite the lack of detectable activity of its dominant phenolic constituents (rosmarinic and chlorogenic acids). This finding suggests that α-amylase inhibition by the extract likely arises from other metabolites present, such as flavonoid glycosides, or results from synergistic interactions among multiple components within the complex phenolic matrix. Such a selective inhibitory profile-characterized by stronger inhibition of α-glucosidase relative to α-amylase, is considered pharmacologically desirable for the control of postprandial hyperglycaemia, as it allows attenuation of the glycaemic response while minimizing gastrointestinal side effects associated with excessive α-amylase inhibition (56).

A particularly significant finding of this study relates to the inhibitory activity of the methanol extract against arginase. According to the available literature data, no previous reports have investigated the arginase inhibitory potential of *H. officinalis*, indicating that the present results provided a novel insight into the pharmacological profile of this plant species. Arginase plays a key role in regulating the bioavailability of *L*-arginine, a common substrate for

nitric oxide (NO) synthesis. Excessive arginase activity competitively reduces the availability of *L*-arginine for nitric oxide synthase, leading to decreased NO production and the development of endothelial dysfunction (64), whereas inhibition of this enzyme is associated with improved endothelial function and vascular homeostasis (65). Although the inhibitory activity of the methanol extract was significantly weaker compared to the potent reference inhibitor (BEC), the clearly measurable arginase inhibition (IC_{50} = 7.47 mg/mL) suggests the presence of components that may contribute to the observed inhibitory activity. Overall, the inhibitory effect of the methanol extract against arginase indicates that *H. officinalis* contains constituents with measurable inhibitory activity toward this enzyme; however, the biological significance of this finding requires further investigation.

The methanol extract of *H. officinalis* exhibited moderate inhibitory activity against elastase (IC_{50} = 26.24 mg/mL). Although the obtained value was significantly higher compared to the reference inhibitor quercetin, this finding is noteworthy because, to the best of our knowledge, it represents the first report on the elastase inhibitory potential of this plant species. Elastase, a neutrophil-derived serine protease, plays a key role in tissue remodelling, and its excessive activity has been associated with extracellular matrix degradation and the progression of chronic inflammatory conditions (66).

Overall, the enzyme inhibition results indicated that the methanol extract of *H. officinalis* exhibits a moderate but clearly differentiated inhibitory potential toward metabolic and proinflammatory enzymes. The relatively more favourable inhibition of *α*-glucosidase compared to *α*-amylase, together with the first-time reported activity against arginase and elastase, indicates that the investigated extract exhibits inhibitory activity toward multiple enzymatic targets.

The different chemical compounds tentatively identified in the extract, such as salvianolic acid B, rosmarinic acid derivatives, danshensu, and apigenin glycosides, may contribute to the overall observed inhibition of *α*-glucosidase, *α*-amylase, arginase, and elastase. However, the contribution of individual constituents to the observed enzyme inhibition cannot be established from the present data and would require further investigation using isolated compounds and/or targeted mechanistic studies. Additionally, the fact that the whole extract inhibits *α*-amylase, while pure chlorogenic and rosmarinic acids do not, suggests that the inhibitory activity may be associated with other bioactive constituents present in the plant matrix.

Molecular docking simulations

Since the investigated *H. officinalis* extract demonstrated measurable inhibitory activity against *α*-glucosidase and arginase in the *in vitro* assays, molecular docking was performed to explore whether its two major phenolic constituents, chlorogenic acid and rosmarinic acid, could contribute to the observed biological effects at the molecular level. Molecular docking is widely used as a computational approach for predicting the preferred binding orientation of small molecules within enzyme active sites and for estimating the relative strength of ligand–protein interactions. However, it should be emphasized that docking provides theoretical predictions rather than direct evidence of enzyme inhibition and therefore should be interpreted in conjunction with experimental findings.

The calculated thermodynamic parameters obtained from the docking simulations are summarized in Table IV. In the present study, acarbose was used as the reference ligand for *α*-amylase and *α*-glucosidase, whereas *S*-(2-boronoethyl)-*L*-cysteine served as the reference inhibitor for arginase. In general, lower predicted binding free energies (ΔG_{bind}) were associated with lower predicted inhibition constants (K_i), indicating internal consistency of the molecular docking calculations.

Among the investigated enzymes, the reference inhibitor exhibited the most favourable predicted binding affinity toward both *α*-amylase (ΔG_{bind} = -11.00 kcal/mol) and *α*-glucosidase (ΔG_{bind} = -10.12 kcal/mol), whereas chlorogenic acid and rosmarinic acid showed moderately higher binding energies. These results indicate that the two phenolic acids are predicted to interact less strongly with the catalytic sites than the reference inhibitor under the applied molecular docking conditions. Nevertheless, both compounds demonstrated considerably more favorable predicted binding toward *α*-glucosidase than toward *α*-amylase. This observation agrees well with the experimental findings, where both phenolic acids inhibited *α*-glucosidase but showed no detectable *α*-amylase inhibition at the tested concentrations. Thus, although docking cannot confirm enzyme inhibition, the computational results provide a plausible structural explanation for the experimentally observed enzyme selectivity.

Rosmarinic acid exhibited the most favorable predicted interaction with *α*-glucosidase (ΔG_{bind} = -7.56 kcal/mol; K_i = 2.88 μM), whereas chlorogenic acid showed a somewhat weaker predicted affinity (ΔG_{bind} = -6.31 kcal/mol; K_i = 23.54 μM). These differences suggest that rosmarinic acid may establish a more stable interaction network within the *α*-glucosidase binding pocket. However, given that both phenolic acids demonstrated comparable *in vitro α*-glucosidase inhibitory potencies, these docking variations should be interpreted as supportive of the overall binding capacity rather than as direct predictors of relative experimental potency. Also, it should be emphasized that the docking results alone do not

allow conclusions regarding absolute inhibitory potency or superiority over acarbose, since reliable comparison with the reference inhibitor requires biochemical and kinetic validation under identical experimental conditions.

A similar relationship was observed for arginase. Both phenolic acids displayed more favorable predicted binding energies than the reference ligand, with rosmarinic acid showing the lowest ΔG_{bind} value (-7.77 kcal/mol), followed by chlorogenic acid (-7.47 kcal/mol). These findings suggest that both compounds are capable of forming stable interactions within the predicted arginase binding site and may contribute to the arginase inhibitory activity observed for the *H. officinalis* extract. However, despite the more favorable docking scores relative to the reference ligand, these computational predictions should not be interpreted as evidence that either compound is a stronger arginase inhibitor than *S*-(2-boronoethyl)-*L*-cysteine. Rather, the docking analysis identifies chlorogenic acid and particularly rosmarinic acid as promising candidates for further biochemical investigation aimed at validating their inhibitory activity and elucidating their mechanism of action.

Table IV

Thermodynamic parameters from molecular docking simulations of tested compounds with selected enzymes

Enzyme – Tested compound	**ΔG_{bind} (kcal/mol)**	**K_i (μM)**	**FIE (kcal/mol)**	**vdW + Hbond + desolv Energy (kcal/mol)**	**Electrostatic Energy (kcal/mol)**	**FTIE (kcal/mol)**	**TFE (kcal/mol)**	**USE (kcal/mol)**
α-amylase – reference inhibitor	-11.00	0.00872	-13.68	-13.56	-0.12	-1.91	+2.68	-1.91
α-amylase – chlorogenic acid	-6.10	33.74	-9.38	-9.42	+0.04	-4.86	+3.28	-4.86
α-amylase – rosmarinic acid	-6.13	31.95	-9.71	-9.52	-0.19	-4.58	+3.58	-4.58
α-glucosidase – reference inhibitor	-10.12	0.03821	-12.80	-12.98	+0.18	-2.63	+2.68	-2.63
α-glucosidase – chlorogenic acid	-6.31	23.54	-9.60	-9.33	-0.26	-4.49	+3.28	-4.49
α-glucosidase – rosmarinic acid	-7.56	2.88	-11.14	-11.70	+0.56	-3.62	+3.58	-3.62
arginase – reference inhibitor	-4.47	533.36	-7.21	-5.72	-1.49	-1.78	+2.74	-1.78
arginase – chlorogenic acid	-7.47	3.35	-10.75	-9.49	-1.26	-4.03	+3.28	-4.03
arginase – rosmarinic acid	-7.77	2.03	-11.35	-9.77	-1.57	-4.11	+3.58	-4.11

* The parameters ΔG_{bind} and K_i represent *in silico* predicted values obtained from molecular docking simulations.

To further rationalize the observed differences in the predicted binding affinities, the structural characteristics of chlorogenic acid and rosmarinic acid were analysed in relation to their interaction with the investigated enzymes. Although both compounds belong to the class of phenolic acids and share several structural features, including multiple hydroxyl groups and aromatic rings, subtle differences in their molecular architecture appear to influence their binding behaviour.

Rosmarinic acid consistently exhibited more favorable predicted binding energies than chlorogenic acid toward both α-glucosidase and arginase. This observation may be explained by its molecular structure, which consists of two catechol moieties connected through an ester linkage, providing a larger aromatic surface together with a greater number of hydrogen bond donor and acceptor groups. Such an arrangement enables the establishment of an extensive net-

work of noncovalent interactions, including conventional hydrogen bonds, π-mediated interactions, and hydrophobic contacts, thereby promoting more stable accommodation of the ligand within the enzyme binding pocket. In contrast, although chlorogenic acid also possesses several hydroxyl groups capable of participating in hydrogen bonding, its molecular geometry appears to restrict the formation of equally extensive interaction networks, resulting in slightly weaker predicted binding affinities.

The molecular docking calculations further indicate that hydrogen bonding represents the dominant driving force governing ligand recognition in all investigated enzyme–ligand complexes. This observation is supported by the favorable van der Waals, hydrogen-bonding, and desolvation energy components, which contributed substantially to the overall binding free energy, whereas electrostatic interactions played a comparatively smaller role. The more negative intermolecular energy (FIE) and final total internal energy (FTIE) values calculated for rosmarinic acid additionally suggest a more stable protein–ligand complex, consistent with its lower predicted inhibition constants.

The predicted binding poses also provide insight into the possible mechanism of enzyme inhibition. Both phenolic acids were located within or near the catalytic binding pockets of α-glucosidase and arginase, where they interact with residues involved in substrate recognition and stabilization. Occupation of these regions could interfere with substrate access to the catalytic site, suggesting a possible competitive mode of inhibition.

To further elucidate the molecular basis of enzyme recognition, the binding modes of the highest-ranked docking poses were analysed in detail (Figures 1 and 2). Rather than relying solely on the calculated binding energies, examination of the ligand–protein interaction patterns provides additional insight into the structural factors that may govern ligand stabilization within the catalytic pockets.

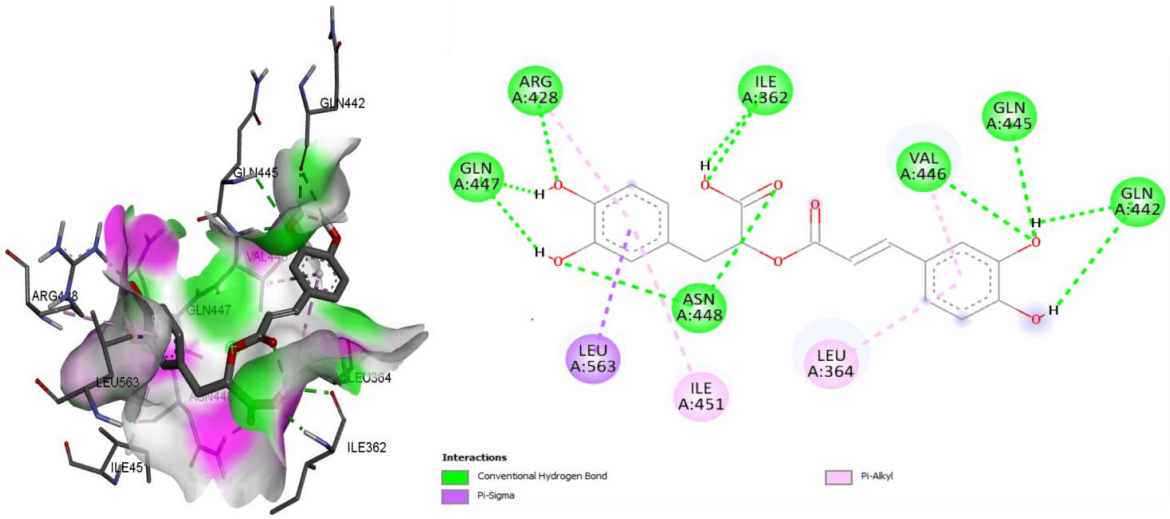


Figure 1.
The recognized interactions of the most stable conformations of protein-ligand complex structures of rosmarinic acid and α-glucosidase

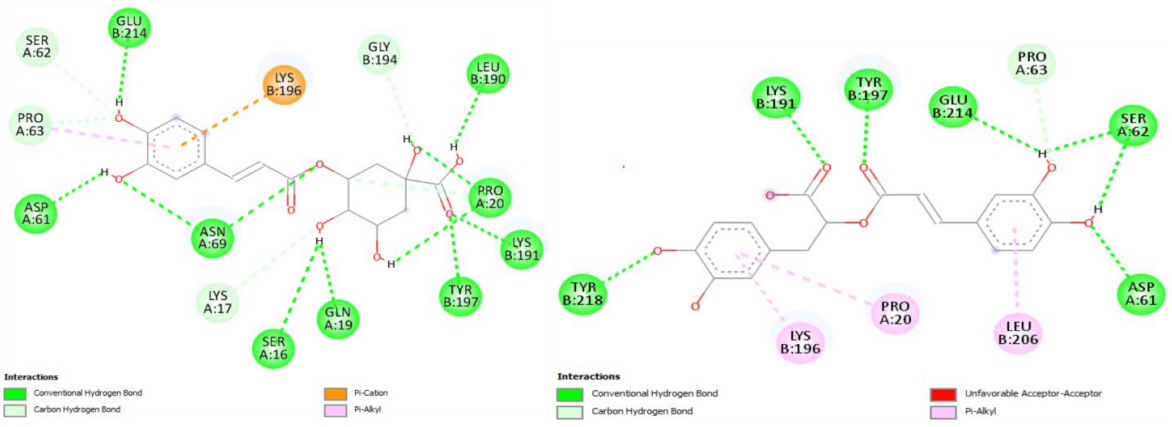


Figure 2.
The recognized interactions of the most stable conformations of protein-ligand complex structures of chlorogenic acid and arginase (left), and rosmarinic acid and arginase (right)

For α-glucosidase, rosmarinic acid adopts a well-defined orientation within the catalytic cavity, where it establishes an extensive network of noncovalent interactions involving residues associated with substrate recognition. Multiple conventional hydrogen bonds are formed with Arg428, Gln445, Gln447, and Asn448, indicating that hydrogen bonding constitutes the principal driving force for ligand anchoring. These interactions are complemented by hydrophobic contacts, including π–alkyl and π–σ interactions with Ile451, Leu364, and Leu563, which facilitate favorable packing of the aromatic rings within the predominantly hydrophobic regions of the binding pocket. The simultaneous contribution of polar and hydrophobic interactions is likely responsible for the relatively stable docking pose and the more favorable predicted binding affinity of rosmarinic acid toward α-glucosidase.

The interaction profile of rosmarinic acid also reflects its structural characteristics. The presence of two catechol rings and multiple hydroxyl groups enables simultaneous engagement with several amino acid residues, thereby increasing the probability of forming cooperative hydrogen-bonding networks while maintaining hydrophobic stabilization through its aromatic framework. Such a balanced interaction pattern is frequently associated with enhanced ligand accommodation within enzyme active sites and provides a plausible structural explanation for the stronger predicted binding of rosmarinic acid relative to chlorogenic acid.

A comparable interaction pattern was observed for arginase, although notable differences in residue involvement were evident. Chlorogenic acid interacted predominantly through an extensive hydrogen-bonding network involving Glu214, Asp61, Asn69, Ser16, and Gln19, while additional carbon hydrogen bonds and π-mediated interactions with Pro63 and Lys196 further contributed to stabilization of the ligand within the catalytic cavity. Rosmarinic acid occupied a similar binding region but established a broader interaction network involving Lys191, Tyr197, Tyr218, Ser62, Asp61, and Glu214, together with multiple hydrophobic contacts involving Leu206, Lys196, and Pro20. The larger number of favorable interactions observed for rosmarinic acid is consistent with its lower predicted binding free energy and inhibition constant obtained from the docking calculations.

Although both phenolic acids occupy similar regions within the arginase active site, the more extensive interaction network formed by rosmarinic acid suggests a greater capacity for stabilizing the enzyme–ligand complex.

Taken together, the interaction analyses support the hypothesis that the inhibitory activity of the investigated phenolic acids is primarily governed by the cooperative action of hydrogen bonding and hydrophobic interactions.

Limitations of the Study

Although this study provides the first integrated phytochemical characterization together with the evaluation of antioxidant, enzyme inhibitory, and molecular docking properties of *Hyssopus officinalis* subsp. *aristatus* from Montenegro, several limitations should be acknowledged. First, the biological activities were evaluated exclusively using *in vitro* assays and therefore do not fully reflect the complexity of biological systems, including bioavailability, metabolism, and pharmacokinetic processes occurring *in vivo*. Second, only the two quantitatively dominant phenolic constituents, rosmarinic acid and chlorogenic acid, were individually evaluated, whereas the possible contribution of other tentatively identified compounds and their synergistic interactions within the extract remains to be clarified. Finally, molecular docking provided theoretical predictions based on static protein structures and estimated binding energies. Consequently, docking scores and predicted inhibition constants cannot be directly interpreted as measures of experimental inhibitory potency. Moreover, the simulations did not fully account for protein conformational flexibility, solvent effects, or the dynamic behaviour of ligand–protein complexes. Therefore, the docking results should be considered supportive of the experimental findings rather than conclusive evidence of the proposed mechanisms of enzyme inhibition. Further studies, including cellular models, *in vivo* investigations, molecular dynamics simulations, and biochemical validation experiments, are required to validate the biological effects and clarify the precise mechanisms of action of this plant extract.

Conclusions

The results highlight *H. officinalis* subsp. *aristatus* as a valuable source of multifunctional phenolic compounds with antioxidant properties and selective enzyme inhibitory potential under *in vitro* conditions. The combination of antioxidant and metabolic activities underscores its relevance for further research in the context of oxidative stress-related and metabolic disorders. Importantly, these preliminary findings suggest that *H. officinalis* subsp. *aristatus* could provide initial insights into its potential as a natural source of bioactive compounds for future studies. Since these findings are based on *in vitro* models, they represent a positive first step, while further studies will help fully understand their therapeutic or nutraceutical potential. Docking simulations provide only structural predictions of ligand–enzyme interactions and should therefore be considered complementary to,

rather than a substitute for, experimental enzyme inhibition studies. Future research is required to isolate individual active constituents, clarify potential synergistic effects, and validate the observed activities in cellular and *in vivo* models.

Acknowledgement

Not applicable.

Funding

The present study was supported by the Ministry of Science, Technological Development, and Innovation of the Republic of Serbia through two grant agreements with the University of Belgrade – Faculty of Pharmacy (Nos. 451-03-33/2026-03/200161 and 451-03-34/2026-03/200161), one grant agreement with the University of Belgrade – Faculty of Biology (No. 451-03-136/2025-03/200178), and one grant agreement with the Institute for Information Technologies, University of Kragujevac (No. 451-03-33/2026-03/200378).

Availability of data and materials

The data generated in the present study are included in the figures and tables of this article.

Ethics approval and consent to participate

Not applicable.

AI use disclosure

During the preparation of this manuscript, the authors used ChatGPT for editing assistance and language improvement, specifically to improve spelling, grammar, word choice, readability, and clarity. The authors reviewed and revised all outputs from these tools and take full responsibility for the final content of the work.

Conflict of interest

The authors declare no conflict of interest.

References

1. Atanasov AG, Waltenberger B, Pferschy-Wenzig EM, Linder T, Wawrosch C, Uhrin P, et al. Discovery and resupply of pharmacologically active plant-derived natural products: a review. Biotechnol Adv. 2015;33(8):1582-1614.
2. Shrivastav D, Kumbhakar SK, Srivastava S, Singh DD. Natural product-based treatment potential for type 2 diabetes mellitus and cardiovascular disease. World J Diabetes. 2024;15(7):1603-1614.
3. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev. 2009;2(5):270-278.
4. Tundis R, Loizzo MR, Menichini F. Natural products as α-amylase and α-glucosidase inhibitors and their hypoglycaemic potential in the treatment of diabetes: an update. Mini Rev Med Chem. 2010;10(4):315-331.
5. Jakimiuk K, Gesek J, Atanasov AG, Tomczyk M. Flavonoids as inhibitors of human neutrophil elastase. J Enzyme Inhib Med Chem. 2021;36(1):1016-1028.
6. Durante W, Johnson FK, Johnson RA. Arginase: a critical regulator of nitric oxide synthesis and vascular function. Clin Exp Pharmacol Physiol. 2007;34(9):906-911.
7. Aboalhaija N, Ameereh H, Hammad A, Sunoqrot S, Syaj H, Abaza I, et al. Phytochemical profile and antiinflammatory potential of Verbascum sinaiticum grown in Jordan: in vitro and in vivo validations. Farmacia. 2025;73(3):614-627.
8. Tleubayeva MI, Datkhayev UM, Malm A, Jozefczyk A, Wojtanowski K, Seitaliyeva AM, et al. Purslane (Portulaca oleracea L.) growing in South Kazakhstan – phytochemical composition of ultrasonic hydroethanolic extract and its pharmacological activity. Farmacia. 2025;73(3):665-675.
9. Vlase L, Benedec D, Hanganu D, Damian G, Csillag I, Sevastre B, et al. Evaluation of antioxidant and antimicrobial activities and phenolic profile for Hyssopus officinalis, Ocimum basilicum and Teucrium chamaedrys. Molecules. 2014;19(5):5490-5507.
10. Salamon I, Kryvtsova M, Bucko D, Tarawneh AH. Chemical characterization and antimicrobial activity of some essential oils after their industrial large-scale distillation. J Microbiol Biotechnol Food Sci. 2019;8(4):984-988.
11. Kizil S, Hasimi N, Tolan V, Kilinc E, Karatas H. Chemical composition, antimicrobial and antioxidant activities of hyssop (Hyssopus officinalis L.) essential oil. Not Bot Horti Agrobot Cluj Napoca. 2010;38(3):99-103.
12. Džamić AM, Soković MD, Novaković M, Jadranin M, Ristić MS, Tešević V, et al. Composition, antifungal and antioxidant properties of Hyssopus officinalis L. subsp. pilifer (Pant.) Murb. essential oil and deodorized extracts. Ind Crops Prod. 2013;51:401-407.
13. Venditti A, Bianco A, Frezza C, Conti F, Bini LM, Giuliani C, et al. Essential oil composition, polar compounds, glandular trichomes and biological activity of Hyssopus officinalis subsp. aristatus (Godr.) Nyman from central Italy. Ind Crops Prod. 2015;77:353-363.
14. Mićović T, Topalović D, Živković L, Spremo-Potparević B, Jakovljević V, Matić S, et al. Antioxidant, antigenotoxic and cytotoxic activity of essential oils and methanol extracts of Hyssopus officinalis L. subsp. aristatus (Godr.) Nyman (Lamiaceae). Plants (Basel). 2021;10(4):711.
15. Mićović T, Stanković JSK, Bauer R, Nöst X, Marković Z, Milenković D, et al. In vitro, in vivo and in silico evaluation of the anti-inflammatory potential of Hyssopus officinalis L. subsp. aristatus (Godr.) Nyman (Lamiaceae). J Ethnopharmacol. 2022;293:115201.

16. Judžentienė A. Hyssop (Hyssopus officinalis L.) oils. In: Preedy VR, editor. Essential oils in food preservation, flavor and safety. London: Academic Press; 2016. p. 471-479.
17. Tucakov J. Lečenje biljem. Beograd: Zapis; 2010.
18. Srivastava A, Awasthi K, Kumar B, Misra A, Srivastava S. Pharmacognostic and pharmacological evaluation of Hyssopus officinalis L. (Lamiaceae) collected from Kashmir Himalayas, India. Pharmacogn J. 2018;10(4):690-693.
19. Aihaiti K, Li J, Yaermaimaiti S, Liu L, Xin X, Aisa HA. Non-volatile compounds of Hyssopus cuspidatus Boriss and their antioxidant and antimicrobial activities. Food Chem. 2022;374:131638.
20. Alves-Silva JM, Pedreiro S, Cavaleiro C, Cruz MT, Figueirinha A, Salgueiro L. Effect of Thymbra capitata (L.) Cav. on inflammation, senescence and cell migration. Nutrients. 2023;15(8):1930.
21. Christaki S, Bouloumpasi E, Lalidou E, Chatzopoulou P, Irakli M. Bioactive profile of distilled solid by-products of rosemary, Greek sage and spearmint as affected by distillation methods. Molecules. 2022;27(24):9058.
22. Fuji Y, Uchida A, Fukahori K, Chino M, Ohtsuki T, Matsufuji H. Chemical characterization and biological activity in young sesame leaves (Sesamum indicum L.) and changes in iridoid and polyphenol content at different growth stages. PLoS One. 2018;13(3):e0194449.
23. Göğer F, Özek G, Tekin M, Yur S, Özek T. Phytochemical profiling and evaluation of Marrubium sivasense Aytaç, Akgül & Ekici for antioxidant activity and inhibition effects on α-amylase, lipoxygenase, xanthine oxidase and tyrosinase enzymes. JOTCSA. 2019;6(3):281-292.
24. Jaiswal R, Kuhnert N. How to identify and discriminate between the methyl quinates of chlorogenic acids by liquid chromatography–tandem mass spectrometry. J Mass Spectrom. 2011;46(3):269-281.
25. Li C, Yang J, Tong X, Zhao C, He Y, Wan H. Precursor ion scan enhanced rapid identification of the chemical constituents of Danhong injection by liquid chromatography–tandem mass spectrometry: an integrated strategy. J Chromatogr A. 2019;1602:378-385.
26. Liu M, Li YG, Zhang F, Yang L, Chou GX, Wang ZT, et al. Chromatographic fingerprinting analysis of Danshen root (Salvia miltiorrhiza Radix et Rhizoma) and its preparations using high performance liquid chromatography with diode array detection and electrospray mass spectrometry (HPLC-DAD-ESI/MS). J Sep Sci. 2007;30(14):2256-2267.
27. Mateos R, Baeza G, Sarriá B, Bravo L. Improved LC-MSn characterization of hydroxycinnamic acid derivatives and flavonols in different commercial mate (Ilex paraguariensis) brands. Quantification of polyphenols, methylxanthines, and antioxidant activity. Food Chem. 2018;241:232-241.
28. Zhang ZC, Xu M, Sun SF, Qiao X, Wang BR, Han J, et al. Metabolic analysis of four phenolic acids in rat by liquid chromatography–tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2008;871(1):7-14.
29. Zeng G, Xiao H, Liu J, Liang X. Identification of phenolic constituents in radix Salvia miltiorrhizae by liquid chromatography/electrospray ionization mass spectrometry. Rapid Commun Mass Spectrom. 2006;20(3):499-506.
30. Zhu L, Ma S, Li K, Xiong P, Qin S, Cai W. Systematic screening of chemical constituents in the traditional Chinese medicine Arnebiae radix by UHPLC-Q-Exactive orbitrap mass spectrometry. Molecules. 2022;27(9):2631.
31. Xiao F, Xu T, Lu B, Liu R. Guidelines for antioxidant assays for food components. Food Front. 2020;1(1):60-69.
32. Lekouaghet A, Boutefnouchet A, Bensuici C, Gali L, Ghenaiet K, Tichati L. In vitro evaluation of antioxidant and anti-inflammatory activities of the hydroalcoholic extract and its fractions from Leuzea conifera L. roots. S Afr J Bot. 2020;132:103-107.
33. Mokrani A, Krisa S, Cluzet S, Da Costa G, Temsamani H, Renouf E, et al. Phenolic contents and bioactive potential of peach fruit extracts. Food Chem. 2016;202:212-220.
34. Zengin G, Sarikurkcu C, Aktumsek A, Ceylan R, Ceylan O. A comprehensive study on phytochemical characterization of Haplophyllum myrtifolium Boiss. endemic to Turkey and its inhibitory potential against key enzymes involved in Alzheimer, skin diseases and type II diabetes. Ind Crops Prod. 2014;53:244-251.
35. Wan LS, Min QX, Wang YL, Yue YD, Chen JC. Xanthone glycoside constituents of Swertia kouitchensis with α-glucosidase inhibitory activity. J Nat Prod. 2013;76(7):1248-1253.
36. Zedet A, Kanga Y, Pudlo M, Senejoux F, Girard C. Arginase inhibitory activities of chemical constituents from Macaranga hurifolia Beille leaves. Nat Prod Res. 2024;38(23):4286-4291.
37. Marchisio L, Gaudillat Q, Muller J, Zedet A, Tissot M, Harakat D, et al. Synthesis and evaluation of piceatannol derivatives as novel arginase inhibitors with radical scavenging activity and their potential for collagen reduction in dermal fibroblasts. Eur J Med Chem. 2025;287:117376.
38. Lee KK, Kim JH, Cho JJ, Choi JD. Inhibitory effects of 150 plant extracts on elastase activity, and their anti-inflammatory effects. Int J Cosmet Sci. 1999;21(2):71-82.
39. Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, et al. AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. J Comput Chem. 2009;30(16):2785-2791.
40. Gilles C, Astier JP, Marchis-Mouren G, Cambillau C, Payan F. Crystal structure of pig pancreatic α-amylase isoenzyme II, in complex with the carbohydrate inhibitor acarbose. Eur J Biochem. 1996;238(2):561-569.
41. Barker MK, Rose DR. Specificity of processing α-glucosidase I is guided by the substrate conformation: crystallographic and in silico studies. J Biol Chem. 2013;288(19):13563-13574.
42. Di Costanzo L, Ilies M, Thorn KJ, Christianson DW. Inhibition of human arginase I by substrate and

product analogues. Arch Biochem Biophys. 2010;496(2):101-108.
43. Dassault Systèmes BIOVIA. Discovery Studio Modeling Environment, Release 2017 R2 [software]. San Diego: Dassault Systèmes; 2017.
44. Zhang Y, Forli S, Omelchenko A, Sanner MF. AutoGridFR: improvements on AutoDock affinity maps and associated software tools. J Comput Chem. 2019;40(32):2882-2886.
45. Shomirzoeva O, Li J, Numonov S, Mamat N, Shataer D, Lu X, et al. Chemical components of Hyssopus seravshanicus: antioxidant activity, activations of melanogenesis and tyrosinase, and quantitative determination by UPLC-DAD. Nat Prod Res. 2019;33(6):866-870.
46. Grzegorczyk-Karolak I, Krzemińska M, Kiss AK, Olszewska MA, Owczarek A. Phytochemical profile and antioxidant activity of aerial and underground parts of Salvia bulleyana Diels. plants. Metabolites. 2020;10(12):497.
47. Taamalli A, Arráez-Román D, Abaza L, Iswaldi I, Fernández-Gutiérrez A, Zarrouk M, et al. LC-MS-based metabolite profiling of methanolic extracts from the medicinal and aromatic species Mentha pulegium and Origanum majorana. Phytochem Anal. 2015;26(5):320-330.
48. Borrelli F, Pagano E, Formisano C, Piccolella S, Fiorentino A, Tenore GC, et al. Hyssopus officinalis subsp. aristatus: an unexploited wild-growing crop for new disclosed bioactives. Ind Crops Prod. 2019;140:111594.
49. Fathiazad F, Mazandarani M, Hamedeyazdan S. Phytochemical analysis and antioxidant activity of Hyssopus officinalis L. from Iran. Adv Pharm Bull. 2011;1(2):63-67.
50. Hamoud NEH, Maammeri Z. Comprehensive analysis of bioactive compounds in Hyssopus officinalis L. and evaluation of its antioxidant, antibacterial, and anti-Alzheimer properties. J Herbmed Pharmacol. 2025;14(1):71-81.
51. Topal M, Gulcin İ. Evaluation of the in vitro antioxidant, antidiabetic and anticholinergic properties of rosmarinic acid from rosemary (Rosmarinus officinalis L.). Biocatal Agric Biotechnol. 2022;43:102417.
52. Popov AM, Osipov AN, Korepanova EA, Krivoshapko ON, Artyukov AA. Study of antioxidant and membrane activity of rosmarinic acid using different model systems. Biophysics. 2013;58(5):607-615.
53. Hatipoğlu G, Sökmen M, Bektaş E, Daferera D, Sökmen A, Demir E, et al. Automated and standard extraction of antioxidant phenolic compounds of Hyssopus officinalis L. ssp. angustifolius. Ind Crops Prod. 2013;43:427-433.
54. Berton-Carabin C, Villeneuve P. Targeting interfacial location of phenolic antioxidants in emulsions: strategies and benefits. Annu Rev Food Sci Technol. 2023;14:63-83.
55. Kiokias S, Oreopoulou V. Review on the antioxidant activity of phenolics in o/w emulsions along with the impact of a few important factors on their interfacial behaviour. Colloids Interfaces. 2022;6(4):79.
56. Lebovitz HE. Alpha-glucosidase inhibitors. Endocrinol Metab Clin North Am. 1997;26(3):539-551.
57. Matsuura H, Miyazaki H, Asakawa C, Amano M, Yoshihara T, Mizutani J. Isolation of α-glucosidase inhibitors from hyssop (Hyssopus officinalis). Phytochemistry. 2004;65(1):91-97.
58. Miyazaki H, Matsuura H, Yanagiya C, Mizutani J, Tsuji M, Ishihara C. Inhibitory effects of hyssop (Hyssopus officinalis) extracts on intestinal α-glucosidase activity and postprandial hyperglycemia. J Nutr Sci Vitaminol (Tokyo). 2003;49(5):346-349.
59. Loizzo MR, Saab AM, Tundis R, Menichini F, Bonesi M, Piccolo V, et al. In vitro inhibitory activities of plants used in Lebanon traditional medicine against angiotensin converting enzyme (ACE) and digestive enzymes related to diabetes. J Ethnopharmacol. 2008;119(1):109-116.
60. Zhu F, Asada T, Sato A, Koi Y, Nishiwaki H, Tamura H. Rosmarinic acid extract for antioxidant, antiallergic, and α-glucosidase inhibitory activities, isolated by supramolecular technique and solvent extraction from Perilla leaves. J Agric Food Chem. 2014;62(4):885-892.
61. Lin YT, Lin HR, Yang CS, Liaw CC, Sung PJ, Kuo YH, et al. Antioxidant and anti-α-glucosidase activities of various solvent extracts and major bioactive components from the fruits of Crataegus pinnatifida. Antioxidants (Basel). 2022;11(2):320.
62. Nyambe-Silavwe H, Williamson G. Chlorogenic and phenolic acids are only very weak inhibitors of human salivary α-amylase and rat intestinal maltase activities. Food Res Int. 2018;113:452-455.
63. Kwon YI, Apostolidis E, Shetty K. Inhibitory potential of wine and tea against α-amylase and α-glucosidase for management of hyperglycemia linked to type 2 diabetes. J Food Biochem. 2008;32(1):15-31.
64. Caldwell RB, Toque HA, Narayanan SP, Caldwell RW. Arginase: an old enzyme with new tricks. Trends Pharmacol Sci. 2015;36(6):395-405.
65. Kovamees O, Shemyakin A, Eriksson M, Angelin B, Pernow J. Arginase inhibition improves endothelial function in patients with familial hypercholesterolaemia irrespective of their cholesterol levels. J Intern Med. 2016;279(5):477-484.
66. Korkmaz B, Horwitz MS, Jenne DE, Gauthier F. Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. Pharmacol Rev. 2010;62(4):726-759.