Macedonian Journal of Chemistry and Chemical Engineering, Vol. **45**, No. 1, pp. xx–xx (2026)

MJCCA9 – ISSN 1857-5552
e-ISSN 1857-5625
Received: January 22, 2026 DOI: 10.20450/mjcce.2026.3426
Accepted: May 12, 2026 *Original scientific paper*

PHYTOCHEMICAL CHARACTERIZATION, ANTIOXIDANT PROPERTIES, AND MOLECULAR DOCKING ANALYSIS OF *CISTANCHE TINCTORIA* FROM THE ALGERIAN SAHARA

Nesrine Sadaoui-Smadhi[1*], Yasser Kadri[2], Souheyla Toubal[1], Djillali El haddad[1], Sihem Akmoussi-Toumi[1], Hamza Aliboudhar[3], Narimen Benhabyles[1] Razika Laoufi[4], Mohammed Tayyib Benaissa[1], Souad Khemili-Talbi[1]

[1]*Laboratoire de Bio-Informatique, Microbiologie Appliquée et Biomolécules, Faculty of Sciences, University M'Hamed Bougara of Boumerdes, Boumerdes 35000, Algeria*
[2]Laboratory of Saharan Natural Resources, African University Ahmed Draia, Adrar 01000, Algeria
[3]*Laboratoire d'Analyse Organique Fonctionnelle, Faculté de Chimie, University of Sciences and Technology Houari Boumediene (USTHB), BP32, El-Alia Bab Ezzouar 16111, Alger, Algeria*
[4]*Laboratory of Technologies Soft and Valorization of Biological Materials and Biodiversity, Faculty of Science University M'hamed Bougara of Boumerdes, Algeria*

n.sadaoui@univ-boumerdes.dz

Cistanche tinctoria is a medicinal plant traditionally used for its tonic and antioxidant properties; however, its bioactive profile remains insufficiently characterized. This study aimed to investigate the chemical composition and antioxidant potential of a hydromethanolic extract of *C. tinctoria*, and to explore the interactions of its major phenolic compounds with enzymes involved in oxidative stress-related processes. The total phenolic and flavonoid contents were determined using spectrophotometric methods, while chromatographic profiling and quantification of individual constituents were performed by high-performance liquid chromatography coupled with a photodiode array detector (HPLC-PDA). The antioxidant activity of the extract was evaluated using the DPPH radical scavenging, ABTS radical scavenging, cupric reducing antioxidant capacity, and ferric reducing antioxidant power assays. HPLC-PDA analysis revealed the presence of phenolic compounds, with acteoside and chicoric acid identified as the major constituents. The extract exhibited strong radical scavenging and reducing activities. Molecular docking studies suggested favorable interactions between the major phenolic compounds and protein targets associated with oxidative stress. In addition, *in silico* absorption, distribution, metabolism, excretion, and toxicity predictions suggested acceptable physicochemical and pharmacokinetic properties. Overall, this integrated chemical and computational approach supports the antioxidant potential of *C. tinctoria* and highlights its value as a promising source of bioactive natural compounds.

Keywords: *Cistanche tinctoria*; acteoside; chicoric acid; antioxidant activity; molecular docking

ФИТОХЕМИСКА КАРАКТЕРИЗАЦИЈА, АНТИОКСИДАЦИСКИ СВОЈСТВА И АНАЛИЗА НА МОЛЕКУЛАРНО ДОКИРАЊЕ НА *CISTANCHE TINCTORIA* ОД АЛЖИРСКАТА САХАРА

Cistanche tinctoria е лековито растение кое традиционално се користи поради своите тонични и антиоксидациски својства; сепак, неговиот биоактивен профил останува недоволно карактеризиран. Целта на оваа студија беше да се испита хемискиот состав и антиоксидацискиот потенцијал на хидрометанолен екстракт од *C. tinctoria*, како и да се истражат интеракциите на неговите главни фенолни соединенија со ензими вклучени во процесите поврзани со оксидациски стрес. Вкупната содржина на феноли и флавоноиди беше определена со спектрофотометриски методи, додека хроматографското профилирање и квантификацијата на поединечните состојки беа

извршени со високоефикасна течна хроматографија поврзана со фотодиоден детектор (HPLC-PDA). Антиоксидацискатa активност на екстрактот беше оценета со тестови за неутрализација на DPPH радикали, ABTS радикали, антиоксидациски капацитет за редукција на бакар и антиоксидациска моќ за редукција на железо. HPLC-PDA анализата откри присуство на фенолни соединенија, при што актеозидот и шикорната киселина беа идентификувани како главни состојки. Екстрактот покажа силна активност за неутрализација на радикали и редукциски својства. Студиите на симулирано молекуларно докирање укажаа на поволни интеракции помеѓу главните фенолни соединенија и протеинските цели поврзани со оксидациски стрес. Дополнително, *in silico* предвидувањата за апсорпција, распределба, метаболизам, екскреција и токсичност покажаа прифатливи физичко-хемиски и фармакокинетички својства. Севкупно, овој интегриран хемиски и компјутерски пристап го потврдува антиоксидацискиот потенцијал на *C. tinctoria* и ја истакнува нејзината вредност како ветувачки извор на биоактивни природни соединенија.

Клучни зборови: *Cistanche tinctoria*; актеозид; шикорна киселина; антиоксидациска активност; молекуларно докирање

1. INTRODUCTION

Reactive oxygen species (ROS) play a dual role in biological systems, acting as essential signaling mediators under physiological conditions while causing oxidative damage when produced in excess. An imbalance between ROS generation and antioxidant defenses leads to oxidative stress, which is strongly implicated in the development of numerous chronic and degenerative diseases, including cancer, cardiovascular disorders, neurodegenerative diseases, and inflammation.[1] Consequently, the search for safe and effective natural antioxidants capable of restoring redox homeostasis has attracted increasing scientific interest.

Medicinal plants constitute an important source of bioactive compounds, particularly phenolic compounds and flavonoids, which are well recognized for their antioxidant and cytoprotective properties. These compounds exert their effects through direct free radical scavenging as well as through modulation of endogenous antioxidant defense systems.[2]

Cistanche tinctoria (Orobanchaceae) is a holoparasitic desert plant distributed in North Africa, the Arabian Peninsula, and parts of Asia. It develops underground by attaching to the roots of specific host plants, including *Tamarix gallica*, *Calligonum comosum*, and *Pulicaria* species.[3] In traditional medicine, the dried whole plant has been used to treat various ailments such as abdominal pain, diarrhea, muscular disorders, gynecological conditions, delayed lactation, and diabetes.[3,4] Despite its ethnomedicinal relevance, there have been very few comprehensive studies addressing its chemical composition and antioxidant mechanisms.

Given the complexity of antioxidant processes, multiple complementary *in vitro* assays such as DPPH radical scavenging, ABTS radical scavenging, cupric reducing antioxidant capacity (CUPRAC), and ferric reducing antioxidant power (FRAP) are required to achieve a reliable assessment of radical scavenging and reducing capacities.[5] In parallel, molecular docking has emerged as a valuable computational tool to explore enzyme–ligand interactions and to predict binding affinities with key oxidative stress–related enzymes.[6] Furthermore, *in silico* absorption, distribution, metabolism, excretion, and toxicity (ADMET) and toxicity predictions provide early insights into the pharmacokinetic behavior and safety profiles of bioactive compounds.[7,8]

To the best of our knowledge, no previous study has combined *in vitro* antioxidant evaluation, high-performance liquid chromatography (HPLC)-based phytochemical profiling, molecular docking analyses, and ADMET-toxicity predictions for *C. tinctoria*. Therefore, this study aimed (i) to characterize the phenolic profile of *C. tinctoria* roots using HPLC-photodiode array detector (PDA), (ii) to evaluate its antioxidant potential through complementary *in vitro* assays, and (iii) to explore the molecular interactions and safety profile of its major compounds using docking and *in silico* ADMET tools.

2. MATERIAL AND METHODS

2.1. *Plant material*

Specimens of *C. tinctoria* were collected from the Adrar region in southwestern Algeria. Taxonomic identification was confirmed by Dr. Yasser Kadri (University of Adrar, Algeria). The collected plant material was carefully cleaned to

remove dust and foreign particles, washed with distilled water, and separated into aerial and underground parts. The roots were then cut into small pieces, air-dried in the dark at room temperature, ground into a fine powder, and stored in opaque containers until analysis.

2.2. *Preparation of the hydromethanolic extract*

Root powder (20 g) was macerated in 100 ml of methanol/water (80:20, v/v) at room temperature for 24 h. After filtration, the extract was concentrated under reduced pressure at 40 °C using a rotary evaporator. The dried extract was stored for further analysis. The extraction yield was calculated using the following equation:

$$\text{Extraction yield (\%)} = (\text{weight of dry extract} / \text{weight of dry plant material}) \times 100.$$

2.3. *Phytochemical analyses*

2.3.1. *Total phenolic content*

The total phenolic content was determined using the Folin–Ciocalteu method as described by Singleton and Rossi.[9] Briefly, 0.5 ml of extract was mixed with 5 ml of distilled water, followed by the addition of 0.5 ml of diluted Folin–Ciocalteu reagent (1:10, v/v). After 3 min, 0.5 ml of sodium carbonate solution (20%) was added. The mixture was incubated in the dark at room temperature for 1 h, and the absorbance was measured at 760 nm against a blank. The total phenolic content is expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract).

2.3.2. *Total flavonoid content*

The total flavonoid content was determined using the aluminum chloride colorimetric method according to Ayoola et al.[10] Briefly, 1 ml of the extract solution was mixed with 1 ml of 2 % aluminum chloride ($AlCl_3$) solution. The mixture was incubated at room temperature for 10 min, and the absorbance was measured at 430 nm against a blank. The total flavonoid content was calculated from a quercetin calibration curve and is expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g extract).

2.3.3. *HPLC-PDA*

Quantitative analysis of phenolic compounds was performed using an HPLC system equipped with a PDA detector (Nexera-i LC-2040C 3D, Shimadzu). Separation was carried out on a phenylhexyl reversed-phase column (3 μm, 4.6 × 150 mm; GL Sciences InterSustain). The mobile phase consisted of solvent A (0.1 % formic acid in water) and solvent B (acetonitrile, HPLC grade; Merck), delivered at a flow rate of 1 mL/min. The injection volume for both samples and standards was 10 μl, and the column temperature was maintained at 30 °C. Stock solutions of standards and extracts were prepared at a concentration of 1000 mg/l. The phenolic profile was investigated using 16 reference standards: vanillic acid, caffeic acid, epicatechin, *p*-coumaric acid, salicylic acid, cinnamic acid, acteoside, rosmarinic acid, quercetin, chlorogenic acid, apigenin-7-*O*-glucoside, rutin, naringenin, 4-hydroxybenzoic acid, gallic acid, and ferulic acid. Detection was performed using the PDA detector by recording ultraviolet (UV) spectra over an appropriate wavelength range, and compound identification was based on comparison of retention times and UV spectra with those of authentic reference standards analyzed under the same chromatographic conditions. Quantification was based on external calibration curves, and the results are expressed as milligrams per gram of extract (mg/g).

2.4. *In vitro antioxidant activity*

Antioxidant activity was evaluated using complementary assays, including DPPH radical scavenging, ABTS radical cation scavenging, CUPRAC, and FRAP. All experiments were performed in technical triplicates. The results were expressed as the mean ± standard deviation.

2.4.1. *DPPH radical scavenging assay*

The DPPH• free radical scavenging activity of the extract and reference standards was evaluated according to the method described by Blois.[11] Briefly, 1.0 ml of the extract at different concentrations (0.05–0.5 mg/ml) was mixed with 4.0 ml of a 0.1 mM DPPH• solution and vortexed for 30 s. The mixtures were then incubated in the dark for 60 min, and the absorbance was measured at 517 nm against a blank (methanol). The percentage of DPPH• radical scavenging was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [(Ac - As) / Ac] \times 100$$

where Ac is the absorbance of the control and As is the absorbance of the sample.

2.4.2. *ABTS scavenging assay*

The $ABTS^{\bullet+}$ radical scavenging activity was evaluated according to the method described by Re et al.[12] The $ABTS^{\bullet+}$ radical solution was generated by incubating 2 mM ABTS with 2.3 mM potassium persulfate ($K_2S_2O_8$) in the dark at room temperature for 4 h. Before use, the $ABTS^{\bullet+}$ solution was diluted with 0.1 mM phosphate buffer (pH 7.4) to obtain an absorbance of 0.700 ± 0.02 at 734 nm. Three-milliliter aliquots of the extract at different concentrations (0.05–0.50 mg/ml) were mixed with 1.0 ml of the diluted $ABTS^{\bullet+}$ solution. After incubation, absorbance was measured at 734 nm against a buffer blank. The percentage of $ABTS^{\bullet+}$ radical scavenging was calculated with the following equation

$$ABTS^{\bullet+} \text{ scavenging effect (\%)} = [(Ac - As) / Ac] \times 100$$

where Ac represents the initial absorbance of $ABTS^{\bullet+}$ and As corresponds to the remaining absorbance of $ABTS^{\bullet+}$ in the sample.

2.4.3. *CUPRAC assay*

The CUPRAC assay was performed to evaluate the reducing power of the extract according to the method described by Apak et al.[13] Briefly, 1.0 mL of the extract (0.05–0.50 mg/ml) or Trolox standard solution was mixed with 1.0 ml of 0.01 M copper(II) chloride ($CuCl_2$), 1.0 ml of 7.5×10^{-3} M neocuproine, and 1.0 ml of acetate buffer. The reaction mixture was incubated at room temperature for 30 min, and the absorbance was measured at 450 nm. Antioxidant capacity was quantified using a Trolox calibration curve and is expressed as micromoles of Trolox equivalents per gram of sample (μmol TE/g).

2.4.4. *FRAP assay*

The FRAP assay was performed as described by Benzie and Strain,[14] with slight modifications. Briefly, the FRAP reagent was freshly prepared by mixing 10 ml of 300 mM acetate buffer (pH 3.6), 1 ml of 10 mM 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) solution prepared in 40 mM HCl, and 1 ml of 20 mM iron(III) chloride ($FeCl_3$) solution. The mixture was warmed to 37 °C before use. Iron(II) sulfate ($FeSO_4$) solutions at different concentrations were prepared to establish the calibration curve. For the assay, 10 μl of extract at various concentrations (0.05–0.50 mg/ml) was added to 300 μl of freshly prepared FRAP reagent in a microplate well. After incubation at 37 °C for 4 min, the absorbance was measured at 593 nm. The FRAP value was calculated from the $FeSO_4$ calibration curve and expressed as millimoles of $FeSO_4$ equivalents per gram of extract (mmol $FeSO_4$/g extract).

2.5. *In silico molecular docking analysis*

To complement the *in vitro* approaches, molecular docking analyses were conducted on key oxidative stress–related enzymes, namely glutathione peroxidase (GPx, PDB ID: 3NRZ) and NADPH oxidase (NOX, PDB ID: 2CDU). These enzymes are directly related to the radical scavenging and reducing activities measured experimentally and thus provide molecular insight into the potential interactions of the extract's bioactive constituents.

The major phenolic compounds of the hydromethanolic extract, chicoric acid (CID: 5281764) and acteoside (CID: 5281800), along with the reference antioxidant Trolox (CID: 40634), were retrieved in the SDF format from PubChem.[15] The structures were converted to the PDB format using the CACTUS Online SMILES Translator (https://cactus.nci.nih.gov/translate/) for subsequent molecular modeling analyses.

Protein structures were downloaded from the Protein Data Bank[16] and prepared in ArgusLab by removing water molecules and bound ligands and by adding missing hydrogen atoms.[17]

Docking simulations were performed using AutoDock Vina,[18] and binding affinities are reported in kcal/mol. The best-ranked poses were selected based on the lowest binding energy and visualized using Discovery Studio Visualizer.[19]

2.6. *In silico pharmacokinetic and toxicity prediction*

The pharmacokinetic properties of the selected molecules, commonly referred to as ADME, were evaluated using SwissADME, a freely available online tool. In addition, drug-likeness predictions were obtained for each compound.[7] Toxicological predictions for acteoside and chicoric acid were performed using the ProTox-II web server, which provides estimates for multiple toxicity endpoints, including acute toxicity, organ-specific toxicity, cytotoxicity, carcinogenicity, and immunotoxicity.[8]

3. RESULTS AND DISCUSSION

3.1. *Extraction yield*

Hydromethanolic extraction of *C. tinctoria* yielded 2.6 % dry extract. This yield may be explained by the selective extraction of polar and moderately polar constituents under the solvent system used. Extraction yield is known to depend on several parameters, including plant species, organ type, solvent polarity, and extraction conditions.[20]

3.2. *Phytochemical studies*

3.2.1. *Determination of the total polyphenolic and flavonoid contents*

The total phenolic and flavonoid contents of the hydromethanolic extract of *C. tinctoria* roots are expressed as mg GAE/g extract and mg QE/g extract, respectively, and were determined using calibration curves of gallic acid ($y = 6.052x$, $R^2 = 0.997$) and quercetin ($y = 0.0025x$, $R^2 = 0.9975$) (Table 1). The extract exhibited a high total phenolic content (210.39 ± 5 mg GAE/g extract), indicating strong enrichment in antioxidant constituents, whereas the flavonoid content was relatively low (3.91 ± 2.17 mg QE/g extract). Phenolic compounds are among the most abundant plant secondary metabolites and are widely recognized for their antioxidant properties through free radical scavenging and redox modulation.[21] Hydroalcoholic solvents are known to efficiently extract phenolic compounds due to their suitable polarity.[22] The high phenolic content observed in the present study is consistent with previous reports on methanolic extracts of *C. tinctoria* and other *Cistanche* species.[23] However, there are limited data on hydromethanolic extracts of *C. tinctoria*, a fact which highlights the originality of the present work.

T a b l e 1

The total phenolic and flavonoid contents of the hydromethanolic (HME) extract of Cistanche tinctoria roots

Extract	Total phenolics (mg GAE/g)	Total flavonoids (mg QE/g)
HME	210.39 ± 5.00	3.91 ± 2.17

The data are expressed as the mean ± standard deviation (SD) for triplicate measurements. GAE: gallic acid equivalents; QE: quercetin equivalents.

3.2.2. *HPLC-PDA analysis*

HPLC-PDA profiling revealed the presence of two major phenolic compounds in the hydromethanolic extract, identified as acteoside and chicoric acid by comparison with reference standards (Table 2, Fig. 1). Acteoside was the predominant compound (2.29 mg/g, t_R = 23.22 min), followed by chicoric acid (0.637 mg/g, t_R = 26.67 min).

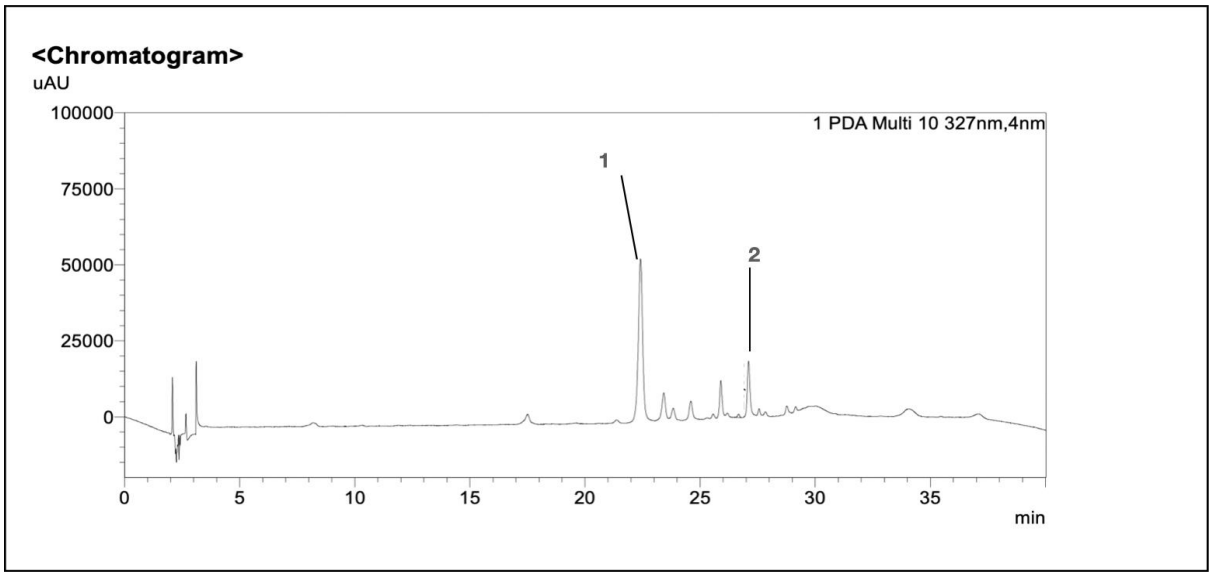

Fig. 1. Chromatogram of the hydro-methanolic extract of *Cistanche tinctoria*

Table 2

Phenolic compounds identified in hydromethanolic root extract of Cistanche tinctoria using high-performance liquid chromatography coupled with a photodiode array detector

Peak	Retention time (min)	Concentration (mg/g)	Compound name
1	23.22	2.29	Acteoside
2	26.67	0.64	Chicoric acid

Acteoside (verbascoside) is a phenylethanoid glycoside widely reported for its antioxidant,[24] anti-inflammatory,[25] and neuroprotective properties.[26] Chicoric acid, a caffeic acid derivative,[27] is also known for its antioxidant, anticancer and anti-inflammatory potential.[28] Unlike previous studies reporting naringin and chlorogenic acid in crude methanolic extracts,[23] these compounds were not detected in the present extract, likely due to differences in solvent composition and extraction conditions.

3.3. *In vitro antioxidant activities*

The antioxidant activity of the hydromethanolic extract of *C. tinctoria* roots was evaluated using four complementary *in vitro* assays (DPPH, ABTS, CUPRAC, and FRAP) in order to cover different antioxidant mechanisms, including radical scavenging and reducing power (Table 3). The use of multiple assays is essential due to the chemical diversity of phenolic compounds and the complexity of antioxidant reactions.[29]

Table 3

Antioxidant activities of the hydromethanolic extract of Cistanche tinctoria and reference standards (Trolox, iron (II) sulfate [$FeSO_4$]) evaluated based on the DPPH radical scavenging, ABTS radical scavenging, cupric reducing antioxidant capacity (CUPRAC), and ferric reducing antioxidant power (FRAP) assays

Sample	DPPH (IC_{50} mg/ml)	ABTS (IC_{50} mg/ml)	CUPRAC (mmol TEAC/g extract)	FRAP (mmol $FeSO_4$ /g extract)
HME	0.88 ± 0.20	0.53 ± 0.07	0.85 ± 0.38	0.89 ± 0.23
Trolox	0.077 ± 0.018	0.155 ± 0.016	0.239 ± 0.050	–
$FeSO_4$	–	–	–	0.39 ± 0.05

The extract exhibited moderate DPPH radical scavenging activity, with a half-maximal inhibitory concentration (IC_{50}) of 0.88 ± 0.20 mg/ml. This assay mainly reflects hydrogen atom-donating ability and is particularly sensitive to lipophilic antioxidants. The higher IC_{50} observed for the extract compared with Trolox suggests a lower hydrogen donating efficiency or a weaker interaction of some phenolic constituents with the DPPH radical in the methanolic medium.[30]

In contrast, the ABTS radical scavenging assay revealed a stronger antioxidant effect: The extract exhibited a lower IC_{50} of 0.53 ± 0.07 mg/ml. Since the $ABTS^{\bullet+}$ assay can be applied in both aqueous and organic systems and is responsive to both hydrophilic and lipophilic antioxidants, this result suggests a substantial contribution of water-soluble phenolic compounds. Similar differences between DPPH and ABTS radical scavenging activities have been reported for plant extracts rich in phenolic compounds and are often attributed to differences in solubility and reaction mechanisms.[30]

The reducing capacity of the extract was further confirmed by the CUPRAC and FRAP assays. The CUPRAC value was 0.85 ± 0.38 mmol TE/g extract, reflecting a moderate to high electron donating ability toward cupric ions. The FRAP assay yielded a value of 0.89 ± 0.23 mmol Fe^{2+}/g extract, indicating notable ferric reducing power. These results support the presence of redox-active phenolic constituents capable of participating in electron transfer–based antioxidant mechanisms.[29]

Overall, the antioxidant activity of the hydromethanolic activity, as evaluated by the four assays, is consistent with previous reports on *C. tinctoria* and related species.[23,31] The antioxidant activity of the extract may be largely attributed to its major phenolic constituents, acteoside and chicoric acid, both of which are well known for their strong radical scavenging and electron-donating properties.[24,32] Minor variations in antioxidant val-

ues compared with those reported in the literature may be related to differences in extraction solvent composition and phenolic profile.

3.4. *Molecular docking analysis*

In addition to the *in vitro* antioxidant findings, molecular docking and *in silico* analyses were performed to further elucidate the potential interaction mechanisms between the phytoconstituents of *C. tinctoria* and key oxidative stress–related enzymes. The two major bioactive compounds identified in the extract, acteoside and chicoric acid, were selected as ligands to evaluate their binding affinities and interaction patterns with these oxidation-related enzymes. GPx (PDB ID: 3NRZ), a crucial enzyme involved in the reduction of hydrogen peroxide and organic hydroperoxides, and NOX (PDB ID: 2CDU), a major source of reactive oxygen species (ROS), were selected as biological targets. The binding energies and key molecular interactions of acteoside and chicoric acid with GPx and NOX are summarized in Table 4 and Figure 2.

T a b l e 4

Docking analysis of bioactive compounds from Cistanche tinctoria with antioxidant enzymes

Ligand	Target enzyme (PDB ID)	Binding affinity (kcal/mol)	Key interacting residues	Interaction type
Acteoside	Glutathione peroxidase (GPx, 3NRZ)	–9.7	Lys1228, Ala338	Hydrogen bond
			Arg426	Hydrophobic (π-alkyl)
	NADPH oxidase (NOX, 2CDU)	–9.6	His10,	Hydrogen bond
			Ala303, Phe245, Ile160	Hydrophobic (π-alkyl),
			Asp282	Electrostatic (π-anion)
Chicoric acid	Glutathione peroxidase (GPx, 3NRZ)	–9.8	Gly260, Ala346,	Hydrogen bond
			Ser347	Hydrophobic (π-alkyl)
	NADPH oxidase (NOX, 2CDU)	–8.6	Gln80, Lys255	Hydrogen bond
			Leu251	Hydrophobic (π-alkyl),
			Val84	Unfavorable donor–donor
Trolox	Glutathione peroxidase (GPx, 3NRZ)	–8.4	Ile1531, Ile1620, Lys1523, Leu1524, Ile1620	Hydrophobic (π-alkyl and Pi-sigma)
	NADPH oxidase (NOX, 2CDU)	–7.8	Pro883, Arg882, Phe14, Tyr62	Hydrophobic (π-alkyl)
			Lys17	Electrostatic, Hydrophobic (π-alkyl),

Based on the molecular docking analysis, both acteoside and chicoric acid display favorable predicted binding affinities toward GPx and NOX, which appears to be consistent with the *in vitro* antioxidant activities observed for the hydromethanolic extract of *C. tinctoria*. Their calculated binding energies were more negative than those of the reference ligand Trolox (Table 4), suggesting more favorable predicted interactions within the docking model used. However, these results should be interpreted cautiously, as docking provides only a theoretical estimation of ligand–protein interactions and does not constitute direct evidence of enzyme modulation or inhibition.

For GPx (3NRZ), chicoric acid exhibited the lowest predicted binding energy (–9.8 kcal/mol), with a binding mode involving hydrogen bonds with Gly260 and Ala346, as well as hydrophobic π–alkyl interactions with Ser347. These interactions may support stable accommodation of the ligand within the binding region. Acteoside also showed a favorable predicted affinity for GPx (–9.7 kcal/mol), forming hydrogen bonds with Lys1228 and Ala338 together with hydrophobic interactions involving Arg426. The presence of multiple hydroxyl groups and glycosidic moieties in both compounds may facilitate polar and hydrophobic contacts, which is in line with their reported antioxidant potential.[24,28,32]

Regarding NOX (2CDU), acteoside showed the most favorable predicted binding energy (–9.6 kcal/mol), with interactions involving His10, Ala303, Phe245, Ile160, and Asp282. Chicoric acid also displayed a favorable predicted affinity for NOX (–8.6 kcal/mol), forming hydrogen bonds with Gln80 and Lys255, together with hydrophobic interactions with Leu251. The less favorable donor–donor interaction observed with Val84 may partly explain its comparatively weaker predicted affinity. These observations are compatible with

previous reports suggesting that polyphenols may interfere with oxidative stress–related pathways, including ROS-producing systems.[33]

Trolox showed less favorable predicted docking scores toward GPx (–8.4 kcal/mol) and NOX (–7.8 kcal/mol) compared with acteoside and chicoric acid. Nevertheless, these differences should not be interpreted as direct evidence of stronger biological activity, but rather as an indication of potentially favorable ligand–target complementarity under the computational conditions applied.

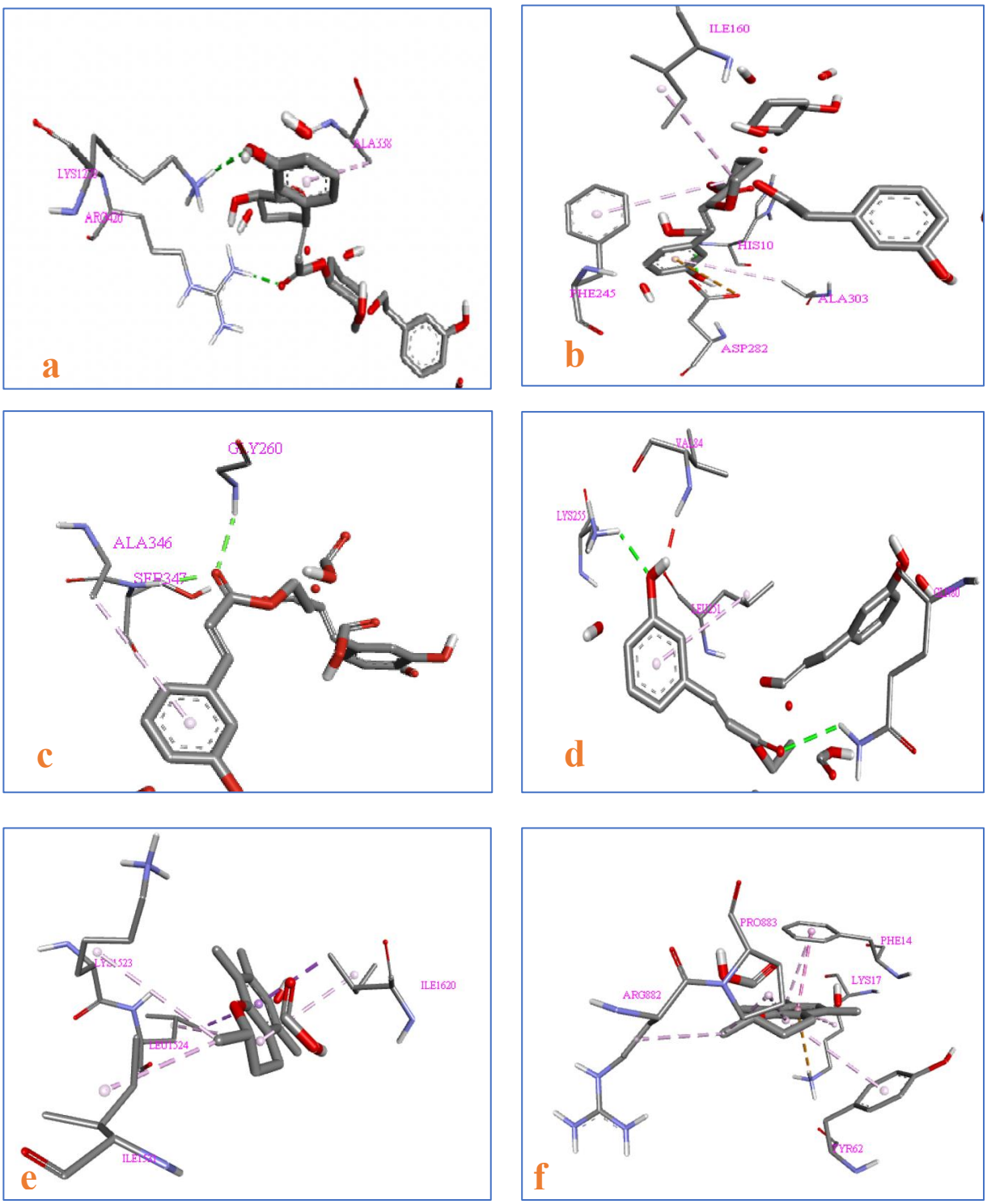

Fig. 2. Molecular docking results: a) acteoside–3NRZ, b) acteoside–2CDU, c) chicoric acid–3NRZ, d) chicoric acid–2CDU, e) Trolox–3NRZ, and f) Trolox–2CDU. Amino acid residues are shown in pink, hydrogen bonds in green dashed lines, and hydrophobic interactions in purple dashed lines.

Overall, the docking findings may provide supportive mechanistic insights that complement the *in vitro* antioxidant results obtained from the DPPH, ABTS, CUPRAC, and FRAP assays. Taken together, these results suggest that acteoside and chicoric acid could be involved in the antioxidant effects of *C. tinctoria*, not only through radical scavenging properties but also through possible interactions with oxidative stress-related enzymes. However, further enzymatic and cellular studies are required to confirm these mechanisms experimentally.

3.5. *Pharmacokinetic analysis*

The *in silico* ADME evaluation revealed distinct pharmacokinetic profiles for acteoside and chicoric acid, both characterized by high polarity and limited oral bioavailability, which are common features of polyphenolic antioxidants (Table 5).[34,35]

Acteoside exhibited a high molecular weight (624.6 g/mol), extensive polarity (topological polar surface area [TPSA] = 245 Å^2), and low lipophilicity (logP = –0.6), resulting in low predicted gastrointestinal absorption and inability to cross the blood–brain barrier. It was also identified as a P-glycoprotein substrate, which may further limit its systemic availability. Nevertheless, acteoside showed good aqueous solubility and no predicted inhibition of major cytochrome P450 (CYP450) enzymes, suggesting a low risk of metabolic drug–drug interactions. These characteristics are consistent with previous reports describing the limited bioavailability of phenylethanoid glycosides.[34]

Chicoric acid displayed a lower molecular weight (474.4 g/mol) and slightly reduced polarity (TPSA = 208 Å^2) compared with acteoside, along with moderate lipophilicity (logP = 1.06). Although it also showed low predicted gastrointestinal absorption, its membrane affinity was slightly higher. SwissADME predictions indicated no blood–brain barrier penetration, no CYP450 inhibition, and high aqueous solubility. Experimental studies have shown that chicoric acid is poorly absorbed intestinally but that its bioavailability may be improved through formulation strategies such as chitosan-based delivery systems.[35]

Both compounds failed to fully comply with conventional drug-likeness rules (Lipinski, Veber, and Muegge), mainly due to their molecular size and high hydrogen bonding capacity. However, these features are consistent with their role as natural antioxidants and enzyme modulators rather than classical oral drug candidates. Overall, the ADMET analysis suggests that acteoside and chicoric acid are safe and biocompatible phytochemicals with limited systemic absorption, supporting their potential use in nutraceutical or topical applications rather than conventional systemic therapies.

T a b l e 5

Comparative SwissADME profile of acteoside and chicoric acid

Category	Parameter	Acteoside	Chicoric acid
Physicochemical Properties	Molecular formula	$C_{29}H_{36}O_{15}$	$C_{22}H_{18}O_{12}$
	Molecular weight (g/mol)	624.59	474.37
	H-bond acceptors / donors	15 / 9	12 / 6
	Rotatable bonds	11	11
	Topological polar surface area (TPSA, Å²)	245.29	208.12
Lipophilicity	Consensus logP	–0.60 (hydrophilic)	1.06 (slightly lipophilic)
Water Solubility	Log S (ESOL)	–2.87 (soluble)	–3.58 (soluble)
Pharmacokinetics	GI absorption	Low	Low
	BBB permeation	No	No
	P-gp substrate	Yes	Yes
	CYP450 inhibition (1A2, 2C19, 2C9, 2D6, 3A4)	No	No
	Skin permeation (log Kp, cm/s)	–10.46	–7.77
	Bioavailability score	0.17	0.11
Drug-likeness	Lipinski rule	No --- 3 violations	No --- 2 violations
	Veber rule	No ---TPSA > 140; Rotors > 10	No --- TPSA > 140; Rotors > 10
	Muegge filter	No --- 4 violations	No --- 3 violations
Medicinal Chemistry	PAINS / Brenk alerts	1 / 2	1 / 3
	Synthetic accessibility	6.37 (moderately difficult)	4.14 (moderately easy)

3.5. *Toxicity analysis*

In silico toxicity prediction of acteoside and chicoric acid was performed using the ProTox-II platform to assess their potential safety profiles (Table 6). Both compounds exhibited a favorable toxicological profile, with predicted oral median lethal dose (LD_{50}) of 5000 mg/kg, corresponding to toxicity class 5 and indicating low acute oral toxicity according to the Organisation for Economic Co-operation and Development (OECD) guidelines.

T a b l e 6

The toxicity properties of acteoside and chicoric acid

Toxicity Category	Endpoint / Target	Acteoside	Chicoric acid
Organ toxicity	Hepatotoxicity	Inactive (0.81)	Inactive (0.58)
Toxicity endpoints	Carcinogenicity	Inactive (0.81)	Inactive (0.51)
	Immunotoxicity	Active (0.99)	Active (0.86)
	Mutagenicity	Inactive (0.87)	Inactive (0.85)
	Cytotoxicity	Inactive (0.77)	Inactive (0.92)
Oral toxicity	LD_{50} (mg/kg)	5000	5000
	Toxicity class	5	5

The values shown in parentheses represent the prediction confidence/probability assigned by ProTox-II to the predicted class (active or inactive) for each specific toxicity endpoint

Neither acteoside nor chicoric acid showed predicted hepatotoxic, carcinogenic, or mutagenic effects, supporting their biocompatibility and low genotoxic risk. These findings are consistent with previous reports describing the generally low toxicity of natural phenolic compounds.[24,28,32]

Both compounds were predicted to display immunotoxic activity, which may reflect their reported immunomodulatory properties rather than adverse toxicity. Importantly, cytotoxicity was not predicted for either compound. Overall, these results suggest that acteoside and chicoric acid are safe, non-mutagenic natural antioxidants suitable for further *in vitro* and *in vivo* investigations.

4. CONCLUSION

This study demonstrated that the hydromethanolic extract of *C. tinctoria* roots is rich in phenolic compounds, with acteoside and chicoric acid identified as the major constituents. The extract exhibited notable antioxidant activity, as evidenced by complementary *in vitro* assays reflecting both radical scavenging and reducing capacities. Molecular docking analyses further supported these findings by suggesting favorable interactions between the major phenolic compounds and key oxidative stress-related enzymes, while *in silico* ADMET and toxicity predictions suggested a favorable safety profile. Overall, these results provide experimental and computational evidence supporting the antioxidant potential of *C. tinctoria* and highlight its relevance as a promising source of natural bioactive compounds.

Data availability statement. The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Acknowledgements. The authors gratefully acknowledge the financial support provided by the PRFU project (Projets de Recherche Formation-Universitaire, Ministry of Higher Education and Scientific Research, Algeria) [DOOLO5UN350120 220001]. The authors also thank the staff of the Laboratory of Bioinformatics, Applied Microbiology and Biomolecules, Faculty of Sciences, M'Hamed Bougara University of Boumerdes, for their technical assistance and valuable support.

REFERENCES

(1) Ansari, W. A.; Srivastava, K.; Nasibullah, M; Khan, F. M. Reactive oxygen species (ROS): sources, generation, disease pathophysiology, and antioxidants. *Discover Chemistry* **2025**, *2*, 191. https://doi.org/10.1007/s44371-025-00275-z

(2) Xu, L.; Wang, X. A Comprehensive Review of Phenolic Compounds in Horticultural Plants. *Int. J. Mol. Sci.* **2025**, *26*, 5767. https://doi.org/10.3390/ijms26125767

(3) Bouzitouna, A.; Ouali, K.; Djeddi, S. Protective Effects of *Cistanche tinctoria* Aqueous Extract on Blood Glucose and Antioxidant Defense System of Pancreatic β-Cells in Experimental Diabetes in Rats. *Int. J. Pharm. Sci. Rev. Res.* **2015**, *32*, 243–249.

(4) Lakhdari, W.; Dehliz, A.; Acheuk, F.; Mlik, R.; Hammi, H.; Doumandji-Mitiche, B.; Gheriani, S.; Berrekbia, M.; Guermit, K.; Chergui, S. Ethnobotanical study of some plants used in traditional medicine in the region of Oued Righ (Algerian Sahara). *J. Med. Plants Stud.* **2016**, *4*, 204–211.

(5) Sadowska-Bartosz, I.; Bartosz, G. Evaluation of the antioxidant capacity of food products: Methods, applications and limitations. *Processes* **2022**, *10*, 2031. https://doi.org/10.3390/pr10102031

(6) Sarı, S.; Kılıç, N.; Yılmaz, M. *In vitro* antioxidant activities and *in silico* molecular docking studies of N-substituted oxime derivatives. *Struct. Chem.* **2023**, *34*, 605–616. https://doi.org/10.1007/s11224-022-01978-0

(7) Daina, A.; Michielin, O.; Zoete, V. SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Sci. Rep.* **2017**, *7*, 42717. https://doi.org/10.1038/srep42717

(8) Banerjee, P.; Kemmler, E.; Dunkel, M.; Preissner, R. ProTox 3.0: A webserver for the prediction of toxicity of chemicals. *Nucleic Acids Res.* **2024**, *52*, W1–W8. https://doi.org/10.1093/nar/gkae303

(9) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158. https://doi.org/10.5344/ajev.1965.16.3.144

(10) Ayoola, G. A.; Coker, H. A.; Adesegun, S. A.; Adepoju-Bello, A. A.; Obaweya, K.; Ezennia, E. C.; Atangbayila, T. O. Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in Southwestern Nigeria. *Trop. J. Pharm. Res.* **2008**, *7*, 1019–1024.

(11) Blois, M. S. Antioxidant determinations by the use of a stable free radical. *Nature* **1958**, *181*, 1199–1200. https://doi.org/10.1038/1811199a0

(12) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3

(13) Apak, R.; Güçlü, K.; Özyürek, M.; Karademir, S. E. Novel total antioxidant capacity index for dietary polyphenols: CUPRAC method. *J. Agric. Food Chem.* **2004**, *52*, 7970–7981. https://doi.org/10.1021/jf048741x

(14) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power. *Anal. Biochem.* **1996**, *239*, 70–76. https://doi.org/10.1006/abio.1996.0292

(15) Kim, S.; Chen, J.; Cheng, T. *et al*. PubChem in 2021: New data content and improved web interfaces. Nucleic Acids Res. 2021;49(D1):D1388–D1395. https://doi.org/10.1093/nar/gkaa971

(16) Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E.; The Protein Data Bank. Nucleic Acids Res. **2000**, *28* (1), 235–242. https://doi.org/10.1093/nar/28.1.235

(17) Morris, G. M.; Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J Comput Chem*. **2009**, *30* (16), 2785–2791. https://doi.org/10.1002/jcc.21256

(18) Trott, O.; Olson, A. J.; AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem*. **2010**, *31* (2), 455–461. https://doi.org/10.1002/jcc.21334

(19) Baroroh, U., Muscifa, Z. S.; Destiarani, W.; Rohmatullah, F. G.; Yusuf, M. Molecular interaction analysis and visualization of protein–ligand docking using BIOVIA Discovery Studio Visualizer. Indones *J Comput Biol*. **2023**, *2* (1):22–30.

(20) Dai, J.; Mumper, R. J. Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. *Molecules* **2010**, *15*, 7313–7352. https://doi.org/10.3390/molecules15107313

(21) Zhang, Y.; Cai, P.; Cheng, G.; Zhang, Y. Q. A brief review of phenolic compounds identified from plants: their extraction, analysis, and biological activity. *Nat Prod Commun.* **2022**, *17* (1), 1–14. https://doi.org/10.1177/1934578X211069721

(22) Sahreen, S.; Khan, M. R.; Khan, R. A. Evaluation of antioxidant activities of various solvent extracts of *Carissa opaca* fruits. *Food Chem.* **2010**, *122*, 1205–1211. https://doi.org/10.1016/j.foodchem.2010.03.120

(23) Chouikh, A.; Ben Ali, A.; Chenguel, A. Antioxidant potential and phenolic composition of *Cistanche tinctoria*: A comparative study of crude, flavonoid, and tannin extracts. Uchenye Zapiski Kazanskogo Universiteta. Seriya Estestvennye Nauki, **2025**, *167*, 242–253. https://doi.org/10.26907/2542-064X.2025.2.242-253

(24) Li, X.; Xie, Y.; Li, K.; Wu, A.; Xie, H.; Guo, Q.; Xue, P.; Maleshibek, Y.; Zhao, W.; Guo, J.; Chen, D. Antioxidation and cytoprotection of acteoside and its derivatives: Comparison and mechanistic chemistry. *Molecules.* **2018**, *23* (2), 498. https://doi.org/10.3390/molecules23020498

(25) Marčetić, M.; Bufan, B.; Drobac, M.; Antić Stanković, J.; Arsenović Ranin, N.; Milenković, M. T.; Božić, D. Multifaceted biological properties of verbascoside/acteoside: Antimicrobial, cytotoxic, anti-inflammatory, and immunomodulatory effects. *Antibiotics* **2025**, *14* (7), 697. https://doi.org/10.3390/antibiotics14070697

(26) Burgos, C.; Muñoz-Mingarro, D.; Navarro, I.; Martín-Cordero, C.; Acero, N. Neuroprotective potential of verbascoside isolated from *Acanthus mollis* L. leaves through its enzymatic inhibition and free radical scavenging ability. *Antioxidants* **2020**, *9* (12), 1207. https://doi.org/10.3390/antiox9121207

(27) Yang, M.; Wu. C.; Zhang, T.; Chen, L.; Yi, X.; Zhang, Y.; Li, D.; Sun, P. Chicoric acid: Natural occurrence, synthesis, biosynthesis and bioactive effects. *Front Chem*. **2022**, *10*, 888673. https://doi.org/10.3389/fchem.2022.888673

(28) Nazir, M. M.; Farzeen, I.; Zafar, S.; Fatima, S.; Zafar, N.; Ashraf, A.; Lebelo, S. L. Biological potential and therapeutic effectiveness with diverse signaling pathways of phyto-product chicoric acid: A comprehensive review with computational evidence. *Naunyn Schmiedebergs Arch Pharmacol*. **2025**, *398*, 8259–8286. https://doi.org/10.1007/s00210-025-03931-4.

(29) Rumpf, J.; Burger, R.; Schulze, M. Statistical evaluation of DPPH, ABTS, FRAP, and Folin–Ciocalteu assays to assess the antioxidant capacity of lignins. *Int J Biol Macromol*. **2023**, *233*, 123470. https://doi.org/10.1016/j.ijbiomac.2023.123470.

(30) Lang, Y.; Gao, N.; Zang, Z.; Meng, X.; Lin, Y.; Yang, S.; Yang, Y.; Jin, Z.; Li, B. Classification and antioxidant as-

says of polyphenols: A review. *J Future Foods*. **2024**, *4* (3), 193–204. https://doi.org/10.1016/j.jfutfo.2023.07.002

(31) Zhou, S.; Feng, D.; Zhou, Y.; Duan, H.; Jiang, Y.; Yan, W. Analysis of active ingredients and health applications of *Cistanche*. *Front Nutr*. **2023**, *10*, 1101182. https://doi.org/10.3389/fnut.2023.1101182

(32) Alharthi, F. Chicoric acid protects against inflammation and apoptosis in a colitis model. *Environ Sci Pollut Res*. **2023**, *30* (57), 119814–119824. https://doi.org/10.1007/s11356-023-30742-y

(33) Obeme-Nmom, J. I.; Abioye, R. O; Reyes Flores, S. S.; Udenigwe, C. C. Regulation of redox enzymes by nutraceuticals: A review of the roles of antioxidant polyphenols and peptides. *Food Funct.* 2024, 22:2158–2178. https://doi.org/10.1039/d4fo03549f

(34) Xue, Z.; Yang, B. Phenylethanoid glycosides: Research advances in their phytochemistry, pharmacological activity and pharmacokinetics. *Molecules* **2016**, *21* (8), 991. https://doi.org/10.3390/molecules21080991

(35) Wang, Y.; Zhang, C.; Zhang, M.; Sun, L.; Zhang, J. Intestinal absorption mechanism of chicoric acid and bioavailability improvement with chitosan. *Heliyon* **2022**, *8* (10), e11232. https://doi.org/10.1016/j.heliyon.2022.e09955