

Valorization of Olive Oil Residues: Phytochemical Analysis and Potential Bioactivity [†]

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Abstract

The valorization of olive oil production residues represents an innovative and sustainable strategy aligned with circular economy principles and the United Nations Sustainable Development Goals. In this study, we aimed to explore the phytochemical composition and neuroprotective potential of organic extracts obtained from olive pomace of the Arbequina and Arbosana cultivars. Extracts were prepared through solid–liquid extraction and analyzed by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS), enabling a comprehensive identification of bioactive metabolites. The analysis revealed a diverse profile of phenolic compounds, including hydroxytyrosol, tyrosol, and multiple oleuropein derivatives, as well as triterpenic acids such as oleanolic and maslinic acids. These compounds are widely recognized for their antioxidant, anti-inflammatory, and neuroprotective activities. The antioxidant potential of the extracts was evaluated in vitro using DPPH and ABTS radical scavenging assays, showing significant activity comparable to standard antioxidants. Moreover, cholinesterase inhibitory assays demonstrated moderate to strong inhibition of acetylcholinesterase, enzyme implicated in neurodegenerative diseases such as Alzheimer's disease. To further elucidate the molecular basis of these effects, in silico molecular docking studies were performed on the most abundant compounds, revealing favorable binding affinities and interactions with key active site residues of acetylcholinesterase. Overall, these findings highlight olive pomace as a promising, underutilized source of bioactive compounds with potential applications in the development of functional foods, nutraceuticals, and neuroprotective therapeutic agents. The integration of in vitro and in silico approaches strengthens the evidence supporting the use of these extracts in future biomedical and industrial applications.

Keywords: HPLC-MS phytochemical profile; agro-industrial waste; olive pomace; neuroprotective compounds; binding free energy; acetyl-cholinesterase

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1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia and is strongly linked to cholinergic deficits, particularly reduced acetylcholine in cortical and hippocampal regions [1,2]. Current treatments rely on cholinesterase inhibitors such as donepezil and rivastigmine, which provide only symptomatic relief and often cause adverse effects [3,4], highlighting the need for safer alternatives [5]. Natural products offer structural diversity and multitarget properties [6], with several studies reporting antioxidant, anti-inflammatory, and enzyme-modulating activities [7,8]. Olive-derived matrices are of particular interest due to their richness in polyphenols and triterpenoids [9,10], yet alperujo—the byproduct of olive oil extraction—remains largely unexplored pharmacologically [11].

In Chile, the olive oil industry has expanded significantly, with Arbequina and Arbosana cultivars widely adopted [12,13], leading to increased alperujo generation [14]. Currently treated as waste [15], this residue contrasts with its chemical richness and potential as a source of bioactives [16]. Characterizing alperujo could thus contribute to both sustainability and health innovation [17]. This study aimed to profile the phytochemical composition of Chilean alperujo and evaluate its major metabolites as acetylcholinesterase (AChE) inhibitors using HPLC-MS and molecular docking [18,19], positioning this material as a biotechnological resource rather than a discarded residue [20].

2. Materials and Methods

2.1. Preparation of Extracts

Alperujo from Arbequina and Arbosana varieties was obtained immediately after oil extraction in the Maule region, Chile. The material was air-dried at room temperature, stored under dark and cool conditions, and later processed by two extraction methods. For sonication-assisted extraction, 200 g of dried pomace were suspended in 800 mL of ethanol and treated with a probe sonicator (400 W, 20 kHz) for 30 min [21]. For reflux extraction, 200 g of pomace were mixed with 800 mL of ethanol:water (7:3 v/v) and heated at 70 °C for 1 h [22]. In both cases, extracts were filtered, concentrated under reduced pressure, and lyophilized to yield dry material for subsequent assays.

2.2. Enzymatic Inhibition Assay

The inhibitory activity of olive *alperujo* extracts against AChE was determined following Ellman's colorimetric method [23]. Briefly, reactions were performed using acetylthiocholine iodide as substrate and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as chromogenic reagent [24]. The reaction mixture was incubated with different concentrations of *alperujo* extracts, and the formation of the yellow 5-thio-2-nitrobenzoate anion was monitored at 405 nm. Galantamine was used as a positive control, showing an IC₅₀ value of 0.101 ± 0.01 µg/mL for AChE inhibition [25]. All assays were performed in triplicate under the same experimental conditions.

IC₅₀ Determination

The half-maximal inhibitory concentration (IC₅₀) values were derived from the enzymatic inhibition data obtained using Ellman's method [23]. Extracts were evaluated at three concentrations (125, 250, and 500 µg·mL⁻¹). For each concentration (C) and its corresponding percentage inhibition (%I), IC₅₀ values were estimated by fitting the data to the Hill–Langmuir equation:

$$y = V_{max} \times x^n / (k^n + x^n)$$

Three IC₅₀ estimates were obtained per sample, one at each tested concentration, and the final IC₅₀ was expressed as the arithmetic mean ± standard deviation of these values.

The fitting followed the Hill–Langmuir equation, where y represents the response expressed as percentage inhibition, x is the concentration, $V_{\max}$ is the maximum response, k corresponds to the IC_{50} value, and n is the Hill coefficient describing the steepness of the curve.

2.3. Sample Preparation for HPLC-MS

Alperujo extracts from Arbequina and Arbosana cultivars were solubilized with 200 μ L of ice-cold 80% acetonitrile. Samples were vortexed and sonicated for 5 min, followed by centrifugation at $10,000\times g$ for 10 min. The supernatant was transferred to HPLC vials and maintained at 8 °C until injection [26].

2.4. HPLC-MS Analysis

Chromatographic separation was carried out on a Luna NH_2 column (2.1×100 mm, 3 μ m; Phenomenex) using hydrophilic interaction chromatography (HILIC) [26]. The mobile phases consisted of:

Solvent A: water + 0.1% acetic acid + 10 mM ammonium acetate.

Solvent B: 99% acetonitrile + 0.1% acetic acid + 10 mM ammonium acetate.

The flow rate was set to 250 μ L $\cdot$ min $^{-1}$, with a column temperature of 40 °C. The injection volume was 3 μ L.

2.5. Mass Spectrometry

Data acquisition was performed in both positive and negative ionization modes, across three acquisition segments: (i) system equilibration, (ii) calibrant injection for post-acquisition recalibration, and (iii) MS/MS acquisition of the sample [27].

2.6. Compound Selection and Molecular Modeling

The ten most abundant compounds identified by HPLC from Arbequina and Arbosana *alperujo* extracts were selected for in silico evaluation [28]. Molecular structures were drawn and optimized in three dimensions using the protein preparation wizard module of the Schrödinger suite. The ligands were prepared through the LigPrep module, ensuring proper protonation states at pH 7.0 and energy minimization using the OPLS_2005 force field [29].

2.7. Protein Preparation

The crystallographic structure of human AChE was retrieved from the RCSB Protein Data Bank (PDB ID: 4BDT). The protein was pre-processed in the protein preparation wizard module, including the assignment of bond orders, addition of missing hydrogen atoms, and optimization of hydrogen bonding networks [30]. Protonation states were assigned at pH 7.0 with PROPKA software [31]. The crystallographic ligand (HuPrine W), and water molecules within 5 Å of the co-crystallized ligand were retained. Energy minimization of the hydrogen atoms was performed using the OPLS_2005 force field to relieve structural strain. Prior to molecular docking calculations, the reference ligand was removed from the binding site.

2.8. Docking Protocol

Molecular docking calculations were conducted using the Induced Fit Docking (IFD) protocol [32]. The binding site was defined as the residues located within 5 Å of the co-crystallized ligand HuPrine W in the AChE crystal structure. These residues included chain A positions: Asp74, Gly80, Gly82, Thr83, Trp86, Gly120, Gly121, Gly122, Tyr124, Ser125, Leu126, Tyr133, Glu202, Ser203, Ala204, Phe295, Arg297, Tyr337, Phe338, Tyr341,

Trp439, Met443, Pro446, His447, Gly448, Tyr449, Ile451. Each ligand was subsequently docked into the catalytic active site (CAS) of AChE, and the resulting complexes were scored based on predicted binding free energy and interaction profiles.

2.9. MM-GBSA Free Energy Calculations

To refine docking results, the binding free energy of each ligand–protein complex was estimated using the Prime MM-GBSA method implemented in Schrödinger [33]. Calculations were performed under the VSGB 2.1 solvation model, and outputs included ΔG_{bind} values, docking scores, and the number of ligand–residue interactions (INT). These results were compared against the docking profile of the reference inhibitor Huprine W to assess relative inhibitory potential.

3. Results and Discussion

3.1. Inhibitory Activity of Alperujo Extracts on AChE

The alperujo extracts from Arbequina and Arbosana displayed concentration-dependent inhibition of AChE across all extraction methods tested (sonication, maceration, reflux). The inhibitory activity at 125, 250, and 500 $\mu\text{g}\cdot\text{mL}^{-1}$ was used to estimate IC_{50} values. Across samples, IC_{50} estimates fell in the range of 120–150 $\mu\text{g}\cdot\text{mL}^{-1}$, indicating consistent potency between varieties and extraction modes.

Table 1 summarizes the percentage inhibition at the three tested concentrations and the corresponding IC_{50} estimates for each extract. Galantamine was included as a positive control.

Table 1. Inhibition of AChE by Arbequina and Arbosana *alperujo* extracts obtained through different extraction methods. IC_{50} values were estimated from inhibition at 125, 250, and 500 $\mu\text{g}\cdot\text{mL}^{-1}$.

Sample	%Inhibition 500 $\mu\text{g}\cdot\text{mL}^{-1}$	%Inhibition 250 $\mu\text{g}\cdot\text{mL}^{-1}$	%Inhibition 125 $\mu\text{g}\cdot\text{mL}^{-1}$	Estimated IC_{50} ($\mu\text{g}\cdot\text{mL}^{-1}$)
Sonicated Arbequina	80.98	63.03	47.50	161.2 $\pm$ 0.30
Sonicated Arbosana	80.81	65.64	47.85	146.7 $\pm$ 1.08
Reflux Arbequina	80.75	63.81	53.13	372.3 $\pm$ 0.44
Reflux Arbosana	83.21	63.97	49.19	233.8 $\pm$ 0.46
Galantamine	-	-	-	0.101 $\pm$ 0.01

Effect of Extraction Method

Comparison between extraction procedures revealed distinct extraction efficiencies for bioactive constituents. Reflux produced extracts with lower IC_{50} values, consistent with the enhanced solubilization of aglycone flavonoids and semi-polar metabolites at elevated temperature [34]. Sonication also yielded potent extracts, likely by disrupting plant tissue and releasing bound compounds [35]. Importantly, Arbequina and Arbosana showed comparable inhibitory capacities, suggesting that varietal differences are secondary to extraction technique in determining inhibitory potency.

3.2. HPLC–MS Metabolite Profiling

High-resolution HILIC–MS analysis revealed a diverse and complex phytochemical profile in both Arbequina and Arbosana *alperujo* extracts. This analysis enabled the identification of a broad spectrum of metabolites and also provided insights into their relative

abundances, which are critical for correlating chemical composition with biological activity. The ten most abundant compounds, ranked by relative intensity, are presented in Table 2.

The compounds identified belong to different chemical families, including flavonoids, phenolic acids, sugars, amines, nucleobases, and iridoid glycosides. Among these, flavonoids such as luteolin and apigenin stand out due to their well-documented capacity to inhibit cholinesterases and their recognized neuroprotective roles in Alzheimer's disease models [36,37]. Phenolic acids like 3,4-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid, although generally weaker inhibitors, may act additively to enhance overall inhibitory activity in complex mixtures. The presence of iridoid glycosides such as loganin further enriches the phytochemical profile, suggesting that the extracts may exert biological effects beyond cholinesterase inhibition, including antioxidant and anti-inflammatory actions.

Table 2. Top Ten most intense compounds identified in Arbequina/Arbosana *alperujo* extracts by HPLC–MS. RT refers to retention time (minutes); *m/z* meas. corresponds to the measured mass-to-charge ratio of the precursor ion; M meas. indicates the measured molecular mass; Formula represents the molecular formula of the putatively identified compound.

Rank	RT (min)	<i>m/z</i> meas.	M meas.	Compound	Formula	Intensity ¹
1	4.59	181.072	182.079	Dulcitol	C ₆ H ₁₄ O ₆	640,879/286,162
2	7.79	285.041	286.048	Luteolin	C ₁₅ H ₁₀ O ₆	212,976/203,961
3	4.38	167.035	168.042	3,4-Dihydroxyphenylacetic acid	C ₈ H ₈ O ₄	118,219/123,467
4	5.75	179.056	180.065	D-Tagatose	C ₆ H ₁₂ O ₆	38,916/68,465
5	0.98	102.127	101.120	Triethylamine	C ₆ H ₁₅ N	41,477/46,029
6	4.53	269.046	270.054	Apigenin	C ₁₅ H ₁₀ O ₅	27,190/45,961
7	0.91	182.118	181.110	N-Phenyldiethanolamine	C ₁₀ H ₁₅ NO ₂	19,824/14,083
8	2.23	136.061	135.054	Adenine	C ₅ H ₅ N ₅	11,019/12,190
9	1.39	153.055	152.047	3-Hydroxyphenylacetic acid	C ₈ H ₈ O ₃	4471/6054
10	1.32	391.158	390.151	Loganin	C ₁₇ H ₂₆ O ₁₀	3925/5062

¹. Values are expressed as intensity in Arbequina/Arbosana order.

Linking Phytochemistry and Inhibitory Activity

Among the top metabolites, luteolin (rank 2) and apigenin (rank 6) are particularly notable, as both flavonoids have been consistently associated with AChE inhibition in previous *in vitro* and *in silico* studies [38,39]. Their relative abundance in the extracts provides a plausible mechanistic explanation for the observed inhibitory activity. Phenolic acids such as 3,4-dihydroxyphenylacetic acid may further contribute via weaker, additive effects. Sugars and sugar alcohols (dulcitol, D-tagatose) likely play no direct inhibitory role but indicate the polar nature of the extracts. The presence of loganin (an iridoid glycoside) suggests possible contributions to neuroprotective effects beyond AChE inhibition, although its direct activity against the enzyme is expected to be limited.

Taken together, these results support a model where flavonoid content, particularly luteolin and apigenin, underpins the inhibitory potency of *alperujo* extracts. Differences in extraction method are therefore expected to reflect the relative enrichment or depletion of these flavonoids, explaining the observed IC₅₀ variations.

3.3. Molecular Docking and MM-GBSA Analysis

To further explore the inhibitory potential of alperujo-derived metabolites, the ten most intense compounds identified by HPLC–MS were individually subjected to molecular docking analysis against AChE [40]. This selection allowed us to assess whether the most intense signals in the chromatographic profile correspond to bioactive molecules or if less abundant compounds are the primary contributors to the inhibitory effect. The docking results, including binding free energy (ΔG_{bind}), number of ligand–protein interactions (INT), and overall ranking, are summarized in Table 3.

Table 3. Docking results of the ten most intense alperujo-derived compounds identified by HPLC–MS against AChE, including Huprine W as reference inhibitor. ΔG_{bind} : calculated binding free energy; INT: number of protein–ligand interactions with residues of the binding site.

Compound	ΔG_{bind}^1	INT
Loganin	−43.81	33
Luteolin	−50.25	27
Apigenin	−43.59	26
Dulcitol	−18.40	28
D-tagatose	−19.58	26
3-Hydroxyphenylacetic acid	−5.64	22
Adenine	−18.49	25
N-Phenyldiethanolamine	−26.49	21
Triethylamine	−24.71	23
3,4-Dihydroxyphenylacetic acid	−15.68	15
Huprine W	−115.67	34

¹. Binding free energy (ΔG_{bind}) values are expressed in kcal·mol^{−1}.

Among them, loganin displayed the most favorable interaction profile ($\Delta G_{\text{bind}} = -43.81$ kcal·mol^{−1}, 33 interactions), ranking sixth overall in the docking dataset. This result is particularly relevant given its relatively high abundance in *alperujo* and its structural similarity to other iridoids previously reported as cholinesterase inhibitors [41].

Flavonoids such as luteolin and apigenin showed intermediate binding affinities (−50.25 and −43.59 kcal·mol^{−1}, respectively), with normalized scores between 0.61 and 0.65. These values, although lower than those of the reference inhibitor huprine (−115.67 kcal·mol^{−1}) are consistent with literature reports where flavonoids contribute significantly to the inhibitory potential of polyphenol-rich extracts [42].

Sugars and sugar alcohols, including dulcitol and D-tagatose, exhibited weaker affinities (ΔG_{bind} between −18.4 and −19.6 kcal·mol^{−1}) and lower rankings, suggesting they are unlikely to contribute directly to cholinesterase inhibition. Similarly, small nitrogen-containing metabolites such as adenine and N-phenyl diethanolamine displayed modest binding energies (−18.5 and −26.5 kcal·mol^{−1}, respectively), reinforcing the notion that their biological role may be indirect or negligible in this context.

Triethylamine and 3-hydroxyphenylacetic acid also ranked low ($\Delta G_{\text{bind}} = -24.71$ and −5.64 kcal·mol^{−1}, respectively), confirming their limited contribution to the overall inhibitory activity. Finally, 3,4-dihydroxyphenylacetic acid (34_DPA) showed very weak interactions ($\Delta G_{\text{bind}} = -15.68$ kcal·mol^{−1}, Rank 83), highlighting its minimal relevance compared to more active phenolics.

This convergence between computational and enzymatic data reinforces the hypothesis that the bioactivity of *alperujo* arises from the synergistic action of phenolic and iridoid constituents rather than from the dominant sugar fraction.

Among the evaluated metabolites, luteolin emerged as one of the most promising ligands in terms of predicted binding affinity and interaction profile. To better understand its binding mode, a two-dimensional ligand interaction diagram was generated (Figure 1).

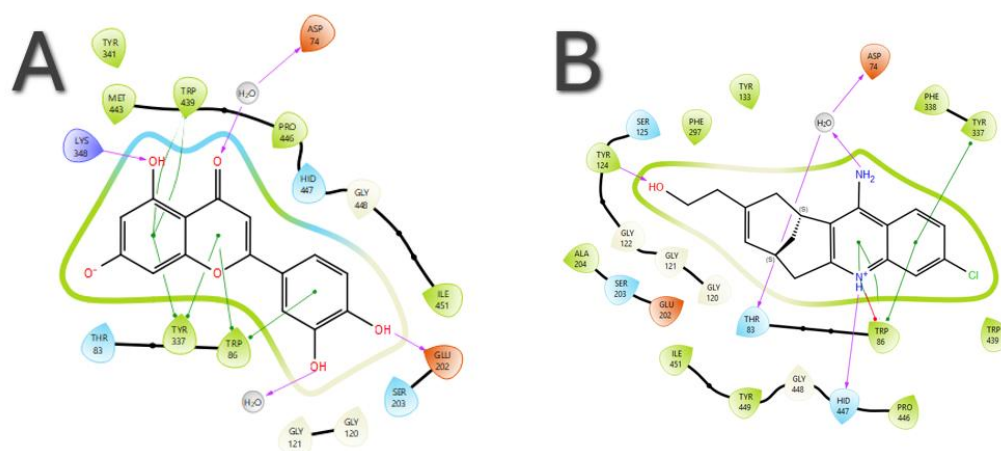


Figure 1. Comparative 2D ligand interaction diagrams of Luteolin (A) and Huprine W (B) bound to AChE.

The analysis revealed that luteolin engages in a dense interaction network within the enzyme gorge, forming hydrogen bonds with Glu202, Ser203, and Tyr337, and establishing π - π stacking interactions with Trp86 and Tyr341 [43]. These residues are of pharmacological relevance because they define both the catalytic anionic site (CAS) and the peripheral anionic site (PAS), two domains that are fundamental for substrate recognition and catalytic turnover. Luteolin's ability to simultaneously contact residues in both the CAS and PAS suggests that it may function as a dual-site ligand, a mechanism associated with enhanced inhibitory potency [44]. Moreover, interactions with Trp439 and Met443 further stabilize the compound within the gorge, reducing the likelihood of displacement by the natural substrate acetylcholine. When compared to crystallographic data of Huprine W, a reference inhibitor with nanomolar potency, remarkable parallels can be observed. Huprine W is known to anchor through π - π stacking with Trp86 and Tyr337, while establishing hydrogen bonding with Glu202, thereby occupying both the CAS and PAS. The fact that luteolin reproduces these key interactions, despite its lower molecular complexity and natural origin, highlights its potential as a pharmacologically relevant cholinesterase inhibitor. The overlap in binding determinants between luteolin and Huprine W strengthens the mechanistic plausibility of luteolin as a contributor to the inhibitory activity of *alperujo* extracts, bridging the gap between computational predictions and experimental enzymatic data.

4. Conclusions

The integration of enzymatic inhibition assays, metabolite profiling, and molecular docking provided a coherent view of the cholinesterase inhibitory potential of *alperujo* extracts. The HPLC-MS results revealed a chemical matrix dominated by sugars, yet docking analyses demonstrated that these abundant metabolites contribute little to the observed activity. Instead, phenolic and iridoid compounds such as luteolin, apigenin, and loganin emerged as the most relevant contributors, showing strong predicted affinities for AChE. Luteolin reproduced several of the critical interactions described for the reference inhibitor Huprine W, including contacts with Trp86, Tyr337, and Glu202, suggesting a dual-site binding mode. This mechanistic alignment between computational predictions and IC_{50} values measured in vitro underscores the pharmacological relevance

of specific secondary metabolites within the extracts; highlighting the potential of *alperujo*-derived molecules as promising natural scaffolds for the development of cholinesterase inhibitors and illustrates how agro-industrial byproducts can serve as valuable resources for biomedical research.

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Conflicts of Interest:

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