



Ajuga vestita Boiss: a “natural gold mine” for the design of novel nutraceuticals and phytopharmaceuticals

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Abstract

Members of the *Ajuga* genus are of significant interest due to their traditional applications in various countries, and have therefore been the subject of phytochemical research investigating their natural bioactive compounds. The aim of this study was to determine the chemical composition and biological activities of various *Ajuga vestita* extracts (ethyl acetate, ethanol, ethanol/water and water). The biological effects assessed included antioxidant, enzyme inhibition and cytotoxic activities. The extracts were chemically profiled using ultra-high-performance liquid chromatography – electrospray ionization – quadrupole time-of-flight mass spectrometry (UHPLC-ESI-QTOF-MS), which identified 121 compounds, including iridoids, flavonoids, and phenolic acids. The ethanol/water extract exhibited superior radical scavenging activity (2,2-Diphenyl-1-picrylhydrazyl (DPPH): 43.13 mg Trolox equivalent (TE)/g; 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS): 45.37 mg TE/g) and reducing power (cupric reducing antioxidant capacity (CUPRAC): 103.43 mg TE/g; ferric reducing antioxidant power (FRAP): 45.29 mg TE/g). Regarding enzyme inhibition, the ethanol extract exhibited the greatest inhibition of acetylcholinesterase (AChE), butyrylcholinesterase (BChE), glucosidase, and tyrosinase, whereas the ethyl acetate extract was most effective against amylase. Regarding cytotoxicity, the ethyl acetate extract exhibited the strongest effect, significantly reducing cell viability in all cell lines and having a particularly strong impact on murine bone marrow stromal (S17) cells, with viability at $30.8 \pm 2.7\%$ at a concentration of 100 $\mu\text{g/mL}$. By contrast, the aqueous extract exhibited low cytotoxicity, maintaining high cell viability (over 94% at 50 $\mu\text{g/mL}$ and over 74% at 100 $\mu\text{g/mL}$). Additionally, network pharmacology, molecular docking and molecular dynamics simulations were employed to investigate the multi-target mechanisms and binding stability of *A. vestita* phytochemicals. These findings provide new, detailed insights into *A. vestita* and suggest its potential as a raw material for developing health-promoting nutraceutical and pharmaceutical products.

Keywords *Ajuga* · phenolic · iridoids · oxidative stress, diabetes mellitus · in silico studies · phyto-pharmaceuticals

Introduction

The exploration of natural products remains a pivotal aspect of contemporary drug discovery and development due to the unique structural diversity and biological activity of naturally derived compounds. These molecules, which have evolved over thousands of years for specific ecological purposes, offer privileged scaffolds that are often unattainable through synthetic chemistry. They act as essential lead compounds or direct treatments for numerous diseases, with

a particular emphasis on cancer and infectious diseases. Recent progress is reshaping the field by addressing traditional obstacles like supply and rediscovery. This includes employing next-generation sequencing and bioinformatics to study the complex biosynthetic gene clusters of unculturable microbes, and utilizing metagenomics to uncover novel chemical entities. Moreover, synthetic biology facilitates the heterologous expression of these gene clusters in manageable host organisms for sustainable production. The integration of AI and machine learning with genomic and

metabolomic data expedites the identification and prediction of bioactive compounds, while advanced analytical methods such as NMR-based dereplication and genome mining enhance the discovery process. This ensures that natural products remain integral to the development of innovative therapeutic agents [1, 2].

The genus *Ajuga* in the Lamiaceae family encompasses around 300 species, showcasing significant morphological diversity and distributed throughout Europe, Asia, Africa, Australia, and North America [3]. Turkey plays a crucial role for this genus, housing 13 species, with six being endemic [3]. *Ajuga* species have a longstanding tradition in ethnomedicine, utilized across various cultures to address ailments such as fever, malaria, hypertension, diabetes, gastrointestinal issues, and toothache [4]. For instance, *A. integrifolia* has long been used in traditional medicine to manage various conditions, especially high blood pressure, for which a decoction of the leaves is taken as a medicinal tea [5]. Additionally, *A. chamaepitys* has been employed in the treatment of menstrual irregularities, hemorrhoids, diabetes, stomach pain, gout, and joint discomfort [6]. The leaves of *A. bracteosa* are also used locally to help treat headaches, acne, measles, gastric acidity, jaundice, high blood pressure, sore throat, burns, and for purifying the blood [7]. These traditional uses are corroborated by pharmacological activities like anti-inflammatory, diuretic, antifungal, anthelmintic, and antimycobacterial effects [4]. The diverse bioactivities of *Ajuga* plants are attributed to a rich array of secondary metabolites such as terpenes, ecdysteroids, flavonoids, iridoids, withanolides, sterols, anthocyanidins, triglycerides, and essential oils [8]. These compounds offer a wide range of biological and pharmacological effects [8]. Despite these promising ethnobotanical insights and chemical diversity, many *Ajuga* species remain understudied in terms of phytochemistry and pharmacology, offering considerable potential for further investigation and novel therapeutic developments [9]. *Ajuga vestita* Boiss., a rare endemic species, is confined to Turkey's Southeastern Anatolia region [10]. Its endemic nature necessitates conservation efforts. Accordingly, Akbaş et al. [10] developed an effective micro-propagation protocol for *A. vestita*, examining the impact of plant growth regulators, specifically benzylaminopurine (BAP) and kinetin (Kin), on its in vitro growth. Additionally, the same team demonstrated that callus cultures from leaf explants—particularly those grown on media enhanced with BAP and Kin—yield extracts rich in unsaturated fatty acids [11]. This underscores the potential of in vitro systems for both conservation and the focused production of valuable metabolites.

The literature data showed that *A. vestita* is not an unexplored species in terms of chemical profile and biological activities. This study aimed to evaluate the pharmaceutical

potential of *A. vestita* by analysing its antioxidant and enzyme inhibitory effects, anticancer activity against the S17, RAW and HepG2 cell lines, and the phytochemical content of its bioactive extracts. We also employed network pharmacology, structure-based molecular docking and all-atom molecular dynamics simulations to elucidate the putative multi-target mechanisms underlying *A. vestita* bioactivity.

Materials and methods

Plant collection

Plant specimens were collected in 2022 from the Mardin province (Between Mardin and Nusaybin, 8. Km, 860 m) in Turkey. Dr Maruf Balos carried out the formal botanical classification of the material. A voucher specimen, under the code 4262, was placed in the Faculty of Science at Harran University. After collection, the aerial parts were separated immediately and air-dried at room temperature in a shaded, well-ventilated area. After complete drying, the plant material was milled to a fine powder. To ensure chemical integrity and prevent deterioration, the powdered samples were sealed in opaque containers and stored under stable conditions.

Plant extract preparation

Four solvent systems were used to obtain bioactive constituents: ethyl acetate, ethanol, 70% (v/v) ethanol-water, and distilled water. For each extraction, 10 g of the prepared plant material was mixed with 200 mL of the corresponding solvent. The methodology was adapted according to the properties of the solvent. Extractions using organic solvents were performed via maceration for 24 h under ambient conditions. In contrast, the water-based extraction utilised a 15-minute infusion with heated water. Following extraction, the products obtained were concentrated using distinct techniques. The aqueous extract was stabilised by lyophilisation, while the organic solvent fractions were recovered by evaporating the solvents under reduced pressure using a rotary evaporator.

Assay for total phenolic and flavonoid content

Total phenolic and flavonoid contents of the extracts were determined using established colorimetric assays, following the referenced procedure. Calibration curves were constructed using gallic acid (mg gallic acid equivalents (GAE)/g) and rutin (mg rutin equivalents (RE)/g) as standards [12].

Assays for in vitro antioxidant capacity

Antioxidant capacity was assessed using a panel of in vitro assays, following a previously described protocol [13]. Reducing power was evaluated using FRAP (ferric reducing antioxidant power) and CUPRAC (cupric reducing antioxidant capacity), while radical scavenging was measured using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assays. Results from these four assays were quantified and standardized to Trolox and reported as mg Trolox equivalents (TE) per g of dried extract (mg TE/g). Trolox and expressed as milligrams of Trolox equivalent per gram of dried extract (mg TE/g). Total antioxidant capacity was additionally determined by the phosphomolybdenum method (PBD assay) and expressed as mmol Trolox equivalents per g (mmol TE/g). Finally, metal-chelating activity was evaluated using a chelation assay and reported as mg EDTA equivalents (EDTAE) per g of extract (mg EDTAE/g).

Inhibitory effects against some key enzymes

The enzyme inhibitory potential of the extracts was evaluated against five key targets; acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase, α -amylase and α -glucosidase; using established colorimetric procedures [13]. To standardise the results, inhibition was quantified using established reference compounds. Cholinesterase inhibitory activity was expressed as mg galanthamine equivalents (GALAE) per g of extract (mg GALAE/g). α -Amylase and α -glucosidase inhibitory activities were expressed as mg acarbose equivalents (ACAE) per g (mg ACAE/g), while tyrosinase inhibition was reported as mg kojic acid equivalents (KAE) per g (mg KAE/g).

UHPLC-ESI-QTOF-MS analysis and data curation

The different extracts obtained from *A. vestita* were prepared at a concentration of 5000 μ g/mL in the same solvent applied during extraction and then filtered through 0.22 μ m. The analyses were carried out using an ACQUITY UHPLC H-Class System (Waters Corp.) coupled to a QTOF-MS (Synapt G2, Waters Corp.) with a reversed-phase column (ACQUITY UHPLC BEH SHIELD RP18, 130 Å, 1.7 μ m, 2.1 mm $\times$ 150 mm) at 22 °C. The multi-step gradient used was previously reported by Ahmed et al., 2025 (<https://doi.org/10.1007/s11130-025-01314-x>) using acidified water with acetic acid (0.5%v/v) (eluent A) and acetonitrile (eluent B). Additionally, the mass spectrometer coupled to UHPLC were set considering a m/z range 50–1200 m/z; negative ionization mode; capillary voltage, 2.2 kV; cone voltage,

30 V; desolvation temperature, 500 °C; desolvation gas flow, 700 L/h; cone gas flow, 50 L/h; source temperature, 100°C; scan duration 0.1s; resolution, 20,000 full width at half maximum. The fragmentation of ion were acquitted using two parallel scan function with different collision energies 4, 20 and 60 eV.

The tentative identification of analytes detected by the analytical platforms were performed using the open-source software MZmine V4.08. First, the raw data were converted to mzML format using the MSConvertGUI software (v3.0.24). The signal filtering was adjusted considering a noise level of 5E3 and the base-peak chromatograms were obtained by an automated data analysis pipeline (ADAP) setting the m/z tolerance at 15ppm and a minimum highest intensity at 1E4. Additionally, the chromatograms were, deconvoluted using the local minimum resolver algorithm setting the following parameters: S/N threshold: 60; minimum feature height: 2E4; coefficient/area threshold: 110; peak duration range: 0.05–0.4 min; RT wavelet range: 0–0.05. Furthermore, an isotope grouper algorithm was also applied, and the obtained features were aligned using a m/z tolerance of 15ppm and a RT tolerance of 0.25 min. Finally, the phytochemical identification of the obtained features was determined using Sirius software (v6.1.0) and comparing the obtained molecular formulas and MS/MS spectra in different databases (MoNA, HMDB, SciFinder).

Cell culture

The human hepatocellular carcinoma (HepG2) and murine bone marrow stromal (S17) cell lines were provided by the Center for Biomedical Research (CBMR) at the University of Algarve, Portugal. The murine leukemic macrophage cell line (RAW264.7) was obtained from CLS Cell Line Service GmbH (Germany). All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% antibiotic solution (50 U/mL penicillin and 50 μ g/mL streptomycin). Cultures were incubated at 37 °C in a humidified atmosphere with 5% CO₂.

Assessment of cell viability

Cytotoxicity was evaluated in HepG2, RAW 264.7, and S17 cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates at densities of 5×10^3 cells/well (HepG2 and S17) and 1×10^4 cells/well (RAW 264.7) in 100 μ L of culture medium and incubated overnight to allow cell adhesion. After attachment, cells were treated with the test extracts at concentrations of 50 and 100 μ g/mL for 72 h. Extracts were dissolved in DMSO and diluted in culture medium so that

the final DMSO concentration did not exceed 0.5% (v/v), which served as vehicle control. After the treatment period, 20 μ L of MTT solution (5 mg/mL) was added to each well and plates were incubated for an additional 2 h at 37 °C to allow the formation of formazan crystals. The medium was then removed, and the crystals were dissolved in 150 μ L of DMSO. Absorbance was measured at 595 nm using a Biochrom EZ Read 400 microplate reader. Cell viability was expressed as a percentage relative to vehicle control.

Network pharmacology

Disease targets and acquitting intersection targets

For target prediction, we evaluated 36 phytochemicals using two complementary in-silico platforms: the Similarity Ensemble Approach (SEA) v3.0 and SwissTargetPrediction. These molecules were selected based on their relative abundance in the source plant, structural uniqueness, and literature-supported pharmacological potential. Target lists obtained from both tools were concatenated and de-duplicated to yield a non-redundant gene set [14, 15]. Disease-associated genes were retrieved from the Comparative Toxicogenomics Database (CTD). The intersection between compound-associated and disease-related gene sets was then computed with EVenn (<https://www.bic.ac.cn/test/venn/#/>), enabling the identification of shared targets that may underlie the putative therapeutic mechanism [16, 17].

Protein-Protein interaction network construction

Predicted target proteins were mapped to a protein-protein interaction (PPI) network using STRING with Homo sapiens specified as the reference organism and a minimum required interaction score of 0.700 to retain high-confidence edges. The interaction table was exported in .txt format and imported into Cytoscape v3.10.3 for visualization and network topology assessment. Regulatory hubs were prioritized with the cytoHubba extension, ranking nodes by Maximal Clique Centrality (MCC) and corroborating with node degree; hub candidates were then carried forward for downstream functional analyses [18].

Functional annotation of common genes

Functional annotation and enrichment of the overlapping targets were performed with DAVID (Database for Annotation, Visualization and Integrated Discovery) v6.8. We queried Gene Ontology (GO) categories, focusing on Biological Process (BP), alongside Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment [17]. Terms and pathways reaching $p < 0.05$ were considered

significant [18]. To emphasize the most informative categories, we selected the top 10 GO BP terms and top 10 KEGG pathways ranked by gene count and visualized them as bar plots [19]. All figures, including detailed KEGG pathway depictions, were generated in R v4.3.3.

Molecular docking

Molecular docking was carried out to assess the binding propensities of key phytochemicals identified from *Ajuga vestita*, including Lamiuside E, Lamiuside D, Lamiuside B, Lamiuside A, Shaftoside, Lamiophlomiside A, Lamiuside C, Lunarioside, Leonoside B, Cistanoside D, Garcinone B, Parvifoline H, Plantamoside, Andrographiside, Eriobioside, Ajugapitin, Phlomuroside, Ipolamiide, Scroside D, Harpagide, Phlorigidoside B, Hispidulin, Auroside, Shanzhiside, Sweroside, Ajugol, Decaloside, Loganic acid, Melilotoside, Kenposide B, Kenposide, Androsin, Sodononin, Erostoside, Lactucopicrin, and Linaride. The docking panel comprised selected therapeutic targets spanning metabolic enzymes, pathogenic proteins, and cancer related biomolecules. Three dimensional crystal structures for targets such as AChE [20], BChE [21], tyrosinase, α -amylase, and α -glucosidase [22–24] and proteins implicated in rheumatoid arthritis, sepsis, and ulcerative colitis were retrieved from the Protein Data Bank PDB. Ligand structures were sourced from PubChem and SpectraBase. Full target details, PDB identifiers, and docking parameters are summarized in Supplementary Table S2. Protein preparation was performed in BIOVIA Discovery Studio and AutoDockTools v4.2.6. Ligand geometries were optimized with Avogadro v1.2.0; hydrogens were merged and Gasteiger partial charges assigned prior to docking. Simulations were executed in AutoDock Vina v1.1.2 with exhaustiveness set to 32. Binding pockets were defined using cavity predictions from the POCASA v1.1 server. For protocol validation, cocrystallized ligands were redocked into their native binding sites and root mean square deviation RMSD values were computed to evaluate pose reproducibility [25, 26]. Protein ligand contact patterns such as hydrogen bonds, hydrophobic contacts, and pi stacking interactions were examined with the Protein Ligand Interaction Profiler PLIP [27, 28]. These steps ensured appropriate structural curation and computational robustness of the docking outcomes.

Molecular dynamic simulation

Molecular dynamics MD simulations were performed to evaluate the dynamic stability and binding behavior of selected protein ligand complexes. Initial system setup was conducted with the CHARMM GUI (<https://charmm-gui.org>) [29, 30]. Proteins and ligands were parameterized with

the CHARMM36m force field [31], solvated in an explicit TIP3P transferable intermolecular potential three point water box, and electrically neutralized by adding counterions. Bulk ionic strength was maintained at 0.15 M NaCl. Nonbonded interactions used the Verlet cutoff scheme, and bonds involving hydrogen atoms were constrained with the LINCS linear constraint solver algorithm. Long range electrostatics were computed with the Particle Mesh Ewald PME method. Energy minimization employed the steepest descent procedure until the maximum force was below 1000 kJ per mol per nm. Equilibration proceeded in two stages, first under an NVT constant number volume temperature ensemble and then under an NPT constant number pressure temperature ensemble at 310 K. Production trajectories were generated for 100 ns with GROMACS 2023. Following the simulations, binding free energies were estimated using gmx_MMPBSA molecular mechanics Poisson Boltzmann surface area to obtain quantitative measures of ligand affinity and complex stability [32, 33]. By coupling extensive configurational sampling with end point free energy analysis, this workflow provided a detailed account of the molecular factors that govern the interactions between the selected phytochemicals and their protein targets.

Statistical analysis

All experiments were conducted in triplicate, and differences among extracts were evaluated for statistical significance. One-way ANOVA was performed in GraphPad Prism (version 9.2), followed by Tukey's post hoc test for pairwise comparisons. Statistical significance was set at $p < 0.05$.

Results and discussion

Chemical composition

Phenolic compounds serve as multifunctional agents, with applications extending from food preservation to exerting biological effects [34]. This makes them appealing for pharmaceutical and nutraceutical uses. In this research, we assessed the total phenolic and flavonoid content of *A.*

vestita extracts using colorimetric techniques, with findings detailed in Table 1. The ethanol extract exhibited the highest total phenolic content at 37.34 mg GAE/g, followed sequentially by water, ethanol/water, and ethyl acetate extracts. Conversely, the ethyl acetate extract demonstrated the highest total flavonoid content at 21.43 mg RE/g. These findings imply that the total phenolic and flavonoid contents in *A. vestita* are influenced by the choice of extraction solvent. Supporting our findings, numerous studies have identified ethanol as an effective solvent for phenolic extraction, also noting its 'green' solvent status due to reduced toxicity. For example, Saidi et al. [35] investigated biological properties of three extracts of *A. iva* and the highest total phenolic level was obtained by the hydroethanolic extract with 69.85 mg GAE/g. Similarly, Mimi et al. [36] reported that the ethanolic extract contained higher level of the total phenolic content compared to water extract. Among *A. integrifolia* extracts, the ethanol and methanol extracts contained higher levels of the total phenolic than water extract [37]. In another study by Rebhi et al. [38], the total phenolic content in the ethanol extract of *A. iva* was found to be 184.66 mg GAE/g extract. The extracts of *A. reptans* were examined by Diziki et al. [39] for their chemical profile and biological effects and the total phenolic contents in the hydroethanolic extracts varied from 204.53 to 315.49 mg GAE/g. Al-Qudah et al. [40] reported that the total phenolic levels in the methanol and butanol fractions were 52.35 mg GAE/g and 217.63 mg GAE/g, respectively. Variations in total phenolic and flavonoid content among *Ajuga* species have been reported, attributable to factors like altitude, soil composition, and annual rainfall [41]. However, the Folin-Ciocalteu and AlCl₃ assays have been criticized for their potential inaccuracy in assessing total bioactive compounds, as the intricate nature of plant matrices might hinder reagent efficacy and result in inaccurate readings [42]. Hence, more advanced chromatographic techniques are required for precise accuracy.

The high-resolution LC MS/MS-based untargeted metabolomic analysis showed the presence of more than 120 compounds (Table 2). Among these substances, iridoids, flavonoids and phenolic acids are the main biologically active components. Iridoids are bioactive substances belonging to the monoterpenoid group and they are considered as acetal derivatives of iridodial [43]. Owing to the presence of very reactive C₁-OH moiety, iridoids often connected with sugar to form glycosides. They are divided into iridoid glycosides and secoiridoid glycosides according to the integrity of the cyclopentane ring. In addition, the dimer of iridoids known as bis-iridoids and non-glycosidic iridoids are also discovered in the nature (Table 2). This class of compounds possess broad spectrum of biological properties such as neuroprotective, hypoglycemic, antioxidant,

Table 1 Extraction yields (%), total phenolic and flavonoid contents in the tested extracts

Solvents	Extraction yields (%)	TPC	TFC
EA	2.26	26.61 ± 1.08 ^c	21.43 ± 1.01 ^a
EtOH	14.10	37.34 ± 0.77 ^a	8.03 ± 0.16 ^b
EtOH/Water	26.27	29.52 ± 0.48 ^b	5.34 ± 0.15 ^c
Water	19.69	30.84 ± 0.76 ^b	6.37 ± 0.18 ^c

*Values are reported as mean ± SD of three parallel measurements

GAE Gallic acid equivalents, RE Rutin equivalents. Different letters indicate significant differences between the tested extracts ($p < 0.05$)

Table 2 Chemical composition of the tested extracts

Peak	m/z	Reten- tion time	Molecular Formula	Name	MS/MS Fragments	Error (ppm)	Ion	Iden- tifica- tion level	CSI: FingerIDScore	Relative peak area			
										EA	Ethanol	Ethanol/Water	Water
1	266.0894	0,913	C ₁₀ H ₁₃ N ₅ O ₄	Mannopy- ranosyl serine	104/72/146	-6,195	M-H	2	-82,330	0	0	0.00070172	0
2	293.1001	0,914	C ₁₀ H ₁₈ N ₂ O ₈	Nglycosyl-L asparagine	114/131/203	8,358	M-H	2	-74,570	0	0	0.00102794	0.00083808
3	225.0628	0,924	C ₆ H ₁₂ O ₆	Hexahy- droxyhepta- noic acid	59/71/89	4,813	M-H	2	-27,230	0.00460081	0.00260159	0.00136154	0
4	89.0256	0,939	C ₃ H ₆ O ₃	Glyceralde- hyde	59/58	20,064	M-H	2	-382,000	0.01136845	0.00408978	0.00224721	0
5	341.1103	0,956	C ₁₂ H ₂₂ O ₁₁	Sucrose	165/71/89	-2,187	M-H	2	-61,536	0.00551801	0.01003942	0.00339153	0.00244506
6	387.1156	0,958	C ₁₂ H ₂₂ O ₁₁	Neotrehalose	59/71/89/101/113/119/179	8,405	M+CH ₂ O ₂ - H	2	-16,437	0.01702935	0.02649371	0.00916733	0.00766829
7	537.1696	0,972	C ₁₈ H ₃₂ O ₁₇	Maltotronic acid	195/75/129	16,256	M+H ₂ O-H	3	-46,063	0	0.00684852	0.00950766	0.01307292
8	195.0525	0,977	C ₇ H ₈ N ₄ O ₃	Hydroxyethyl xanthine	75/59/129	3,246	M-H	3	-169,540	0.00135759	0.01197632	0.02326535	0.03088551
9	179.0570	0,981	C ₆ H ₁₂ O ₆	Fructose	59/71/89	-1,31	M-H	3	-25,532	0.0217762	0.0087013	0.00767826	0.00841213
10	533.1735	1,008	C ₁₉ H ₃₄ O ₁₇	Glycero manno hep- topyranosyl glucopyrano- syl glucopy- ranoside	191/85/75	2,039	M-H	3	-150,368	0	0.00099831	0	0
11	165.0419	1,010	C ₅ H ₁₀ O ₆	L-Xylonic acid	87/59/87	8,625	M-H	3	-8,473	0	0.00199211	0.00704642	0.00511669
12	135.0312	1,018	C ₄ H ₈ O ₅	Threonate	75/72/59	9,652	M-H	3	-21,969	0	0.00127458	0.00488615	0.00509246
13	267.0735	1,025	C ₈ H ₁₄ O ₇	Acetylglucose	59/71/101	8,679	M+CH ₂ O ₂ -H	2	-46,888	0.01824324	0	0.00082929	0
14	209.0327	1,032	C ₇ H ₁₄ O ₇	Heptose	57/71/85/129/159	7,355	M-H	3	-41,113	0	0	0.00366124	0.06975368
15	161.0464	1,060	C ₆ H ₁₀ O ₅	Glucan	85/73/71	4,591	M-H	3	-44,838	0.02183364	0	0	0
16	133.0154	1,083	C ₄ H ₆ O ₅	Malic acid	115/99/71	8,647	M-H	3	-120,999	0.0018875	0.01372378	0.07609649	0.09383911
17	275.0239	1,098	C ₁₀ H ₁₂ O ₇ S	Dihydroi- soferuicil acid sulfate	159/115/96/71/61	4,177	M-H	3	-353,883	0	0	0.00194999	0.0100614
18	317.0567	1,154	C ₉ H ₁₈ O ₁₀ S	Glyceryl sulfoquinovo- side	80/94/71	8,963	M-H	2	-108,194	0	0	0.00303852	0.00113426
19	128.0365	1,164	C ₃ H ₇ NO ₃	Pidolate	51/95	11,643	M-H	2	-59,482	0.00057891	0.00164283	0.00549645	0.00074819
20	191.0215	1,186	C ₆ H ₈ O ₇	Citric acid	111/57/87	11,698	M-H	2	-11,441	0	0	0.03247835	0.07444163

Table 2 (continued)

Peak	m/z	Retention time	Molecular Formula	Name	MS/MS Fragments	Error (ppm)	Ion	Identification level	CSI: FingerIDScore	Relative peak area			
										EA	Ethanol	Ethanol/Water	Water
21	267,0724	1,191	C ₉ H ₁₆ O ₉	Mannosylglycerate	59/71/101	10,163	M-H	2	-48,089	0.01084622	0	0	0
22	199,8063	1,192	C ₈ H ₈ O ₆	Fumarylacetate	78/71/125	0,552	M-H	2	-83,640	0	0.00320934	0.00289282	0.00121701
23	409,1344	1,284	C ₁₅ H ₂₄ O ₁₀	Physoside	85/201/157	-5,902	M+CH ₂ O ₂ -H	3	-75,973	0.00553769	0.0100064	0	0.00137846
24	203,0213	1,302	C ₇ H ₈ O ₇	Daucic acid	79/97/69	8,643	M-H	3	-53,875	0	0	0.00618363	0.00877638
25	125,0017	1,321	C ₂ H ₇ O ₄ P	Simethylphosphate	78/62	25,011	M-H	2	-7,170	0	0	0.00300809	0
26	217,0365	1,361	C ₈ H ₁₀ O ₇	Dimethylgar-cinate lactone	83/55/127	4,766	M-H	3	-109,341	0	0	0.02564171	0.04889875
27	311,0980	1,400	C ₁₁ H ₂₀ O ₁₀	Vicianose	59/265/205	-4,688	M-H	2	-53,651	0.00396172	0	0	0
28	199,0378	1,433	C ₃ H ₁₃ O ₆ P	Dihydropropylethylhydrogen phosphate	125/78	-1,282	M-H	2	-27,826	0	0	0.01112262	0
29	391,1219	1,525	C ₁₅ H ₂₂ O ₉	Aucubin	183/59/165	-0,156	M+CH ₂ O ₂ -H	2	-32,875	0.00183766	0	0	0
30	216,9103	1,559	C ₂ H ₂ O ₈ S ₂	Dihydrodioxathiole tetraoxide	96/79	10,364	M-H	3	-112,454	0	0	0	0.00323677
31	393,1376	1,962	C ₁₆ H ₂₆ O ₁₁	Lamiidol	71/89/97	-6,409	M-H	3	-136,417	0.0177336	0.00391064	0	0.00559183
32	409,1324	2,023	C ₁₅ H ₂₄ O ₁₀	Harpagide	59/71/89	-4,778	M+CH ₂ O ₂ -H	3	-74,313	0.03331649	0.05349976	0.0623392	0.05913467
33	393,1384	2,220	C ₁₅ H ₂₄ O ₉	Ajugol	127/167/149	-4,203	M+H	3	-72,886	0.0049664	0	0	0.00392135
34	391,1246	2,281	C ₁₅ H ₂₂ O ₉	Decaloside	85/59/165	-6,263	M+CH ₂ O ₂ -H	3	-41,426	0.08045169	0	0.02687156	0
35	405,1354	2,370	C ₁₇ H ₂₆ O ₁₁	Auroside	359/81/165	-4,589	M-H	3	-67,178	0.00672198	0	0	0
36	391,1226	2,663	C ₁₆ H ₂₄ O ₁₁	Shanzhiside	85/165/183	-3,908	M-H	2	-42,308	0	0.10775633	0.04668412	0.08151598
37	315,0703	2,737	C ₁₃ H ₁₆ O ₉	Genticic Acid Glucoside	108/152	-7,674	M-H	2	-27,205	0	0	0	0.00144344
38	373,1132	3,083	C ₁₆ H ₂₂ O ₁₀	Secologanin	89/119/121	-5,901	M-H	2	-85,389	0	0	0	0.00255794
39	431,1184	3,087	C ₁₈ H ₂₄ O ₁₂	Apiosylglucosylhydroxybenzoate	93/137/225	-5,652	M-H	3	-84,727	0	0	0	0.00101142

Table 2 (continued)

Peak	m/z	Retention time	Molecular Formula	Name	MS/MS Fragments	Error (ppm)	Ion	Identification level	CSI: FingerIDScore	Relative peak area			
										EA	Ethanol	Ethanol/Water	Water
40	153,0190	3,213	C ₇ H ₆ O ₄	Protocatechuic acid	109/108/65	-2,58	M-H	2	-4,797	0	0	0	0.00110727
41	375,1274	3,304	C ₁₆ H ₂₄ O ₁₀	Loganic acid	213/151/71	-2,069	M-H	2	-25,260	0	0	0.00269294	0.00372199
42	593,2082	3,397	C ₂₅ H ₃₈ O ₁₆	Leonuride B	461/135	0,493	M-H	3	-73,483	0	0	0	0.00162304
43	369,0461	3,626	C ₁₅ H ₁₄ O ₁₁	Caffeoylhydroxydic acid	209/191/147	-3,507	M-H	3	-129,202	0	0	0	0.00213942
44	175,0605	3,627	C ₇ H ₁₂ O ₅	Isopropylmalic Acid	85/115/113	-1,912	M-H	2	-9,468	0	0	0	0.00138663
45	341,0868	4,266	C ₁₅ H ₁₈ O ₉	Caffeoylglucose	161/133/179	3,249	M-H	2	-31,820	0	0.0061998	0.0045786	0.00540912
46	373,1132	4,627	C ₁₅ H ₂₀ O ₈	Androsin	165/150/122	-4,488	M+CH ₂ O ₂ -H	2	-48,671	0	0	0.00318278	0.00409373
47	325,0913	4,775	C ₁₅ H ₁₈ O ₈	Melilotoside	119/163	-2,8	M-H	2	-26,012	0	0.00245139	0.0066154	0.0079614
48	487,1432	4,855	C ₂₁ H ₂₈ O ₁₃	Caffeoyl rutinoside	179/135/161	-5,4	M-H	3	-85,851	0	0	0.00392672	0.0019501
49	463,1459	4,929	C ₁₉ H ₂₈ O ₁₃	Phlorigidin B	99/117/363	-2,224	M-H	3	-101,104	0	0	0	0.00179682
50	487,1426	5,024	C ₂₁ H ₂₈ O ₁₃	Caffeoyl rutinoside isomer	179/161/135	-1,412	M-H	3	-61,820	0	0	0.00174542	0.00176281
51	613,1985	5,508	C ₂₃ H ₃₆ O ₁₆	Eriobioside	507/179/165	-0,566	M+CH ₂ O ₂ -H	3	-137,117	0	0	0	0.00168835
52	385,0771	5,552	C ₁₆ H ₁₈ O ₁₁	Feruloylglucaric acid	85/191/147	-5,626	M-H	3	-80,325	0	0	0	0.00241294
53	451,1427	6,015	C ₁₇ H ₂₆ O ₁₁	Shanzhiside methyl ester	345/183/139	-2,683	M+CH ₂ O ₂ -H	3	-93,023	0	0	0.13904332	0
54	457,1338	6,115	C ₂₀ H ₂₆ O ₁₂	p-Coumaric acid [apiosylglucoside]	119/163	-0,59	M-H	3	-46,781	0	0	0.00481826	0.00433684
55	325,0916	6,121	C ₁₅ H ₁₈ O ₈	Coumaroyl hexoside	145/117	-4,22	M-H	2	-32,889	0	0.00420337	0.00501624	0.00223937
56	405,1377	6,215	C ₁₇ H ₂₆ O ₁₁	Ipolamiide	361/199/89	2,055	M-H	3	-118,165	0.01877583	0.07384131	0.06922834	0.06293275
57	431,1910	6,658	C ₂₀ H ₃₂ O ₁₀	Hexoside hexyl tetraacetate	385/205/153	-4,957	M-H	3	-331,379	0	0	0.00351572	0.00255172
58	355,1028	6,932	C ₁₆ H ₂₀ O ₉	Feruloyl glucose	175/160/132	-2,56	M-H	2	-25,997	0.00294762	0.01120635	0.00961284	0.00812474

Table 2 (continued)

Peak	m/z	Retention time	Molecular Formula	Name	MS/MS Fragments	Error (ppm)	Ion	Identification level	CSI: FingerIDScore	Relative peak area		
										EA	Ethanol	Ethanol/Water
59	435,1492	7,136	C ₁₈ H ₂₈ O ₁₂	Eleganaside C	59/147/71	-0,245	M-H	3	-193,209	0.0019762	0.0039206	0.00611706
60	469,1910	7,303	C ₁₈ H ₁₈ O ₁₁	Hexenyl glucopyranosyl glucopyranoside	221/179/161/113/101	-0,244	M+CH ₂ O ₂ -H	3	-75,424	0	0.00385918	0.00641866
61	385,0776	7,398	C ₁₆ H ₁₈ O ₁₁	Feruloylglucuronic acid isomer	85/191/147	-4,435	M-H	3	-67,810	0	0	0.00402475
62	375,1292	7,665	C ₁₆ H ₂₂ O ₉	Sweroside	85/191/147	0,451	M+H ₂ O-H	3	-162,539	0	0	0.0042411
63	433,2073	7,823	C ₁₉ H ₃₂ O ₈	Phlomoside glycoside	59/71/113	-2,656	M+CH ₂ O ₂ -H	3	-136,694	0	0.00307342	0.00630604
64	375,1290	8,109	C ₁₆ H ₂₂ O ₉	Synargen-tolide D	85/147/57	1,435	M+H ₂ O-H	3	-166,258	0	0	0.00279322
65	593,1531	8,157	C ₂₇ H ₃₀ O ₁₅	apigenin diglucoside isomer	353/473/297/269	-2,861	M-H	2	-2,211	0	0.00169934	0.0061745
66	589,2504	8,291	C ₂₆ H ₄₀ O ₁₂	Agnucastoside B	499/337/307	-4,804	M+CH ₂ O ₂ -H	3	-270,669	0	0.00287131	0.00340039
67	375,1299	8,327	C ₁₅ H ₂₂ O ₈	Linaride	85/191/147	-4,753	M+CH ₂ O ₂ -H	3	-181,200	0	0	0.0035398
68	785,2468	8,453	C ₃₅ H ₄₆ O ₂₀	Lamiuside isomer	161/623	-4,296	M-H	3	-68,483	0	0.00231778	0.00184024
69	509,2228	8,472	C ₂₁ H ₃₆ O ₁₁	Linalool oxide	89/101/131	2,699	M+CH ₂ O ₂ -H	3	-104,859	0	0.00686672	0.00490745
70	359,1345	8,507	C ₁₆ H ₂₄ O ₉	primeveroside	135/153/197	-7,699	M-H	2	-15,917	0	0.0037805	0.00269727
71	435,1504	8,510	C ₁₇ H ₂₆ O ₁₀	Deoxyloganic acid	59/123/179	-4,278	M+CH ₂ O ₂ -H	2	-78,347	0.00218049	0.00326022	0.00172479
72	563,1393	8,608	C ₂₆ H ₂₈ O ₁₄	Shaftoside	353/297/383	-2,401	M-H	2	-3,110	0	0.01418007	0.01964367
73	621,1787	8,641	C ₂₉ H ₃₆ O ₁₆	Plantamoside (Cin-namic acid derivative)	461/161	-5,01	M-H ₂ O-H	2	89,123	0	0	0.00138285
74	377,1798	8,687	C ₁₇ H ₃₀ O ₉	Scabroside B	331/85/135	-4,248	M-H	3	-231,296	0	0.00556507	0.00487612
75	785,2458	8,797	C ₃₅ H ₄₆ O ₂₀	Lamiuside isomer	161/623/461	-7,727	M-H	2	-50,723	0	0.0020385	0.00179107
76	563,1400	8,859	C ₂₆ H ₂₈ O ₁₄	Shaftoside isomer	353/297/383	-3,948	M-H	2	-13,584	0	0.00105256	0.00242277

Table 2 (continued)

Peak	m/z	Retention time	Molecular Formula	Name	MS/MS Fragments	Error (ppm)	Ion	Identification level	CSI: FingerIDScore	Relative peak area			
										EA	Ethanol	Ethanol/Water	Water
77	163,0398	8,957	C ₉ H ₈ O ₃	Coumaric acid	65/93/119	-2,464	M-H	2	-19,079	0	0.00172089	0.00127457	0.00105385
78	799,2265	9,010	C ₃₅ H ₄₂ O ₂₀	Kaempferol (rhamnosyl-acetyl-galactoside) rhamnoside	623/285/161	-2,917	M+H ₂ O	3	-166,454	0	0	0.00147921	0.00322629
79	755,2343	9,015	C ₃₄ H ₄₄ O ₁₉	Forsythoside B isomer	161/593/179	-2,339	M-H	2	-54,161	0	0.03168121	0	0
80	699,2160	9,083	C ₃₀ H ₃₈ O ₁₆	Serostide D	537/331/179	-0,488	M+CH ₂ O	3	-168,969	0	0.00165384	0.00253521	0.00210517
81	565,1899	9,113	C ₃₄ H ₃₀ O ₈	Dehydrodicoumaroyl alcohol dibenzoate	329/175/160	2,781	M-H	3	-279,870	0	0	0.00127003	0.00120418
82	533,1301	9,125	C ₂₅ H ₂₆ O ₁₃	Apigenin diglucoside	383/353/269	-0,907	M-H	2	-25,220	0	0.002414	0.0039631	0.00283772
83	755,2376	9,213	C ₃₄ H ₄₄ O ₁₉	Forsythoside B isomer	593/461/161	-1,239	M-H	2	-47,372	0.00304057	0.01342434	0.01921295	0.01998183
84	621,1811	9,323	C ₂₉ H ₃₄ O ₁₅	Dehydro Verbascoside	621/161	-2,937	M-H	3	-102,876	0.00229274	0.00467967	0.00146029	0.00280789
85	623,1994	9,346	C ₂₉ H ₃₆ O ₁₅	Verbascoside isomer	461/161/133	-1,103	M-H	2	-48,514	0.02668329	0.07862736	0.01832429	0.03355564
86	623,1908	9,517	C ₂₉ H ₃₆ O ₁₅	Verbascoside isomer	461/161/133	-2,571	M-H	2	-54,460	0	0.03268017	0.00390379	0.00299875
87	637,1020	9,568	C ₂₇ H ₂₆ O ₁₈	Luteolin diglucuronide	285	-2,34	M-H	3	-60,491	0	0	0.01529143	0.0301652
88	629,2615	9,607	C ₂₅ H ₄₄ O ₁₅	Lunarside	421/289/161	-0,088	M+CH ₂ O	3	-176,455	0	0.01038606	0.0113556	0.00612502
89	755,2407	9,679	C ₃₄ H ₄₄ O ₁₉	Forsythoside B isomer	593/461/161	-1,866	M-H	2	-47,372	0	0.00754118	0.00217775	0.00599596
90	667,2203	9,688	C ₃₁ H ₄₀ O ₁₆	Hemiphroside A	161/179/135	-2,689	M-H	3	-151,153	0	0	0.00619686	0
91	769,2539	9,745	C ₃₅ H ₄₆ O ₁₉	Verpectoside A	175/593/161	-5,155	M-H	2	-14,629	0.00226466	0.01570176	0.00513856	0.00410738
92	187,0971	9,769	C ₉ H ₁₆ O ₄	Monomethyl suberate	123/97/57	-2,189	M-H	3	-55,260	0.00379147	0	0.00149581	0.00096079
93	623,1936	9,779	C ₂₉ H ₃₆ O ₁₅	Verbascoside isomer	461/161/133	-8,65	M-H	2	-43,226	0	0.02578216	0.00700146	0.01959681
94	637,2102	9,937	C ₃₀ H ₃₈ O ₁₅	Leucosceptoside A	461/285/161	-3,65	M-H	2	49,974	0.00376673	0.02509557	0.00558213	0.0062365

Table 2 (continued)

Peak	m/z	Retention time	Molecular Formula	Name	MS/MS Fragments	Error (ppm)	Ion	Identification level	CSI: FingerIDScore	Relative peak area			
										EA	Ethanol	Ethanol/Water	Water
95	537,1611	10,009	C ₂₃ H ₂₄ O ₁₂	Hydroxyluteolin-dimethylether hexoside	329/285/271	-4,254	M+CH ₂ O ₂ -H	2	-122,641	0	0.00305572	0.00214823	0.00193307
96	467,2121	10,124	C ₁₉ H ₃₄ O ₁₀	Octen primeveroside	71/101/289	-0,5	M+CH ₂ O ₂ -H	2	-12,480	0	0.02470764	0.01369382	0.01179562
97	541,1377	10,153	C ₂₆ H ₃₈ O ₁₂	Agneucastosi- side A	151/169/357	-4,199	M-H	2	-40,635	0.02063439	0	0.00359827	0.0022525
98	491,1558	10,292	C ₂₄ H ₂₈ O ₁₁	Eurostoside	307/163/145	-2,119	M-H	3	-177,733	0.0036872	0.01124025	0.00446386	0.00441458
99	783,2692	10,358	C ₃₆ H ₄₈ O ₁₉	Lamiophlomisioside A	607/193/175	-5,82	M-H	3	-81,078	0.00269816	0.0077184	0.00679196	0.00492842
100	651,2262	10,549	C ₃₁ H ₄₀ O ₁₅	Cistanoside isomer	175/161/475	-5,192	M-H	3	-120,578	0.01387677	0.01354321	0.00753412	0.00571467
101	493,2280	10,694	C ₂₁ H ₃₆ O ₁₀	Kenposide isomer	59/71/89/101	-0,31	M+CH ₂ O ₂ -H	2	-40,354	0.0122401	0.0280599	0.01604816	0.01672186
102	783,2637	10,849	C ₃₆ H ₄₈ O ₁₉	Leonoside B	193/175	0,667	M-H	3	-83,500	0	0	0.00073897	0.00138613
103	453,2118	11,102	C ₂₂ H ₃₂ O ₇	Sodoponin	59/83/97/317	-3,334	M+CH ₂ O ₂ -H	3	-205,660	0	0.00261407	0	0
104	451,1954	11,195	C ₂₂ H ₃₀ O ₇	Parvifolin H	405/375/97	-3,164	M-CH ₂ O ₂ -H	3	-236,156	0.09593581	0.05865909	0.01425072	0.01248467
105	557,2593	11,274	C ₂₆ H ₄₀ O ₁₀	Andrographi- side	349/303/179/89	-0,633	M-CH ₂ O ₂ -H	3	-132,806	0.05450274	0.02293958	0.01701729	0.01308981
106	327,2165	11,933	C ₁₈ H ₃₂ O ₅	Trihydroxyocta- decenoic acid	211/69/85/183	-2,585	M-H	2	-9,235	0.02128736	0.01164458	0.0104936	0.01129075
107	287,2216	12,782	C ₁₆ H ₃₂ O ₄	Dihydroxy palmitic acid	269/242/129/71	-1,769	M-H	3	-30,709	0	0.00165212	0.00107491	0.00094668
108	299,0552	12,980	C ₁₆ H ₃₂ O ₆	Hispidulin	65/117/137/256/284	-4,812	M-H	2	-26,197	0.00139626	0.0010302	0.00070523	0
109	553,2642	13,609	C ₂₈ H ₄₂ O ₁₁	Dihydroxyhydroxyajugachin A	507/463/101/59	-0,589	M-H	3	-366,529	0.11514488	0.0582416	0.03564429	0.02675857
110	311,2210	14,255	C ₁₇ H ₃₀ O ₂	Dihydroxy linoleic acid	223/119/57	-5,305	M-CH ₂ O ₂ -H	2	-66,026	0.02218214	0.00945444	0.00586268	0.00444199
111	567,2811	14,446	C ₂₉ H ₄₂ O ₁₀	Ajugapitin	521/477/301/115	-4,769	M+H ₂ O-H	3	-201,568	0.03340968	0.01396896	0.00650582	0.00210043
112	356,2432	15,448	C ₁₉ H ₃₃ NO ₅	Myristoyl glutamic acid	292/198/57	-0,73	M-H	3	-207,943	0.00785317	0.00134792	0.00139432	0
113	393,1366	15,684	C ₂₃ H ₃₂ O ₆	Garcinone B	375	-4,052	M-H	3	-238,518	0.01918212	0.00303306	0.00219982	0

Table 2 (continued)

Peak	m/z	Reten- tion time	Molecular Formula	Name	MS/MS Fragments	Error (ppm)	Ion	Iden- tifica- tion level	CSI: FingerIDScore	Relative peak area		
										EA	Ethanol	Ethanol/Water
114	293,2112	15,722	C ₁₈ H ₃₀ O ₃	Hydroxyocta- decatricenic acid	71/275/183	-2,941	M-H	2	-37,400	0.07227534	0.0240764	0.0155949
115	309,2059	16,001	C ₁₈ H ₃₀ O ₄	Octadecatri- enoic acid	291/195/119/71/59	-4,159	M-H	2	-31,549	0.03256589	0.0034329	0.00328094
116	409,1313	16,035	C ₂₃ H ₂₂ O ₇	hydroperoxy Lactucopirin	99/116/323	2,91	M-H	3	-166,425	0.01510051	0.00177987	0.00156906
117	243,1959	16,138	C ₁₄ H ₂₈ O ₃	Hydroxytet- radecanoic acid	59	-2,066	M-H	3	-7,768	0.02379548	0.00956574	0.00574947
118	373,2590	16,466	C ₂₀ H ₃₈ O ₆	Fatty acid derivative	249/195/133/109	0,692	M-H	3	-298,884	0.03138157	0.00842844	0.00561668
119	313,2375	17,238	C ₁₈ H ₃₄ O ₄	Octadecane- dioic acid	295/269/251/183/155/125/71/59	-4,64	M-H	3	-51,277	0.01477139	0.00194428	0
120	471,2378	18,717	C ₂₇ H ₃₆ O ₇	Simpterpe- noid A	59/162	-5,539	M-H	3	-267,735	0.0461174	0.00531923	0.00315503
121	913,5847	21,780	C ₄₉ H ₈₆ O ₁₅	Digalactosyl- diacylglyc- erol	257/605/277	-2,981	M-H	3	-590,629	0.00267487	0	0
EA Ethyl acetate												

hepatoprotective, anti-inflammatory, anticancer and hypolipidemic effects [44]. Additionally, they are applicable in some cosmetic products [45]. Nevertheless, the analysis of iridoids regarding their dietary and health-related importance remains insufficient. Among commonly consumed fruits, *Olea europaea* L. (Olive) and *Morinda citrifolia* L. (Noni) have been thoroughly investigated regarding the bioactive iridoid content. Olive oil secoiridoids such as oleuropein have a well-founded antioxidant potential and other related activities, involving prevention against diabetes, metabolic, cardiovascular and neurodegenerative disease. In addition, they displayed anticancer, bactericidal, antiaging, antiviral, antidepressant and skin-care effects ([46]. Deacetyloasperulosidic acid and asperulosidic acid isolated from noni demonstrate anti-inflammatory, anti-clastogenic, anti-arthritis, anti-atherogenic, antitumor, anesthetic, antimicrobial, antiviral and genoprotective effects. Deacetyloasperulosidic acid is the antiglycation agent and could relieve atopic dermatitis. The compound shows antioxidant and hypotensive activity. Asperulosidic acid has hypotensive and hypoglycemic activity [47]. The antioxidant and enzyme inhibitory capacity of sweroside - a secoiridoid glycoside isolated from *Schenkia spicata* and present in water extract of *Ajuga vestita* was elucidated. The antioxidant effect was demonstrated by ABTS, CUPRAC, FRAP and phosphomolybdenum (PBD) assay. The results showed that sweroside exhibited inhibitory effects on the enzymes analyzed (BChE, tyrosinase, α -amylase and glucosidase) with the exception of AChE. The compound demonstrated good tyrosinase inhibitory potential. Concerning the antidiabetic activity, it displayed both amylase and glucosidase inhibitory action [48].

Antioxidant effects

The term ‘antioxidants’ is very popular in everyday life, with most people associating it with health-promoting properties, such as protection against harmful diseases. In this sense, antioxidant compounds have a wide range of applications, from medicine to nutraceuticals. In these areas, synthetic antioxidants are mainly used, but many of them have unpleasant side effects. For this reason, many researchers are seeking safe and effective alternatives to synthetic antioxidants [49]. The antioxidant properties of *A. vestita*

extracts were examined using various assays, such as radical quenching, reducing power, and metal chelation. The results are shown in Table 3. DPPH and ABTS radicals are commonly used to evaluate the radical-quenching ability of plant extracts. In both assays, the ethanol extract exhibited the strongest radical scavenging ability, with values of 43.13 mg TE/g and 45.37 mg TE/g in the DPPH and ABTS assays, respectively. The weakest radical scavenging ability was determined for the ethyl acetate extract. Reducing power is an important antioxidant mechanism that reflects electron donation ability. CUPRAC and FRAP assays were performed to measure reducing power and included the transformation of Cu^{2+} to Cu^+ and Fe^{3+} to Fe^{2+} . The ethanol extract exhibited the highest reducing power in both assays (CUPRAC: 103.43 mg TE/g; FRAP: 45.29 mg TE/g), followed by the water extract, the ethanol/water extract and the water extract. The results of the radical scavenging and reducing power assays are consistent with the total phenolic levels in the extracts. Thus, we conclude that phenolic compounds are the main contributors to radical scavenging and reducing power. Phosphomolybdenum assays also contain a reduction of Mo (VI) to Mo (V) by antioxidants and are mostly preferred in antioxidant studies due to its simplicity and low cost [12]. In contrast, the ethyl acetate extract exhibited the greatest reduction ability in this assay, with a value of 2.42 mmol TE/g, followed by the ethanol, ethanol/water and water extracts. The ability to chelate metals is linked to managing the production of hydroxyl radicals in the Fenton reaction. As can be seen in Table 3, the water extract exhibited the greatest metal chelation capacity, at 26.16 mg EDTAE/g. This was followed by the ethanol/water, ethyl acetate and ethanol extracts. Contradictory results from the metal chelation assay can be explained by the presence of non-phenolic chelators, including polysaccharides, peptides and sulfides [12]. Several studies have reported the antioxidant properties of members of the *Ajuga* genus in literature. Saidi et al. [35] reported that the hydroethanolic extract of *A. iva* exhibited a greater DPPH radical scavenging and FRAP ability as compared to water extract obtained by decoction. Similarly, according to Singh et al. [50], the methanol extract exhibited superior DPPH radical scavenging and phosphomolybdenum activities when compared to the extracts obtained using n-hexane and water. Aydın et al. [6] also documented that the methanol extract of

Table 3 Antioxidant properties of the tested extracts

Solvents	DPPH (mg TE/g)	ABTS (mg TE/g)	CUPRAC (mg TE/g)	FRAP (mg TE/g)	Chelating (mg EDTAE/g)	PBD (mmol TE/g)
EA	17.34±0.04 ^d	22.76±1.19 ^c	38.50±1.16 ^c	16.68±0.82 ^c	7.00±1.78 ^c	2.42±0.13 ^a
EtOH	43.13±0.23 ^a	45.37±1.05 ^a	103.43±1.84 ^a	45.29±1.11 ^a	3.30±0.28 ^d	2.28±0.08 ^a
EtOH/Water	37.87±1.24 ^c	41.62±1.18 ^b	79.20±0.86 ^b	39.03±1.26 ^b	13.66±0.62 ^b	1.85±0.10 ^b
Water	40.35±0.49 ^b	43.97±0.78 ^{ab}	79.44±2.91 ^b	40.07±1.55 ^b	26.16±0.50 ^a	1.62±0.04 ^b

^aValues are reported as mean±SD of three parallel measurements. TE: Trolox equivalents, EDTAE EDTA equivalent. Different letters indicate significant differences between the tested extracts ($p < 0.05$)

A. chamaepitys subsp. chia exhibited superior scavenging activities against DPPH and ABTS. The antioxidant potential of water and butanol extracts from *Ajuga turkestanica* is remarkable, demonstrated through DPPH assay with IC₅₀ values of 7.24 µg/mL and 14.57 µg/mL, respectively [51]. According to Toiu et al. (2018), ethanol and methanol extracts from the aerial parts of *A. laxmannii* exhibit the strongest DPPH radical scavenging activity in vitro, with IC₅₀ values of 22.64 µg/mL and 24.89 µg/mL. Additionally, the ethanol extract of *A. bracteosa* shows significant free radical scavenging capability with an IC₅₀ of 14.40 µg/mL [52]. Moreover, methanol root extracts of *A. bracteosa* exhibit significant DPPH radical scavenging activity, with an IC₅₀ value of 36.90 µg/mL [53].

Enzyme inhibitory effects

The increasing prevalence of global health issues underscores the urgent need for therapeutic strategies to address these conditions. One of the most effective of these is the enzyme inhibitory theory. By inhibiting key enzymes, we can mitigate the adverse effects of disease. For example, inhibiting cholinesterase in Alzheimer's disease can increase acetylcholine levels in the synaptic cleft, thereby improving memory function, a concept known as the cholinergic hypothesis [54]. Additionally, inhibiting amylase and glucosidase can significantly delay the rise in blood glucose levels in diabetic patients [55]. Several chemical enzyme inhibitors, such as galantamine and acarbose, have been developed in this context. However, the use of these inhibitors is often limited due to toxic concerns, creating a demand for safer, natural alternatives.

In light of the above information, we examined the inhibitory effects of *A. vestita* extracts on cholinesterase, amylase, glucosidase and tyrosinase enzymes. The results are summarised in Table 4. The ethanol extract exhibited the most potent AChE and BChE inhibitory effects, with values of 2.54 mg GALAE/g and 2.94 mg GALAE/g, respectively. The water extract was inactive against both cholinesterases. Similarly, the ethanol extract displayed the best tyrosinase inhibition, while the water extract showed no tyrosinase

inhibition. The highest amylase inhibition value was displayed by the ethyl acetate extract (0.71 mmol ACAE/g), followed by the ethanol extract, the ethanol/water extract and the water extract. However, the ethyl acetate extract was inactive against glucosidase, whereas the ethanol extract exhibited the greatest glucosidase inhibitory activity at 2.50 mmol ACAE/g. To the best of our knowledge, AChE, BChE, tyrosinase, amylase and glucosidase inhibitory activities of the member of the genus *Ajuga* have been little investigated. In a earlier study conducted by Fettach et al. [56], the methanol extract of *A. iva* exhibited stronger amylase (IC₅₀: 0.172 mg/ml) and glucosidase (IC₅₀: 0.130 mg/ml) inhibition ability when compared to water extract. Llorent-Martínez et al. [57] reported that the ethyl acetate and methanol extracts of *A. chamaepitys* subsp. chia var. chia and *A. bombycina* demonstrated stronger inhibitory effects on AChE and BChE compared to their water extracts, as well as on tyrosinase and amylase. Movahhedini et al. [58] examined the enzyme inhibitory effect of the ethanol and water extracts of *Ajuga chamaecistus* subsp. scoparia collected from Iran and ethanol extracts were more active on AChE, BChE and amylase than water extract. The chloroform and n-hexane extracts from *Ajuga bracteosa* exhibited notable enzyme inhibition capabilities, with IC₅₀ values measured at 29.92 µg/ml and 131.7 µg/ml, respectively [59]. Dżiki et al. [39] also reported a remarkable tyrosinase inhibitory effect of root extract of *A. reptans*. Riaz et al. [60] investigated three withanolides naming bracteosin A, bracteosin B and bracteosin C present in the whole plants of *A. bracteosa* and proved their strong inhibitory effect against cholinesterase enzymes in a concentration-dependent manner.

Cytotoxicity

Among the tested extracts, ethyl acetate showed the highest cytotoxicity, significantly reducing cell viability across all cell lines, particularly in S17 (30.8 ± 2.7%) cells at 100 µg/mL (Table 5). In contrast, the aqueous extract was the least toxic, maintaining high cell viability in all cases (> 94% at 50 µg/mL and > 74% at 100 µg/mL) (Table 5). These results provide a preliminary indication of the cellular safety

Table 4 Enzyme inhibitory properties of the tested extracts

Solvents	AChE (mg GALAE/g)	BChE (mg GALAE)	Tyrosinase (mg KAE/g)	Amylase (mmol ACAE/g)	Glucosidase (mmol ACAE/g)
EA	2.29 ± 0.35 ^a	2.41 ± 0.61 ^{ab}	36.66 ± 4.50 ^{ab}	0.71 ± 0.01 ^a	na
EtOH	2.54 ± 0.03 ^a	2.94 ± 0.23 ^a	38.28 ± 1.00 ^a	0.49 ± 0.01 ^b	2.50 ± 0.16 ^a
EtOH/Water	1.37 ± 0.04 ^b	1.46 ± 0.34 ^b	31.76 ± 1.15 ^b	0.45 ± 0.01 ^c	1.01 ± 0.08 ^b
Water	na	na	na	0.06 ± 0.01 ^d	0.65 ± 0.02 ^c

^aValues are reported as mean ± SD of three parallel measurements.

GALAE Galantamine equivalent, KAE Kojic acid equivalent, ACAE Acarbose equivalent; na: not active. Different letters indicate significant differences between the tested extracts ($p < 0.05$)

Table 5 Cellular viability (%) of mammalian RAW, HepG2 and S17 cell lines, after application of extracts of *Ajuga vestita*

Cell line	S17 cells		RAW cells		HepG2 cells	
Extract	100 µg/mL	50 µg/mL	100 µg/mL	50 µg/mL	100 µg/mL	50 µg/mL
Ethyl acetate	30.8 ± 2.7 ^c	83.5 ± 6.1 ^a	37.8 ± 3.4 ^c	103.1 ± 7.2 ^a	41.3 ± 5.3	81.3 ± 6.0 ^a
Ethanol	59.2 ± 1.2 ^b	73.6 ± 5.7 ^{ab}	87.6 ± 5.5 ^a	104.3 ± 7.5 ^a	55.5 ± 7.9	88.1 ± 6.2 ^a
Ethanol/water	56.1 ± 7.0 ^b	76.4 ± 5.7 ^a	69.3 ± 4.7 ^b	103.8 ± 8.3 ^a	55.7 ± 5.1	89.1 ± 5.7 ^a
Water	74.3 ± 3.9 ^a	94.6 ± 8.2 ^a	93.0 ± 7.9 ^a	97.7 ± 8.4 ^a	99.4 ± 8.9	100.7 ± 6.5 ^a

Values represent the mean ± SD (*n* = 6)

profile of the different extracts. Although the EA extract presented the lowest total phenolic content, it contained the highest flavonoid concentration (21.43 mg RE/g). Flavonoids, depending on their structure and concentration, can exert cytotoxic effects through oxidative stress induction, cell cycle arrest, and apoptosis in mammalian cells [61]. Therefore, the elevated flavonoid content may explain the selective cytotoxicity observed for the EA extract. On the other hand, the aqueous extract, while showing lower flavonoid content, was rich in phenolic compounds (second only to ethanol). These compounds are typically associated with antioxidant and protective effects rather than cytotoxicity [62], which aligns with the high viability observed in cells exposed to this extract. LC-MS/MS analysis further supports these trends: the ethyl acetate extract was particularly enriched in lipophilic flavonoids and iridoids, which are known to exhibit cytotoxic and other bioactive effects in cellular systems, likely due to their higher membrane permeability and consequent intracellular accumulation [63, 64]. In contrast, the aqueous extract was characterized by the presence of polar iridoids and their glycosides, such as sweroside, which has demonstrated antioxidant, antidiabetic, and tyrosinase-inhibitory activities [48], but limited cytotoxic effects. This low cytotoxic profile suggests a favourable cellular safety profile and supports the potential use of this extract in applications where minimal toxicity is required.

These findings point to different possible applications for the ethyl acetate and aqueous extracts of *A. vestita*. Given its marked cytotoxicity and richness in flavonoids and iridoids, the ethyl acetate extract could be explored further as a potential source of cytotoxic bioactive compounds. Follow-up studies might focus on isolating the most active constituents, investigating their mechanisms of action in different cellular systems, and assessing their selectivity and safety in more complex biological systems. On the other hand, the aqueous extract, which showed high cell viability and a relatively high content of phenolic compounds, might be more suitable for applications where low cytotoxicity and antioxidant potential are desired. Its composition suggests possible roles in the development of nutraceuticals, anti-inflammatory formulations, or dermocosmetic products, though these uses would need to be validated in specific biological or clinical contexts. Altogether, the contrasting

profiles of these extracts suggest that both may be worth investigating further, depending on the intended therapeutic or functional goal.

Identification of disease-associated and intersecting targets

Potential molecular targets for the thirty-six *Ajuga vestita* phytochemicals were predicted with the Similarity Ensemble Approach SEA and SwissTargetPrediction. After merging outputs from the two algorithms and removing duplicates, 438 unique compound related genes were obtained. Disease relevance was assessed for immune inflammatory indications using only CTD curated records, yielding rheumatoid arthritis 185 genes, ulcerative colitis 69 genes, sepsis 48 genes, and Crohn disease 54 genes. To define therapeutic intersections, the *Ajuga vestita* target catalogue was compared with each CTD Curated disease set in R. This analysis identified 18 shared genes with rheumatoid arthritis *ABCG2*, *F2*, *ATIC*, *ABCB1*, *AHR*, *MMP2*, *PON1*, *CTSD*, *PTGS1*, *VEGFA*, *PTK2*, *PRKCQ*, *PTGS2*, *MMP12*, *IGFBP3*, *CDK6*, *GRK6*, *BLK*, 3 shared genes with ulcerative colitis *JAK2*, *CXCR1*, *STAT3*, 4 shared genes with sepsis *MAPK1*, *NOS2*, *MAPK3*, *ADORA2B*, and 2 shared genes with Crohn disease *PTAFR*, *TLR9*. These overlapping targets delineate the core molecular interfaces linking the *Ajuga vestita* phytochemical space to the selected disease contexts based exclusively on CTD Curated data (Fig. 1).

Protein-protein interaction (PPI) network analysis

Protein protein interaction PPI analysis was performed in STRING and visualized in Cytoscape to evaluate the overlapping targets between the *Ajuga vestita* catalogue and each disease set, with topology metrics computed in cytoHubba and nodes color coded by Maximal Clique Centrality MCC from red high to yellow low. The integrated view showed that rheumatoid arthritis yielded a connected network with 18 nodes and 19 edges, where MCC ranking highlighted *MMP2*, *PTGS2*, *IGFBP3*, *COL18A1*, and *CTSD* as the top five hubs that concentrate matrix remodeling, eicosanoid synthesis, growth factor control, and lysosomal proteolysis; sepsis formed a sparse graph with 4 nodes and 2 edges comprising *MAPK1*, *MAPK3*, *NOS2*, and *ADORA2B*,

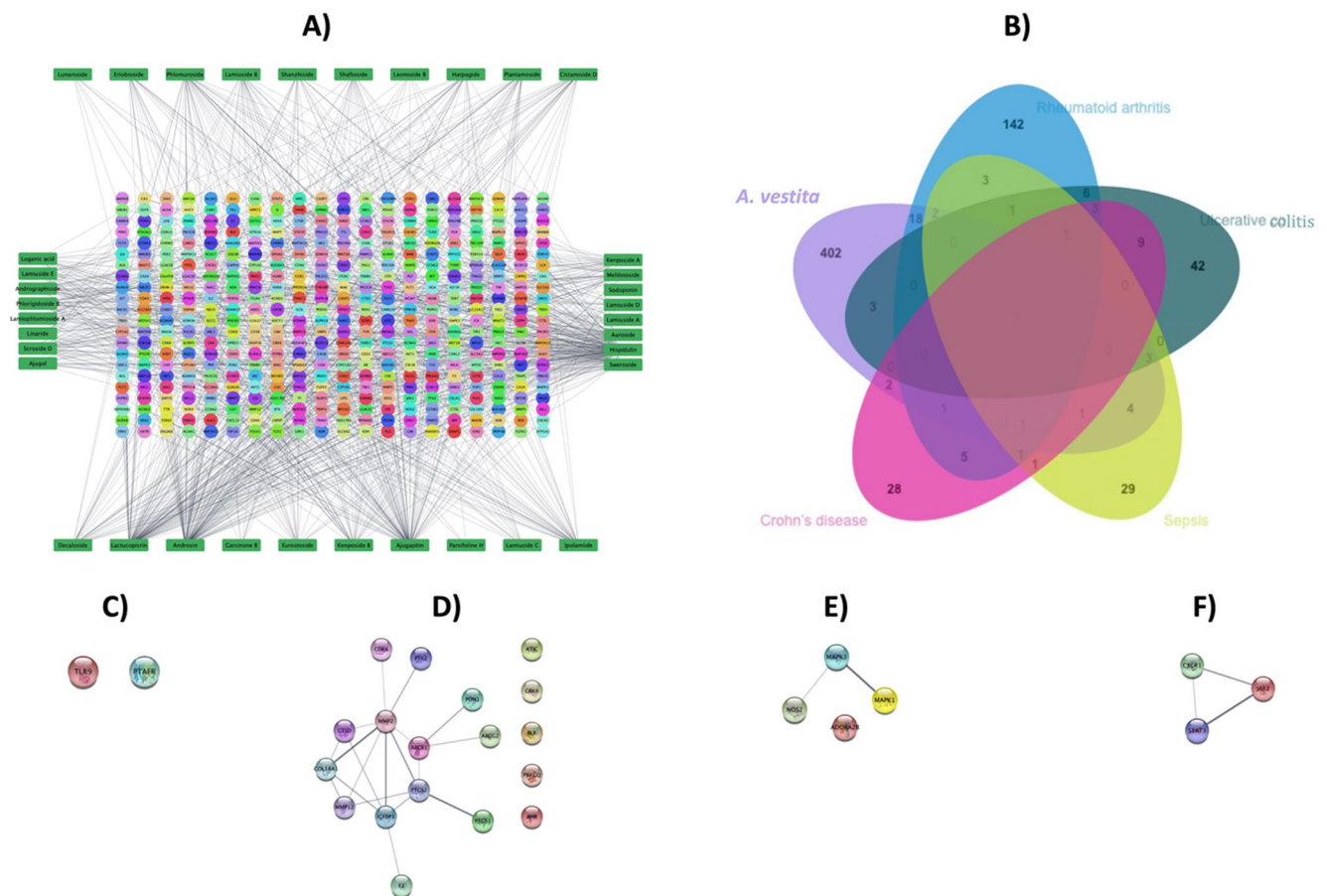


Fig. 1 PPI networks of disease-associated intersecting genes

pointing to stress kinase and nitric oxide biology; ulcerative colitis produced a fully connected triad with 3 nodes and 3 edges linking *JAK2*, *STAT3*, and *CXCR1* and converging on JAK-STAT cytokine signaling and chemokine guided leukocyte trafficking; and Crohn disease projected 2 nodes and 0 edges for *PTAFR* and *TLR9*, indicating parallel but not directly interacting modules at the applied confidence threshold (Fig. 1). Taken together, this single network level summary prioritizes high MCC hubs in rheumatoid arthritis while outlining compact yet mechanistically coherent motifs in the other indications, thereby providing a systems level foundation for downstream validation.

GO enrichment analysis

Gene Ontology GO enrichment was performed on the intersecting targets for each disease with a focus on Biological Process (BP) terms. Chord plots were generated to map term to gene linkages for each disease, confirming multi term hubs and validating the BP selections. The analysis identified 3 significant BP terms for Crohn disease, 37 for rheumatoid arthritis, 37 for sepsis, and 14 for ulcerative colitis. For Crohn disease, the top terms were positive regulation of

interleukin 6 production, positive regulation of tumor necrosis factor production, and inflammatory response. The chord view indicated that *TLR9* and *PTAFR* acted as the principal bridges connecting these cytokine production terms. For rheumatoid arthritis, the top ten terms were response to xenobiotic stimulus, angiogenesis, xenobiotic transport across blood brain barrier, positive regulation of protein phosphorylation, regulation of cell shape, bone trabecula formation, cyclooxygenase pathway, transepithelial transport, export across plasma membrane, and cellular response to fluid shear stress. The chord plot highlighted *PTGS2*, *VEGFA*, *ABCG2*, *PTK2*, and *F2* as multi term hubs linking angiogenesis, transport, and phosphorylation control. For sepsis, the top ten terms were cardiac neural crest cell development involved in heart development, regulation of Golgi inheritance, caveolin mediated endocytosis, response to epidermal growth factor, positive regulation of macrophage proliferation, regulation of cellular pH, trachea formation, regulation of early endosome to late endosome transport, ERBB2 ERBB3 signaling pathway, and regulation of stress activated MAPK cascade. In sepsis, *MAPK1*, *MAPK3*, *NOS2*, and *ADORA2B* jointly bridged endocytosis, growth factor response, pH regulation, and stress kinase cascades

in the chord representation. For ulcerative colitis, the leading terms were interleukin 23 mediated signaling pathway, growth hormone receptor signaling pathway via JAK-STAT, interleukin 6 mediated signaling pathway, growth hormone receptor signaling pathway, negative regulation of cytokine production involved in inflammatory response, cell surface receptor signaling pathway via JAK-STAT, positive regulation of interleukin 1 beta production, positive regulation of tumor necrosis factor production, and cytokine mediated signaling pathway. A compact triad of *JAK2*, *STAT3*, and *CXCR1* spanned these cytokine and JAK-STAT related terms in the chord plot (Figs. 2 and 3). Collectively, these disease specific BP signatures converge on cytokine and growth factor signaling, barrier and transport functions, and stress responsive trafficking and kinase cascades, providing a coherent functional context for the intersecting targets.

KEGG pathway enrichment analysis

KEGG enrichment was performed on the intersecting targets for each disease, and significance was evaluated with DAVID at p smaller than 0.005. At this threshold, the number of enriched pathways were rheumatoid arthritis 14, sepsis 115, ulcerative colitis 17, and Crohn disease none. Chord plots were generated to map pathway to gene connections for each disease, confirming multi pathway hubs

and validating the KEGG selections. Consistent with the top ten selections for each disease, the leading pathways were as follows. For rheumatoid arthritis the top ten were: Pathways in cancer, VEGF signaling pathway, Human cytomegalovirus infection, small cell lung cancer, MicroRNAs in cancer, Human papillomavirus infection, Fluid shear stress and atherosclerosis, Kaposi sarcoma associated herpesvirus infection, Proteoglycans in cancer, Antifolate resistance. In the chord view, *VEGFA*, *PTGS2*, *PTK2*, *CDK6*, *MMP2*, *ABCB1*, *ABCG2*, *ATIC*, and *F2* appeared as recurrent connectors across these pathways, indicating convergence on angiogenesis, eicosanoid signaling, adhesion and drug transport axes. For sepsis the top ten were: Pertussis, Leishmaniasis, Chagas disease, HIF 1 signaling pathway, Toxoplasmosis, Relaxin signaling pathway, Vascular smooth muscle contraction, Apelin signaling pathway, Tuberculosis, Alcoholism. The sepsis chord plot showed *MAPK1*, *MAPK3*, *NOS2*, and *ADORA2B* jointly bridging infection related and vascular signaling routes, consistent with stress kinase and nitric oxide mediated responses. For ulcerative colitis the top ten were: Chemokine signaling pathway, Adipocytokine signaling pathway, Prolactin signaling pathway, EGFR tyrosine kinase inhibitor resistance, *PD-L1* expression and *PD-1* checkpoint pathway in cancer, AGE-RAGE signaling pathway in diabetic complications, Th17 cell differentiation, Toxoplasmosis, Growth hormone

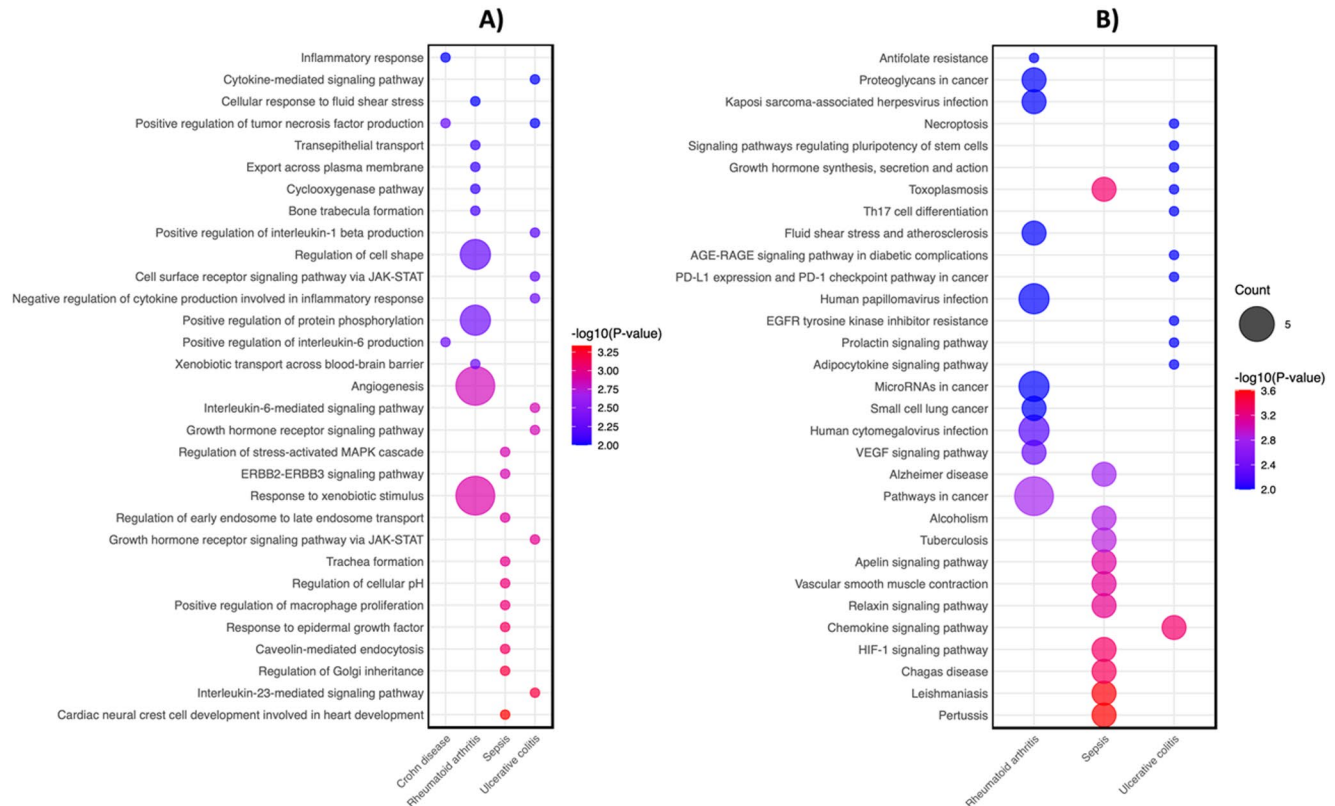


Fig. 2 Functional enrichment diseases: **A** Gene Ontology, **B** KEGG enrichment

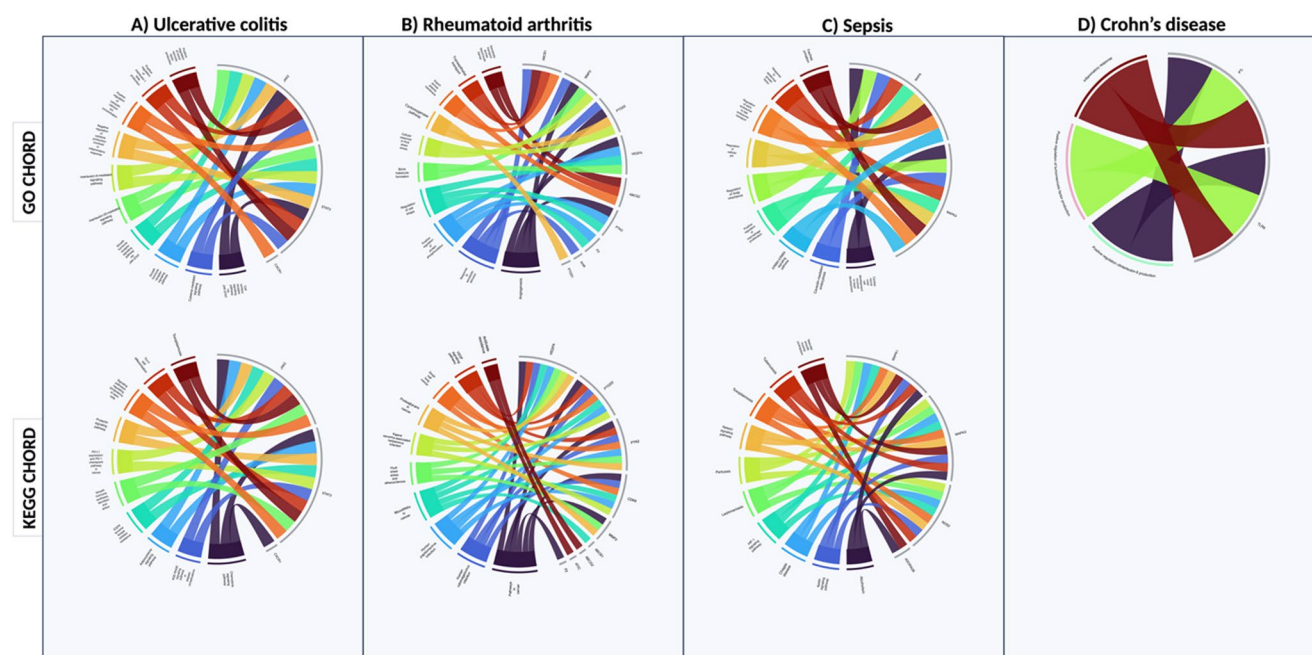


Fig. 3 Chord diagram of GO-Biological Process and KEGG enrichment for intersecting targets

synthesis secretion and action, Signaling pathways regulating pluripotency of stem cells. A compact triad of *JAK2*, *STAT3*, and *CXCR1* spanned these pathways in the chord plot, underscoring the centrality of JAK-STAT cytokine signaling and chemokine trafficking in this indication (Figs. 2 and 3). Taken together, the KEGG profile highlights inflammatory and infection related signaling in sepsis, cytokine and checkpoint signaling in ulcerative colitis, and growth factor and oncogenic signaling axes in rheumatoid arthritis, while no pathway level signal was detected for Crohn disease at the stated DAVID threshold.

Molecular docking results

A library of 36 *Ajuga vestita* phytochemicals was docked against five standard enzyme targets and six network pharmacology-related therapeutic targets (Table S1). Only poses that met the prespecified selection criterion of $\Delta G_{\text{dock}} \leq -7$ kcal/mol were considered for downstream analysis (Table S3). For each target, the distribution was as follows: for the EGFR, all evaluated pairs were at or below the threshold. Among the pairs under consideration, eight exhibited a RMSD of at least 2.0 Å. For MAPK1, all evaluated pairs were at or below the threshold. Among these, 11 pairs exhibited a RMSD ≥ 2.0 Å. For amylase, all evaluated pairs were at or below the threshold. Among these, eight pairs exhibited a RMSD of at least 2.0 Å. For ERK1, thirty-five pairs met this threshold. Among these, 13 pairs exhibited a RMSD of at least 2.0 Å. For butyrylcholinesterase (BChE), 32 pairs met this threshold. Among these, seven

pairs exhibited RMSD of at least 2.0 Å. For PTGS2, thirty-one pairs met this threshold. Among these, 24 pairs exhibited a RMSD ≥ 2.0 Å. For PTK2, 28 pairs met this threshold. Among these, nine pairs exhibited a RMSD of at least 2.0 Å. For JAK2, twenty-five pairs met this threshold. Among these pairs, 14 exhibited a RMSD of at least 2.0 Å. For glucosidase, 25 pairs met this threshold. Among these, six pairs exhibited a RMSD of at least 2.0 Å. For acetylcholinesterase (AChE), twenty-one pairs met this threshold. Among the aforementioned pairs, three exhibited a RMSD value of at least 2.0 Å. For the tyrosinase pair, one pair satisfied this threshold. Within the retained universe, the docking scores ranged from -11.3 to -7.0 kcal/mol, with the most favorable value recorded for Lamiuside D against PTGS2. Scores near the threshold clustered again on PTGS2 at -7.0 kcal/mol. generally acceptable, although a minority of poses showed high RMSD and require caution, as evidenced by a lowest root mean square deviation of 0.0058 angstrom for Decaloside on PTK2. Conversely, the largest deviation of 54.5 angstrom for Sodoaponin on PTGS2 indicates a minority of poses that necessitate cautious interpretation in subsequent molecular dynamics (Table S1–S3, and Fig. 4).

Across the set that was retained, coherent interaction motifs and reproducible placements emerged. On PTGS2, the scores ranged from approximately -11.3 to -7.0 kcal/mol, and involved multi-point H-bonding and π -stacking within the catalytic channel. There were frequent contacts to ALA199, PHE210, THR212, ASN222, ASN382, and catalytic histidines. In the PTK2 model, high-scoring poses in the ATP site exhibited a combination of backbone

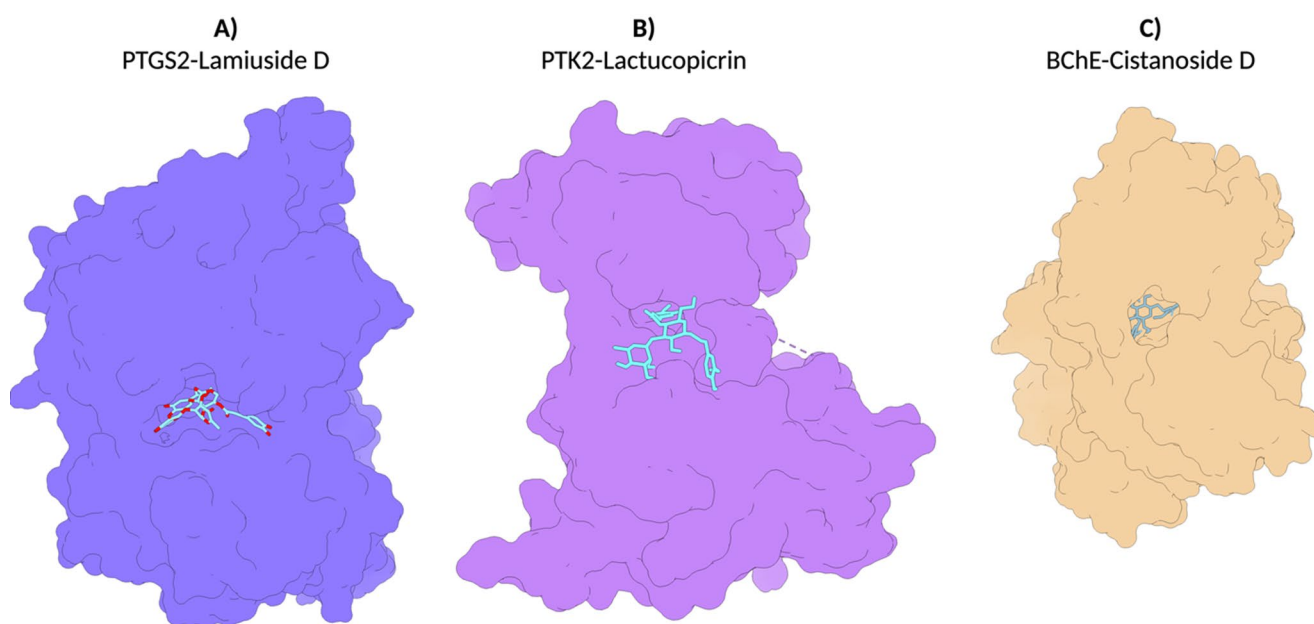


Fig. 4 Representative docked poses in canonical pockets. **A** PTGS2-Lamiuside D **B** PTK2-Lactucopicrin **C** BChE-Cistanoside D

H-bonds and hydrophobic packing in adjacent subpockets, recurrently engaging ARG426, GLN438, CYS502, ARG550, ASN551, ASP564, SER568, and ILE428, VAL436, ALA452, LEU553, and LEU567. Kinase targets such as EGFR, ERK1, and MAPK1 exhibited the canonical ATP-site pattern within the -10 to -7 kcal/mol band, with consistent contacts observed around GLU50, TYR53, LYS71/131, MET125, THR127, ASP184, and, for MAPK1, GLU33, SER41, ASP111, LYS114/54. This pattern was supplemented by aliphatic interactions within the hydrophobic cleft. JAK2 presented a similar arrangement over ~ -9.9 to -7.1 kcal/mol, with recurrent interactions at GLN553, THR555-THR557, VAL629, LYS630, LYS677, ARG715, TRP718. Cholinesterases demonstrated a predilection for gorge-spanning placements: AChE demonstrated a repeated involvement of TRP86, TYR124, GLY120-121, TYR133, PHE297, TYR337, PHE338, TYR341, HIS447. BChE exhibited a concentration around TRP82, GLY115-117, THR120, PHE329, TYR332, HIS438, with balanced polar and π -stacking contributions (~ -8.7 to -7.6 kcal/mol). A study of carbohydrate-processing enzymes revealed a mixture of polar and aromatic contacts surrounding catalytic-barrel residues. α -Amylase exhibited interactions with TRP59, TYR62, ARG195, ASP197, ASP300, and HIS305, with a range of -9.8 to -9.1 kcal/mol. In contrast, glucosidase demonstrated associations with TYR291, PHE350, and ARG352 (frequently involving a π -cation or salt bridge), supported by GLU248, GLY/SER290-294, and TRP331, with a range of -9.7 to -9.1 kcal/mol. TYR yielded borderline values around -7.2 to -7.0 kcal/mol, with the retained pose mainly involving GLU195 and HIS208 (Table S1).

Collectively, these docking and pose-quality results delineate a coherent, pharmacologically credible landscape in which PTGS2 and PTK2 emerge as primary anchors, combining strong scores with dense multi-point interactions in canonical pockets and a predominance of well-placed poses despite small $\text{RMSD} \geq 2.0$ Å subsets. The kinase nodes (EGFR, ERK1, MAPK1, JAK2) demonstrate the anticipated ATP-site engagement, accompanied by backbone hydrogen bonding and hydrophobic sub-pockets. This observation is consistent with the presence of tractable chemotypes that are amenable to selective optimization. Cholinesterases (AChE, BChE) display gorge-spanning solutions that reconcile polar contacts near the catalytic triad with π -stacking along the aromatic corridor. In contrast, carbohydrate-processing enzymes (α -amylase, glucosidase) favor mixed polar-aromatic interactions around catalytic-barrel residues, indicative of feasible inhibition modes within pocket constraints. Tyrosinase, on the other hand, remains borderline at the selection threshold and, given single-pair coverage, is lower priority at this stage. The minority of placements with $\text{RMSD} \geq 2.0$ Å should undergo resampling by explicit-solvent MD prior to rank-ordering, while the majority $\text{RMSD} \leq 2.0$ Å supports the structural reliability of the retained solutions. On this basis, experimental follow-up should prioritize PTGS2 and PTK2, alongside top-ranked complexes on EGFR, ERK1, MAPK1, JAK2, AChE, and BChE. This should be accomplished through orthogonal redocking, MD-based free-energy estimation, and targeted biochemical assays to confirm pose persistence, rank order, and selectivity.

Molecular dynamics simulations

To validate pose stability and interfacial persistence beyond static docking, 100-ns explicit-solvent molecular dynamics simulations were performed on six docked protein-ligand best complexes. For the sake of brevity, complexes are henceforth designated as C1–C6: C1 (Cistanoside D-BChE), C2 (Decaloside - PTK2), C3 (Lamiuside C-EGFR), C4 (Garcinone B-ERK1), C5 (Lactucopicrin-AChE), and C6 (Garcinone B-MAPK1). The trajectories were evaluated using RMSD, root-mean-square fluctuation (RMSF), minimum distance between ligand and protein, contact fraction, mean hydrogen-bond count per frame, and solvent-accessible surface area (SASA) (Figs. 5 and 6).

C1, C2, C3, and C5 demonstrated the most robust dynamics. C1 demonstrated stability by reaching a stable backbone C-alpha root-mean-square deviation plateau early on and maintaining that plateau thereafter. The minimum heavy-atom distance remained low, with a contact fraction of 1.00, and the mean hydrogen-bond count was approximately 3. C3 exhibited progressive tightening, with the minimum heavy-atom distance converging to approximately 1.0–1.2 Å while the mean hydrogen-bond count increased to approximately 5. Once established, all metrics remained stable. C2 and C5

exhibited constant root-mean-square deviation and uninterrupted contact, with mean hydrogen-bond counts of approximately 2 and 1, respectively. These values are consistent with cohesive interfaces. In contrast, C4 exhibited a late-time increase in root-mean-square deviation, ranging from approximately 0.8 Å to around 1.69 Å. This was accompanied by persistently low mean hydrogen-bond counts of approximately 1, indicative of a borderline stabilization pattern despite continuous contact at ≤ 3.0 Å. C6 demonstrated comparatively lability, characterized by an increase in root-mean-square deviation to ~ 3.18 Å, a shift in the minimum heavy-atom distance of ~ 1.77 Å, a slight decrease in contact fraction (~ 0.98), and a persistent weakness in mean hydrogen-bond count. These observations were corroborated by root-mean-square fluctuation analysis, which revealed significant fluctuations in peripheral loops, such as residues 338–340 in C6 at ~ 3.6 Å and residues 977–982 in C3 at ~ 3.0 Å. In contrast, the core regions exhibited compactness. SASA profiles remained within a relatively narrow range across all systems (approximately 178–262 nm²), arguing against pronounced global unfolding during the simulations (Figs. 5 and 6).

The MM-GBSA binding free energies were broadly consistent with these dynamic observations. The raw

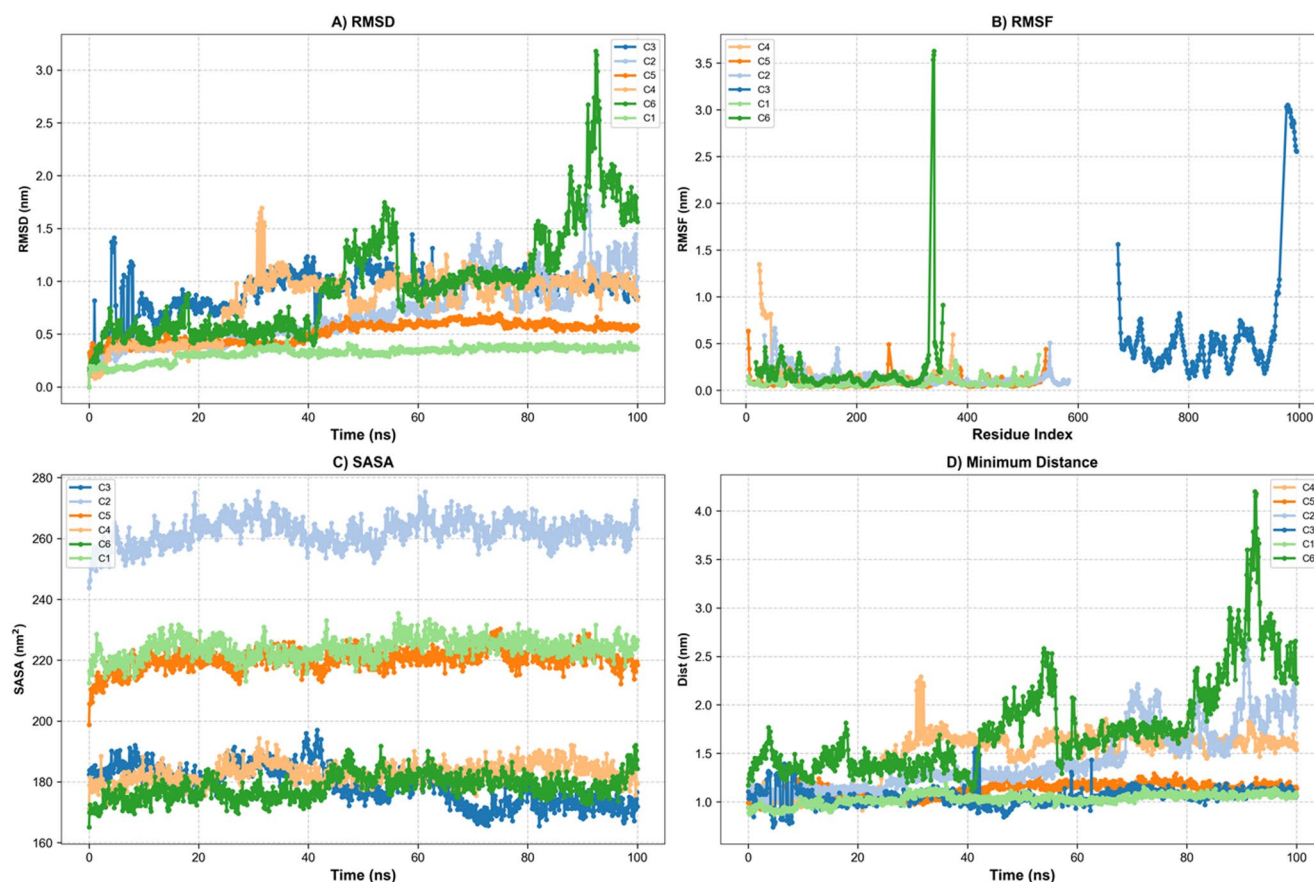


Fig. 5 Molecular-dynamics stability and proximity metrics; **A** RMSD, **B** RMSF, **C** SASA, **D** Minimum distance

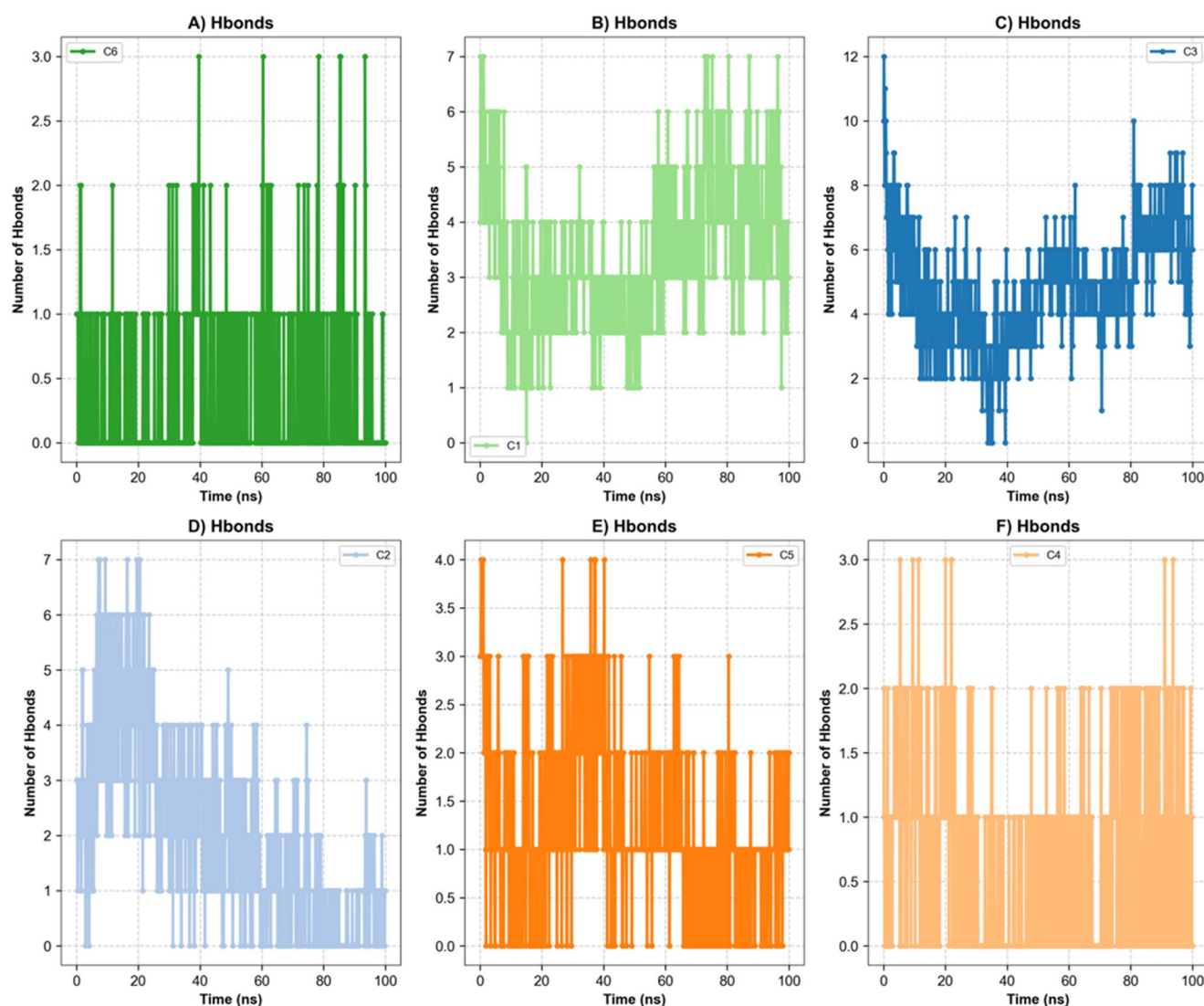


Fig. 6 Time-resolved hydrogen bonding at the protein-ligand interface

frame-wise total MM-GBSA energy values and per-residue energy decomposition data for the six simulated complexes (C1–C6) are provided in Supplementary Tables S4 and S5, respectively. The C1, C3, and C5 complexes were identified as the most favorable, with C2 following closely behind. C4 was classified as intermediate, and C6 was designated as the least favorable. In the MM-GBSA per-residue map, stabilization is dominated by the aromatic gorge, TRP82, PHE329, TYR332, with consistent support from ASP70, GLY116/117/121, GLN119, THR120, TRP231, VAL288, ALA328, and MET437. The protonated catalytic histidine HSP438 (HIS438) provides additional electrostatic and hydrogen-bond stabilization. The total MM-GBSA energy fluctuates within the range of ~ -80 to ~ -45 kcal mol⁻¹ during the initial ~ 150 frames, subsequently oscillating within the -50 to -35 kcal mol⁻¹ range. This oscillatory behavior is in alignment with the previously observed RMSD plateau and

contact fraction of 1.00 for C1 (Fig. 7A). For interpretative clarity, the MM-GBSA results are discussed below according to the relative stability ranking of the complexes rather than strictly in the numerical order C1–C6. For C2, the MM-GBSA trace indicated a moderately stable energetic profile, consistent with its relatively constant RMSD and uninterrupted contact observed during MD. Accordingly, C2 was ranked just below the top-performing group (C1, C3, and C5) and above the intermediate and weakly stabilized systems. In the case of C5, the stabilization is predominantly attributed to aromatic stacking within the gorge, involving TRP86, TYR337, PHE338, and TYR341. This process is complemented by polar support from GLY121, TYR124, GLU202, and HIS447, while additional peripheral contacts (TYR72, VAL73, THR75, and LEU76) serve to reinforce the pose. The MM-GBSA total energy stabilizes from approximately -36 to -23 kcal mol⁻¹ by 100–150 frames,

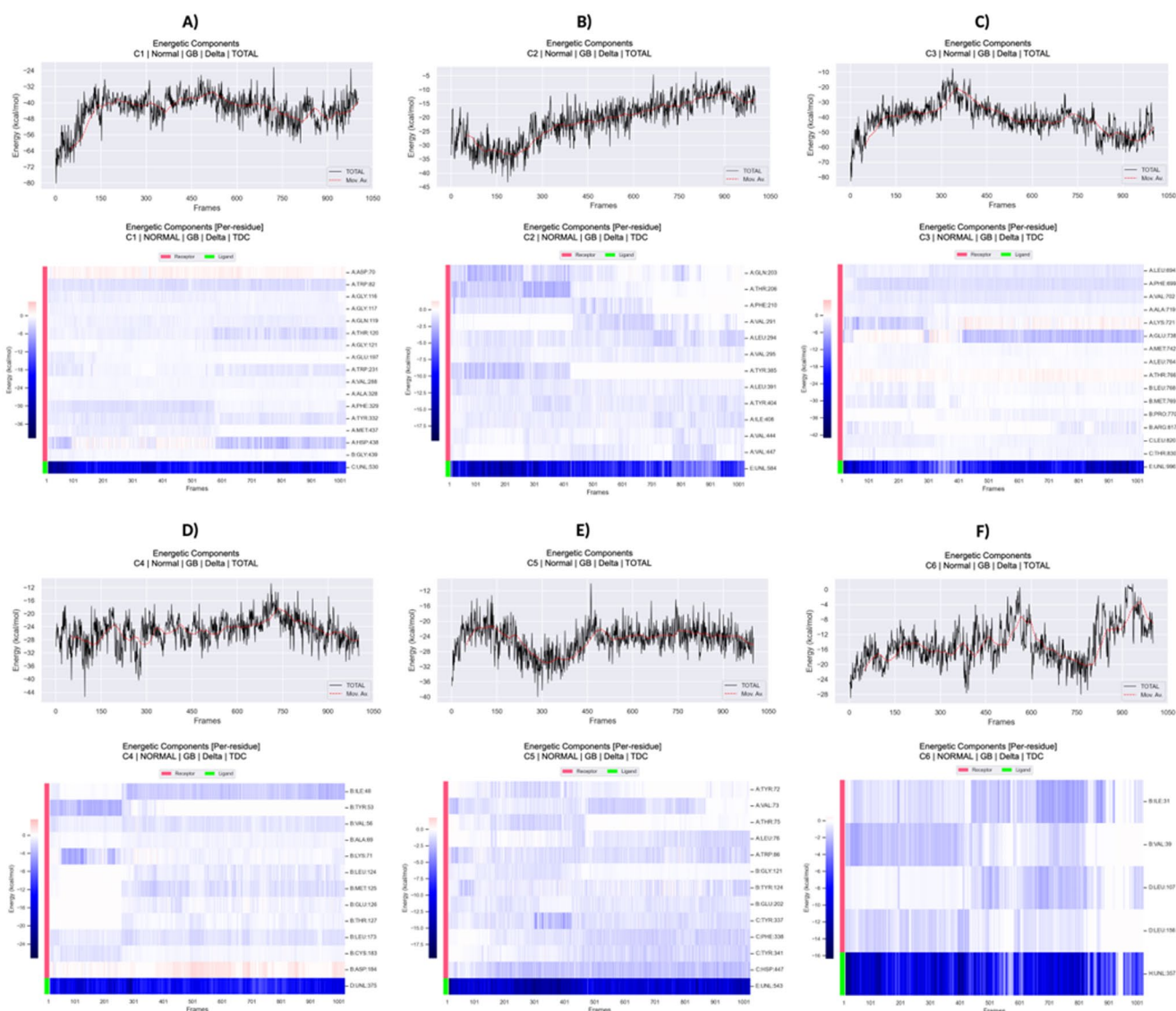


Fig. 7 Time-resolved MM-GBSA binding-energy profiles and per-residue energy decomposition maps of the six simulated protein-ligand complexes. **A** C1, Cistanoside D-BChE; **B** C2, Decaloside-PTK2; **C** C3, Lamiuside C-EGFR; **D** C4, Garcinone B-ERK1; **E** C5, Lactucopirin-AChE; and **F** C6, Garcinone B-MAPK1. For each complex, the upper panel shows the total MM-GBSA binding energy as a function of

simulation frames, while the lower panel shows the per-residue energy decomposition map across the same trajectory. Negative energy contributions indicate stabilizing residue-wise interactions, whereas less negative or positive values indicate weaker or destabilizing contributions. Energy values are expressed in kcal/mol

exhibits a transient deepening around 250–350 frames, and subsequently maintains a -25 to -22 kcal mol $^{-1}$ plateau, consistent with the observed geometric stability despite a modest mean hydrogen-bond count (Fig. 7E).

The MM-GBSA time series for C3 demonstrates an initial stabilization to approximately -40 kcal/mol by approximately 150 frames, followed by a transient destabilization peak around 300 to 340 frames (with less negative energy). This is then followed by a recovery to a steady band near -45 to -55 kcal/mol for the remainder of the trajectory. Residue-level stabilization was predominantly attributable to A: GLU738, A: MET742, A: THR766, and the B/C-chain

pocket rim (B: LEU768, B: MET769, B: PRO770, B: ARG817; C: LEU820, C: THR830), with supplementary vdW support from A: LEU694, A: PHE699, A: VAL702, A: ALA719, A: LYS721. These findings suggest that binding is facilitated by a downstream cleft/peripheral pocket rather than the conventional hinge, thereby aligning with the observed reduction in the protein-ligand minimum distance following the transient excursion (Fig. 7C). In contrast, C4 showed an intermediate stabilization pattern. The contributions are concentrated on B: TYR53, B: VAL56, B: ALA69, B: LYS71, B: LEU124, B: MET125, B: GLU126, and B: THR127, with support from B: LEU173, C: CYS183, and

B: ASP184. A brief late-time weakening is observed, followed by partial stabilization (Fig. 7D). C6 (Garcinone B-MAPK1) showed the weakest stabilization profile. The stabilization is intermittent and predominantly hydrophobic, as evidenced by the presence of B: ILE31, B: VAL39, D: LEU107, and D: LEU156. There is an absence of a persistent electrostatic focus. As the process progresses, the energy curve exhibits a drift towards less negative values, which corresponds to its weaker dynamic stability, as illustrated in Fig. 7F.

A comprehensive examination revealed that the integration of static docking, molecular dynamics, MM-GBSA, and residue-level decomposition identified C1, C3, C5, and C2 as the highest-confidence candidates. These candidates combined stable backbone C α -RMSD, tight minimum heavy-atom distances with high contact fractions, cohesive target-specific hydrogen-bond patterns, and favorable binding free energies. C4 warrants cautious advancement, a short repeat simulation, and confirmatory MM-GBSA. In addition, C6 should undergo resampling and/or ligand optimization prior to experimental prioritization. This integrative readout serves to reinforce the rationale for the implementation of targeted biochemical assays, which are instrumental in confirming activity, elucidating persistence, and determining selectivity.

Conclusion

To the best of our knowledge, this is the first comprehensive study to report on the chemical profile and biological effects of *A. vestita* extract. The chemical profile and biological effects of the plant depend on the extraction solvents used. Generally, the antioxidant and enzyme inhibitory effects of ethanol/water and ethanol extracts were more potent than those of ethyl acetate and water extracts. The chemical profile revealed 120 compounds, most of which were classified as iridoids, flavonoids, or phenolic acids. Notably, the water extract displayed low toxicity, maintaining high cell viability in the tested cell lines. Network pharmacology and molecular docking prioritized PTGS2 and PTK2 as key targets of *A. vestita* phytochemicals, whereas molecular dynamics and MM-GBSA analyses of the six selected complexes further supported C1, C3, C5, and C2 as the most stable candidates. These findings suggest that protein-protein interaction centrality, in conjunction with GO/KEGG enrichment, supports the complexes as rational candidates for designated disease contexts. In summary, *A. vestita* is a valuable source of natural bioactive compounds for use in functional applications in the nutraceutical and pharmaceutical industries. However, further research is needed to

understand the toxicity and bioavailability properties of isolated compounds from *A. vestita*.

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Data availability The data that support the findings of this study are available on request from the corresponding author.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

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