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LC-ESI-MS/MS-based phytochemical profiling and pharmacological validation of *Centaurium erythraea* Rafn (Gentianaceae) traditionally used for pain and inflammation

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ABSTRACT

Ethnopharmacological relevance: These findings provide scientific support for the traditional use of *Centaurium erythraea* Rafn (Gentianaceae) in Algerian folk medicine and suggest its potential for further pharmacological exploration in the management of pain and neuroinflammatory conditions.

Aim of the study: The present study aimed to scientifically validate these traditional uses through phytochemical profiling and pharmacological evaluation of the aqueous extract (*C. erythraea* aqueous extract, CEAE).

Materials and methods: The polyphenolic composition was characterized, and *in vitro* antioxidant and DNA protection activities, enzyme inhibitory effects, as well as *in vivo* analgesic activity using the acetic acid–induced writhing and hot plate tests, in addition to acute oral toxicity, and molecular docking studies were evaluated.

Results: LC-ESI-MS/MS analysis revealed the presence of several phenolic and flavonoid constituents, including trans-ferulic acid (278.74 μg/g), syringic acid (172.81 μg/g), polydatin (33.72 μg/g), vanillin (13.05 μg/g), quercetin (12.44 μg/g), and isoquercitrin (9.32 μg/g), along with minor levels of kaempferol and trans-cinnamic acid. CEAE exhibited moderate antioxidant activity, with the strongest effect in the FRAP assay ($IC_{50} = 148.73 \pm 8.50$ μg/mL). The extract showed dual inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), demonstrating stronger activity against BChE ($IC_{50} = 31.27 \pm 2.68$ μg/mL) than the reference drug galantamine. In plasmid DNA protection assays, CEAE (14.69 % protection) and quercetin preserved supercoiled DNA (Form I) from oxidative damage. Oral administration of CEAE (2000 mg/kg) produced no signs of toxicity, indicating a wide safety margin. In vivo, CEAE significantly reduced acetic acid-induced writhing (51.23 % inhibition at 300 mg/kg) and increased latency times in the hot plate test, showing greater analgesic efficacy than paracetamol. The binding affinity of the trans-ferulic/syringic acid adduct (−7.1 kcal/mol) in molecular docking interactions with AChE was determined to be higher than that of the compounds (−6.7 kcal/mol) used alone.

Conclusions: Overall, the aqueous extract of *C. erythraea* demonstrates notable antioxidant, cholinesterase-inhibitory, DNA-protective, and analgesic activities, consistent with its traditional use for pain and inflammation.

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Abbreviations

$ABTS^{+}$	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation
ACh	Acetylcholine
AChE	Acetylcholinesterase
ANOVA	Analysis of variance
BChE	Butyrylcholinesterase
BHT	Butylated hydroxytoluene
BPC	Base peak chromatogram
CE	Collision energy
CEAE	*Centaurium erythraea* aqueous extract
CRediT	Contributor Roles Taxonomy
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
DTNB	5,5′-Dithiobis-(2-nitrobenzoic acid)
ESI	Electrospray ionization
FRAP	Ferric reducing antioxidant power
H_2O_2	Hydrogen peroxide
HPLC	High-performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
LC-ESI-MS/MS	Liquid chromatography–electrospray ionization tandem mass spectrometry
MRM	Multiple reaction monitoring
OECD	Organization for Economic Co-operation and Development
PDB	Protein Data Bank
RT (Rt)	Retention time
SD	Standard deviation
SEM	Standard error of the mean
UV	Ultraviolet
WHO	World Health Organization

1. Introduction

Herbal remedies have been fundamental to traditional medicine systems across the globe. These natural substances, sourced from plants and botanical sources, contain various bioactive compounds with medicinal properties (Gogoi et al., 2023). Utilizing the potential of herbal medicines in drug discovery, particularly through ethnobotanical surveys, offers the promise of discovering new therapeutic agents. These agents can address unmet medical needs, improve treatment efficacy, and enhance the overall well-being of individuals and communities (Anand et al., 2019). These surveys involve systematically documenting indigenous knowledge about local communities' medicinal use of plants. By rigorously studying the efficacy and safety of traditional remedies, researchers can validate centuries-old practices and integrate them into modern healthcare systems (Boudjelal et al., 2013). Medicinal plants synthesize and accumulate bioactive compounds of diverse chemical nature, such as alkaloids, flavonoids, terpenoids, and phenolic compounds. These compounds have demonstrated many therapeutic properties, including anti-inflammatory, antidiabetic, anticancer, hypotensive, analgesic, neuroprotective, and cardiotonic effects … (Marques et al., 2023; Mirzaei and Masumi, 2022).

By harnessing the potency of bioactive compounds abundant in plants, researchers aim to discover new avenues for innovative therapies that prioritize both efficacy and safety, thereby advancing the frontiers of modern medicine (Atalar et al., 2023).

Centaurium erythraea is an herbaceous plant that grows 20–60 cm tall and can be annual or biennial. According to Plants of the World Online (https://powo.science.kew.org), the genus *Centaurium* currently comprises 31 accepted species. *C. erythraea* is distributed across several countries, including Algeria, Morocco, Italy, Spain, Portugal, and parts of the Balkan Peninsula (Chabane et al., 2024). According to some ethnobotanical surveys in Algeria, this plant has been used historically to treat a variety of human illnesses, such as skin diseases, pain, fever, infections, inflammation, digestive disorders, edema, diabetes, kidney, and urinary ailments (Boudjelal et al., 2013; Demirtas and Sahin, 2013; Hamza et al., 2019; Meddour and Meddour-Sahar, 2015; Taïbi et al., 2020).

Experimental research using *in vitro* and *in vivo* assays has demonstrated that *C. erythraea* possesses antioxidant, anti-inflammatory, analgesic, and antipyretic properties; it is also used to treat a number of conditions, including diabetes, edema, high blood pressure, and spasms in the smooth muscles of the gastrointestinal system (Berkan et al., 1991; Chabane et al., 2024; Chda et al., 2016; Mansar-Benhamza et al., 2013; Matekalo et al., 2018; Sefi et al., 2011). Many secondary metabolites, including iridoids, flavonoids, alkaloids, phenolic acids and their derivatives, terpenoids, xanthones, and fatty acids, have been found in previous phytochemical investigations on *C. erythraea* (El Menyiy et al., 2021; Jerković et al., 2012; Trifunović-Momčilov et al., 2016). According to reports, many of these substances have significant pharmacological and biological properties (Matekalo et al., 2018).

The present study aimed to chemically characterize the aqueous extract of the aerial parts of *C. erythraea* (CEAE), focusing on secondary metabolites, and to evaluate its biological potential using both *in vitro* and *in vivo* models, including antioxidant, anticholinesterase, DNA-protective, and analgesic activities. Additionally, the acute oral toxicity of CEAE was assessed to establish its safety profile. In LC-ESI-MS/MS analysis, trans-ferulic acid and syringic acid were detected at high levels. Their synergistic interaction was evaluated, and their binding affinities toward acetylcholinesterase (AChE) were determined through molecular docking studies. This comprehensive evaluation seeks to provide new insights into the therapeutic potential of *C. erythraea* and highlight its value as a bioactive natural resource.

2. Materials and methods

2.1. Sample

The aerial parts of *Centaurium erythraea* were collected from the northern part of M'Sila, Algeria (35°42′21″ N, 4°32′31″ E), at an altitude of approximately 470 m, in a dry grassland habitat characterized by sparse herbaceous vegetation and low soil moisture, in June 2023. The plant was taxonomically identified and authenticated by Prof. A. Boudjelal (Department of Microbiology and Biochemistry, University of M'sila) using standard regional floras and taxonomic keys. A voucher specimen (AB-35) has been deposited in the herbarium of the Biology: Applications in Health and Environment Laboratory, University of M'sila.

The plant material (leaves, stems, and flowers) was carefully dried in a shaded, well-ventilated area and finely ground into powder using a grinding mill.

2.2. Extraction

The aqueous extract of *C. erythraea* (CEAE) was prepared by infusing 20 g of dried, powdered plant material in 200 mL of boiling distilled water, following the method described by Chda et al. (2016). The infusion was allowed to cool to room temperature, after which it was filtered using Whatman No. 1 filter paper. The resulting filtrate was subsequently lyophilized to obtain the crude extract in powdered form. This extract was stored at 4 °C until use in phytochemical, toxicity, and pharmacological studies. For each experimental procedure, the extract was freshly reconstituted in distilled water. The extraction yield was

calculated to be 22.50 %.

2.3. Characterization of the polyphenolic profile

Quantification of phenolic compounds was conducted using liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) employing an Agilent 1260 Infinity II system connected to a 6460 Triple Quadrupole Mass Spectrometer (Agilent Technologies). Chromatographic separation was achieved on a reversed-phase Agilent Poroshell 120 EC-C18 column (50 mm × 4.6 mm i.d., 2.7 μm particle size), maintained at a constant temperature of 30 °C. The mobile phase consisted of solvent A (water containing 0.1 % formic acid and 5 mM ammonium formate) and solvent B (methanol with 0.1 % formic acid), delivered using the following gradient elution profile: 0 min, 85 % A/15 % B; 5 min, 75 % A/25 % B; 15 min, 25 % A/75 % B; 16 min, 0 % A/100 % B; 22 min, 85 % A/15 % B; and 40 min, 85 % A/15 % B. The total run time was 40 min (Erenler et al., 2018).

Mass-to-charge ratios (m/z) of the analytes were determined under both positive and negative electrospray ionization (ESI) modes, as described by Köktürk et al. (2023). The ESI source parameters were set as follows: capillary voltage, 4000 V; nitrogen nebulizing gas flow rate, 11 L/min; and gas temperature, 350 °C. Quantitative analysis was performed using Multiple Reaction Monitoring (MRM) mode, with compound-specific precursor-to-product ion transitions optimized for dwell time, fragmentor voltage, and collision energy (CE). Identification of compounds was confirmed by comparing retention times, UV–Vis spectra, and mass spectral data with those of 35 certified HPLC-grade reference standards, as well as published literature and entries in spectral databases.

2.4. In vitro studies

2.4.1. Antioxidant assays

The antioxidant potential of CEAE was investigated using three complementary methods. Free radical scavenging activity was assessed via the $DPPH^{\cdot}$ scavenging assay following the procedure of Li et al. (2009), while $ABTS^{+\cdot}$ scavenging activity was evaluated following the protocol of (Thomas et al., 2008). Ferric reducing ability was assessed using Oyaizu (1986) method. Butylated hydroxytoluene (BHT) served as the standard antioxidant for all assays.

2.4.2. Cholinesterases inhibitory activity

The tested sample was evaluated using the method established by Ellman et al. (1961). To each reaction mixture, 150 μL of sodium phosphate buffer (100 mM, pH 8.0), 10 μL of the test sample, and 20 μL of either acetylcholinesterase (AChE) or butyrylcholinesterase (BChE) were added. The mixture was then incubated at 25 °C for 15 min. Following this, 10 μL of DTNB (5,5′-Dithiobis (2-nitro-benzoic acid)) and 10 μL of acetylthiocholine iodide (0.71 mM, AChE substrate) or butyrylthiocholine iodide (0.2 mM, BChE substrate) were introduced. Absorbance was recorded at 412 nm at 0 min and 15 min. Galantamine was used as the reference standard. The IC_{50} values (μg/mL) for the extract and galantamine were subsequently calculated.

2.4.3. DNA protection activity

The protective effect of CEAE against DNA damage was evaluated using agarose gel electrophoresis. The capacity of the extract to prevent oxidative degradation of plasmid DNA (pBR322, Thermo Fisher) caused by hydrogen peroxide (H_2O_2) and UV irradiation was assessed by monitoring DNA strand integrity, as described by Ozen et al. (2022). Briefly, 5 μL of the extract was mixed with 3 μL of plasmid DNA (1:3, v/v) and 1 μL of 30 % H_2O_2. For the negative control (C1), 6 μL of distilled water and 3 μL of plasmid DNA were combined. The positive control (C2) included 6 μL of distilled water, 3 μL of plasmid DNA (1:3, v/v), and 1 μL of H_2O_2 (30 %). A standard antioxidant, quercetin, was also tested under the same conditions.

The mixtures were exposed to UV light for 5 min to initiate oxidative stress. Following irradiation, 2 μL of loading dye was added, and the samples were loaded onto a 1 % agarose gel. Ethidium bromide (2 μL) was incorporated into the gel for DNA visualization. Electrophoresis was performed at 90 V for 60 min, and the gels were visualized under a UV transilluminator at 320 nm and 8000 $\mu W/cm^2$. ImageJ software was used to quantify the degree of protection conferred by the extract and quercetin by analyzing the intensity of supercoiled (Form I) and the broken DNA form (Form II) (Başar et al., 2024a).

2.5. In vivo studies

Healthy Wistar albino rats weighing 180 and 210 g were obtained from the Pasteur Institute in Algiers (Algeria) to evaluate acute oral toxicity and analgesic activity. The animals were kept under controlled environmental conditions, including a temperature of 25 °C and a 12-h light/dark cycle. They had free access to water and food. All experimental procedures were conducted in compliance with Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the National Committee for the Evaluation and Programming of University Research under the Algerian Ministry of Higher Education and Scientific Research (Approval No: DO1N01UN280120200002).

Experimental design: A total of 40 rats were randomly assigned into five groups of five animals each (n = 5 per group) for each analgesic test. The groups included a negative control (distilled water), a positive control (paracetamol, 150 mg/kg body weight), and three treatment groups receiving CEAE at doses of 100, 200, and 300 mg/kg body weight, respectively.

2.5.1. Acute oral toxicity

Acute toxicity of the CEAE was assessed according to OECD Guideline 420. A single oral dose of CEAE (2000 mg/kg body weight) was administered by gavage to three rats, while the control group received only distilled water. Animals were closely monitored for the first 3 h post-administration and at 4-h intervals over the following 72 h for behavioral changes, signs of morbidity or mortality, body weight, and food and water intake. In addition, animals were observed daily for up to 14 days to detect any delayed toxic effects. At the end of the observation period, all animals underwent macroscopic examination (necropsy) of major organs to identify any gross pathological alterations.

2.5.2. Determination of analgesic effects

2.5.2.1. Acetic acid-induced writhing test. Five groups of five rats each were used (**n = 5**) in this study. The negative control group received distilled water, while the positive control group was treated with paracetamol (150 mg/kg body weight). The remaining three groups received CEAE at doses of 100, 200, and 300 mg/kg body weight, respectively. Thirty minutes after treatment, writhing was induced by intraperitoneal injection of 0.6 % acetic acid (10 mL/kg body weight), and the number of writhes was recorded over a 20-min period (Chabane

et al., 2024). The results were evaluated by calculating the mean number of writhes per group, and antinociceptive activity was expressed as percentage inhibition of abdominal writhes calculated according to the formula described by De Garde et al. (2022):

$$\textit{Inhibition}\ (\%) = \frac{\textbf{Mean N°. of writhing (control)} - \textbf{Mean N°. of writhing (test)}}{\textbf{Mean N°. of writhing (control)}} \times \mathbf{100}$$

2.5.2.2. Hot plate test. Five groups of five rats each (**n = 5**) were randomly assigned for this test. The negative control group received distilled water, while the positive control group was treated with paracetamol (150 mg/kg body weight). The other three groups received CEAE at doses of 100, 200, and 300 mg/kg body weight, respectively. Each rat was then placed individually on a hot plate maintained at 55 ± 0.5 °C. The latency time for paw licking or jumping was recorded as the reaction time. Measurements were taken 30, 60, and 90 min after treatment (Chabane et al., 2024).

2.6. Molecular docking study

A computer method called molecular docking simulation studies aids in the discovery of ligands that can efficiently and energetically fit into the binding sites of target proteins. Another benefit is predicting the energetic interactions between ligands and target proteins (Meng et al., 2011). AutoDock Vina tools version 4.2 programs were used to accomplish molecular docking in this work (Başar et al., 2024b, 2025). The Discovery Studio visualizer 2021 was used to assess and display the docking poses with the lowest interaction energy.

2.6.1. Protein preparation

All the molecules in the collection were found in the AChE active site, one of the most druggable sites with Alzheimer effects. The RCSB protein data bank (www.rcsb.org) provided the AChE 3D crystallographic structures based on X-ray diffraction, with PDB ID 1C2O. BIOVIA

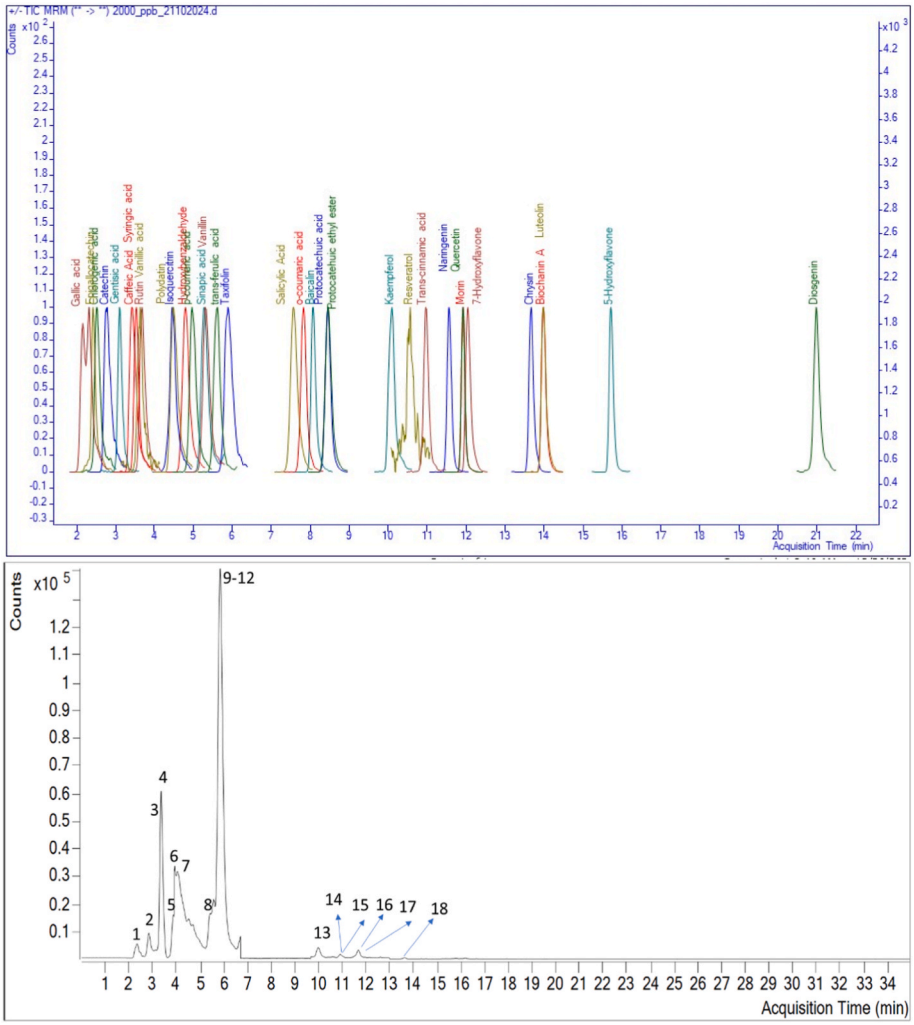

Fig. 1. Comparative LC–ESI-MS/MS chromatograms of reference standards (upper chromatogram) and the CEAE aerial parts extract (lower chromatogram). Peak assignment: (1) Catechin, (2) Gallic acid, (3) Syringic acid, (4) Chlorogenic acid, (5) Gentisic acid, (6) Polydatin, (7) Isoquercitrin, (8) p-Coumaric acid, (9) Sinapic acid, (10) Hydroxybenzaldehyde, (11) trans-Ferulic acid, (12) Vanillin, (13) Quercetin, (14) trans-Cinnamic acid, (15) Naringenin, (16) Kaempferol, (17) Morin, and (18) Biochanin A.

Discovery Studio was used to further optimize the protein structure. Remainders and missing hydrogen atoms were added. We carried out energy minimization and eliminated any water molecules that were not necessary for ligand binding and co-crystallization. Swiss PDB Viewer 4.10 was used to assess the target proteins' final 3D structure.

2.6.2. Protein-ligand docking

The virtual screening software interface AutoDock Vina tools version 4.2 was used to conduct the protein-ligand docking investigation of the chosen protein-ligand complex. The ligands were maintained flexibly, and the protein structures were stiff throughout the docking investigation (Edache et al., 2022). The software's Open Babel tool then stored the protein structures and ligands in the ".pdbqt" format. We surrounded the active binding site with a grid box. By observing the box's border, we modified the grid box's dimensions and coordinates. The conformational search technique used by AutoDock Vina is the Lamarck genetic algorithm. The docking technique employed in this investigation was semi-flexible docking. After docking, the program recorded the binding energy in the ".txt" format and showed it with several conformers. AutoDock Vina divides the docking results into distinct conformers. In order to investigate the interactions between ligands and amino acids found in the target proteins' active sites, we then used Discovery Studio Visualizer 2021 to examine the docking output files. After loading each protein and conformer into Discovery Studio Visualizer, we looked at how they interacted. We chose the optimal conformer based on the docking score and improved non-covalent bond interaction.

2.7. Statistical analysis

Data were analyzed using GraphPad Prism version 8. Results are expressed as mean ± standard deviation (SD) for *in vitro* assays and as mean ± standard error of the mean (SEM) for *in vivo* experiments. Group comparisons were performed using one-way analysis of variance (ANOVA), followed by Tukey's test. Differences were considered statistically significant at $p \leq 0.05$.

3. Results and discussion

3.1. The CEAE chemical composition

The chemical composition of the aqueous extract of *Centaurium erythraea* (CEAE) was characterized using LC-ESI-MS/MS analysis. The base peak chromatogram (BPC) obtained from the LC-ESI-MS/MS analysis is presented in Fig. 1, illustrating the overall profile of the detected compounds. Quantitative data for the identified phenolic and flavonoid constituents are summarized in Table 1, detailing their respective concentrations and retention times.

The LC-ESI-MS/MS analysis of the aqueous extract of *Centaurium erythraea* (CEAE) revealed numerous chromatographic peaks corresponding primarily to flavonoids and phenolic acid derivatives. As presented in Table 1, a total of eighteen distinct compounds were identified, with retention times ranging from 2.73 to 13.62 min. Among the identified phytochemicals, phenolic acids emerged as the most abundant class, followed by flavonoids, highlighting the extract's richness in polyphenolic content.

Notably, the analysis confirmed the presence of polydatin—a naturally occurring and biologically active stilbenoid polyphenol structurally related to resveratrol. In addition, aromatic aldehydes such as hydroxybenzaldehyde and vanillin were detected, contributing to the chemical diversity of the extract. These findings are summarized in Table 1, which details the concentration and retention time of each identified compound.

This comprehensive phytochemical profiling significantly enhances the current understanding of the chemical composition of *C. erythraea*, particularly its aqueous extract, which remains underexplored in the scientific literature. While previous studies have reported only a limited number of phenolic constituents, the present analysis demonstrates a broader and more detailed spectrum of bioactive compounds.

Among the major constituents, trans-ferulic acid was the most abundant (278.736 μg/g extract), followed by syringic acid (172.808 μg/g), polydatin (33.722 μg/g), and vanillin (13.048 μg/g). Flavonoids such as quercetin (12.444 μg/g) and isoquercitrin (9.317 μg/g) were also present in significant amounts. Additional compounds identified in lower concentrations included kaempferol, trans-cinnamic acid, gentisic acid, and *p*-coumaric acid. Furthermore, trace levels of morin, chlorogenic acid, sinapic acid, catechin, and gallic acid were also detected, indicating a diverse and pharmacologically relevant phytochemical profile.

Valentão et al. (2001) identified the main phenolic compounds in *C. erythraea* (CE) extract as several esters of hydroxycinnamic acids, including p-coumaric, ferulic, and sinapic acids, in a hydrolyzed aqueous extract. In contrast, Kachmar et al. (2019) characterized the aqueous extract of flowering tops from Morocco as being rich in twenty-two flavonoid glycosides, particularly acylated derivatives of quercetin, kaempferol, and isorhamnetin. Similarly, Stefkov et al. (2014) reported the presence of seven flavonoid glycosides (mainly derivatives of quercetin and kaempferol) in North Macedonian samples, which accounted for 25 % of the total compounds. Additionally, Hatjimanoli et al. (1988) described the presence of hydroxyterephthalic and 2,5-dihydroxyterephthalic acids in both methanol and aqueous extracts from France. More recently, Valentina and Wulandari (2022) highlighted the predominance of secoiridoid glycosides (swertiamarin,

Table 1
Quantitative analysis of natural compounds in *CEAE* by LC-MS/MS (μg/g extract).

No	Compounds	Rt (min)	Conc. (μg/g)	(m/z) MS	(m/z) MS/MS	CE(V)	Ion mode
1	Catechin	2.738	0.480	291.0	165, 139, 123	12	pos
2	Gallic acid	3.281	0.336	168.9	124.9	12	neg
3	Syringic acid	3.350	172.808	199.0	154.9, 140.2	6	pos
4	Chlorogenic acid	3.715	0.668	353.0	191.0	12	neg
5	Gentisic acid	3.814	2.191	152.8	108.9, 108.0	13	neg
6	Polydatin	3.991	33.722	391.0	229.0	8	pos
7	Isoquercitrin	4.690	9.317	464.9	302.8	8	pos
8	*p*-Coumaric acid	5.526	1.137	163.1	119.2	12	neg
9	Sinapic acid	5.601	0.466	224.9	206.9, 174.8	3	pos
10	Hydroxybenzaldehyde	5.689	0.219	121.0	92.0	24	neg
11	*trans*-Ferulic acid	5.838	278.736	195.1	176.9, 144.7	10	pos
12	Vanillin	5.994	13.048	152.9	124.9, 93.1, 65.2	10	pos
13	Quercetin	10.008	12.444	302.8	152.9, 136.9	34	pos
14	*trans*-Cinnamic acid	11.003	2.222	149.1	131.1, 103.0	10	pos
15	Naringenin	11.447	0.159	270.9	150.8, 119.1	26	neg
16	Kaempferol	11.570	4.835	287.0	212.9, 164.7, 162.7	34	pos
17	Morin	11.679	0.937	302.9	136.8, 153.0	32	pos
18	Biochanin A	13.621	0.236	284.9	151.9	19	pos

gentiopicroside, sweroside), flavonoids (rutin and quercetin-pentoside), and xanthone derivatives in *C. erythraea* infusions from Eastern Serbia, as well as other acids such as syringic and 5-O-caffeoylquinic acids. Compared to these studies, our results offer a more comprehensive profile of phenolic constituents, especially with identifying compounds such as polydatin, morin, vanillin, catechin, and gallic acid, which were not previously reported in aqueous extracts. Such differences are likely influenced by geographical origin or harvesting conditions. Overall, while our findings partially agree with previous studies, they expand the phytochemical spectrum of *C. erythraea*, particularly by identifying polydatin and aromatic aldehydes, and provide a comprehensive profile of phenolic constituents in the traditionally consumed aqueous extract.

Among polyphenols (flavonoids, stilbenes, and phenolic acids), phenolic acids are the major constituents in *C. erythraea* aqueous extract (CEAE). Notably, trans-ferulic acid and syringic acid exhibit a wide range of therapeutic applications, including preventing diabetes, cardiovascular diseases, cancer, and cerebral ischemia. These compounds also possess antioxidant, antimicrobial, anti-inflammatory, neuroprotective, and hepatoprotective activities. They act as effective free radical scavengers and help alleviate oxidative stress markers (Cheemanapalli Srinivasulu et al., 2018; Du et al., 2013; Kumar and Goel, 2019; Marcato et al., 2022; Zheng et al., 2025).

3.2. Antioxidant activity

The antioxidant potential of CEAE was assessed by measuring its radical scavenging ability using the FRAP, DPPH$^{\cdot}$ and ABTS$^{+\cdot}$ scavenging assays. The results obtained are presented in Table 2.

The antioxidant potential of *C. erythraea* aqueous extract (CEAE) was evaluated using three complementary *in vitro* assays: FRAP, DPPH$^{\cdot}$ and ABTS$^{+\cdot}$ scavenging. The IC_{50} values obtained were 192.50 ± 7.62 μg/mL for DPPH$^{\cdot}$ scavenging, 170.37 ± 6.68 μg/mL for ABTS$^{+\cdot}$ scavenging, and 148.73 ± 8.50 μg/mL for FRAP. In comparison, the synthetic antioxidant BHT demonstrated significantly stronger activity, with IC_{50} values of 30.34 ± 0.96 μg/mL, 30.17 ± 0.93 μg/mL, and 32.05 ± 0.11 μg/mL, respectively ($p < 0.001$ for all assays).

These results are consistent with previous findings. For example, Kachmar et al. (2019) reported DPPH$^{\cdot}$ scavenging activity for a Moroccan *C. erythraea* aqueous extract with an IC_{50} of 63.00 μg/mL, indicating stronger activity than in our study, possibly due to differences in phytochemical composition and geographical origin. Similarly, Merghem and Dahamna (2020) observed a much weaker antioxidant response (DPPH$^{\cdot}$ scavenging, IC_{50} = 208 μg/mL; FRAP IC_{50} = 1310 μg/mL), supporting the variability in antioxidant efficacy based on extraction method and plant source. Despite being less potent than BHT, our CEAE results demonstrate a notable capacity to neutralize free radicals, supporting its traditional use as a medicinal plant. The antioxidant activity of *C. erythraea* aqueous extract (CEAE), as evidenced by its IC_{50} values, can be attributed to the synergistic effect of its phytochemical constituents. The extract was found to contain a rich variety of phenolic acids (such as trans-ferulic acid, syringic acid, *p*-coumaric acid, gentisic acid, sinapic acid, chlorogenic acid, gallic acid, and *trans*-cinnamic acid) and flavonoids (including quercetin, isoquercitrin, kaempferol, morin, and catechin), as well as other phenolic compounds like polydatin and vanillin. These molecules are well-recognized for their ability to neutralize free radicals (as measured by DPPH$^{\cdot}$ and ABTS$^{+\cdot}$ scavenging assays) and to reduce ferric ions (as measured by the FRAP assay).

Table 2
Antioxidant activity of *CEAE*.

Assay	CEAE	BHT
DPPH$^{\cdot}$ scavenging	192.50 ± 7.62***	30.34 ± 0.96
ABTS$^{+\cdot}$ scavenging	170.37 ± 6.68***	30.17 ± 0.93
FRAP	148.73 ± 8.50***	32.05 ± 0.11

Values are expressed as **IC_{50} values** in μg/mL (mean ± SD, $n = 3$).
***$p < 0.001$ compared **to BHT (reference standard).**

Multiple hydroxyl groups in these compounds enhance their electron-donating ability, directly contributing to radical scavenging and reducing power. Furthermore, several of these phenolics, particularly flavonoids and hydroxycinnamic acid derivatives, are known to modulate intracellular antioxidant defenses via the Nrf2/HO-1 pathway, promoting the expression of antioxidant enzymes (increasing the activity of antioxidant enzymes, such as superoxide dismutase, catalase, or glutathione peroxidase) while downregulating pro-oxidant factors. This dual action, both direct and indirect, supports the significant antioxidant potential observed for CEAE across all three *in vitro* methods (Cheemanapalli Srinivasulu et al., 2018; Karami et al., 2022; Kumar et al., 2014; Marcato et al., 2022; Park et al., 2021; Pyrzynska, 2024; Srinivasulu et al., 2018; Wang et al., 2024).

Table 3
Anticholinesterase activity of *C. erythraea* aqueous extract.

Sample	AChE	BChE
CEAE	44.62 ± 7,77***	31.27 ± 2.68
Galantamine	6.21 ± 0.4	35.78 ± 1.45

Values are expressed as **IC_{50}** in μg/mL (mean ± SD, $n = 3$).
***$p < 0.001$ compared to galantamine (reference standard).

3.3. Cholinesterases inhibitory activity

Using Ellman's colorimetric method, the inhibitory effects of CEAE on both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were evaluated, with galantamine serving as the reference standard (Ozen et al., 2022). The results for each sample are summarized in Table 3.

Cholinesterase exists in two main forms: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), both essential for regulating acetylcholine (ACh) levels in the nervous system. Excessive cholinesterase activity reduces ACh, leading to memory and concentration impairments typical of Alzheimer's disease (Chen et al., 2022). Therefore, inhibiting cholinesterase is a promising strategy to slow or prevent AD progression. The aqueous extract of *C. erythraea* (CEAE) was evaluated for its inhibitory potential against cholinesterase enzymes, namely acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which are key therapeutic targets in the management of Alzheimer's disease. The results indicate that CEAE exhibits moderate inhibition of AChE, with an IC_{50} value of 44.62 ± 7.77 μg/mL, which is significantly less potent than the reference inhibitor galantamine (IC_{50} = 6.21 ± 0.40 μg/mL). This statistically significant difference ($p < 0.001$) suggests that although CEAE possesses some inhibitory capacity against AChE, its effectiveness is relatively weak compared to the standard drug. Interestingly, CEAE demonstrated a slightly stronger inhibition of BChE (IC_{50} = 31.27 ± 2.68 μg/mL) compared to galantamine (IC_{50} = 35.78 ± 1.45 μg/mL). The results demonstrate a moderate inhibitory effect on AChE and a slightly stronger effect on BChE compared to galantamine. This observation indicates a preferential inhibition of BChE by the extract, which could be therapeutically relevant in the later stages of Alzheimer's disease, where BChE activity increases and partially compensates for the decline in AChE function.

These findings are in agreement with the only available study on the aqueous extract of *C. erythraea* by Guedes et al. (2019), which showed that a decoction of the plant inhibited AChE by 56 % at a higher concentration (500 μg/mL), likely due to the presence of xanthone compounds.

The observed inhibitory activity may be attributed to bioactive compounds in the aqueous extract of *C. erythraea*, including phenolic acids, flavonoids, stilbenoid polyphenols, and aromatic aldehydes. These compound classes are well-documented for their neuroprotective properties and their ability to inhibit cholinesterase enzymes, thereby

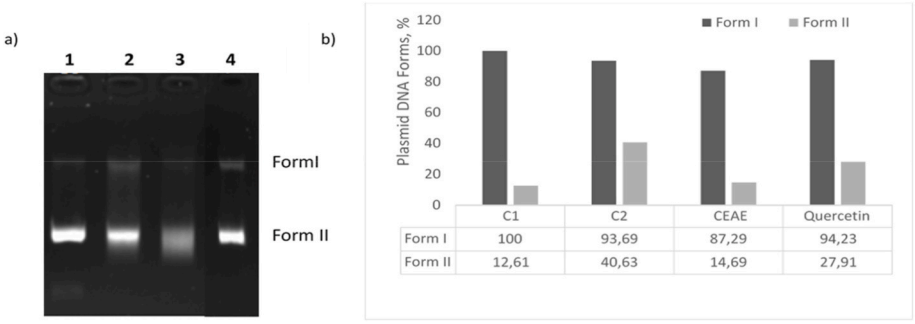

Fig. 2. DNA protective activity of *C. erythraea* aqueous extract.

reducing acetylcholinesterase (AChE) activity and potentially lowering amyloid-beta plaque formation associated with Alzheimer's disease (Budryn et al., 2022; Fakhri et al., 2021; Iannuzzi et al., 2023; Mugundhan et al., 2024; Tuzimski and Petruczynik, 2022).

3.4. DNA protection activity

The aqueous extract from the aerial parts of *C. erythraea* was examined under anaerobic conditions to evaluate its influence on plasmid DNA forms I and II, there by determining its potential DNA protective activity (Fig. 2).

The positive control, quercetin, and CEAE showed higher DNA protective activity in form I compared to form II. Adding CEAE to the H_2O_2 reaction mixture effectively protected supercoiled circular DNA from oxidative damage. However, the DNA protection activity of CEAE (14.69 %) was significantly lower than that of quercetin, as illustrated in Fig. 2. Notably, this study is the first to evaluate the DNA protective effect of CEAE, thereby providing valuable new information in this research area. DNA damage that disrupts normal cellular processes can result in cell death or the onset of cancer. Multiple factors, including biological agents, toxic substances, and chemical compounds may cause such damage. To counteract this, cells employ DNA repair systems that work to restore the original nucleotide sequence (Gajendra et al., 2024). Polyphenols, a broad group of phenolic compounds, are known for preserving DNA integrity (Andrés et al., 2023; Azqueta and Collins, 2016; Kada et al., 2017).

3.5. Acute oral toxicity

An acute oral toxicity study of CEAE was conducted in female mice at a dose of 2000 mg/kg body weight, following OECD Guideline 420. Animals were closely monitored for gross behavioral changes such as mortality, diarrhea, respiratory distress, Straub tail, piloerection, and convulsions during the first 3 h post-administration and then at 4-h intervals over the following 72 h. No mortality or noticeable behavioral alterations were observed at the administered dose. The animals were monitored for two weeks post-treatment, during which no significant differences in food or water consumption were observed between treated and control groups. These results align with previous ethnopharmacological data indicating a wide margin of safety for the therapeutic use of *C. erythraea* (Tahraoui et al., 2010).

Table 4
Analgesic effects of *C. erythraea* aqueous extract on acetic acid-induced abdominal writhing in rats.

Groups	Dose (mg/kg)	Average number of writhes	% Inhibition
Control	–	307.20 ± 15.10	–
CEAE	100	172.00 ± 2.12***	11.45
	200	173.80 ± 3.63***	43.42
	300	149.80 ± 9.88***	51.23
Paracetamol	150	167.80 ± 6.90***	45.30

Values are expressed as mean ± SD, $n = 5$.
***$p < 0.001$ when treated groups are compared to the negative control group.

3.6. Analgesic activity

3.6.1. Acetic acid-induced writhing test

The acetic acid-induced writhing test is commonly employed to assess pain responses resulting from chemically induced tissue injury. When administered intraperitoneally, acetic acid provokes characteristic abdominal constrictions, known as writhing, due to the sensitization of chemo-sensitive nociceptors by prostaglandins, which subsequently activate peripheral nociceptive neurons (Jang et al., 2020). The effects of the aqueous extract of *C. erythraea* on acetic acid-induced writhing in rats are presented in Table 4.

The results showed that the CEAE exhibited a significant, dose-dependent analgesic effect. CEAE reduced acetic acid-induced writhing by 11.45 %, 43.42 %, and 51.23 % at doses of 100, 200, and 300 mg/kg body weight, respectively, with the highest dose demonstrating the most potent effect ($p < 0.001$). Notably, this effect at 300 mg/kg was greater than that of the reference drug paracetamol. These findings contrast with those of Berkan et al. (1991), who reported no analgesic activity for a centaury aqueous extract in mice using the writhing and hot plate models. In contrast, more recent studies have demonstrated that the ethanolic extract of *C. erythraea* possesses antinociceptive activity at both peripheral and central levels. Furthermore, docking studies confirmed the analgesic potential of swertiamarin, the major compound in the extract, which exhibited strong binding interactions with COX-2 (Chabane et al., 2024).

This discrepancy may be due to differences in the type of extract, dosage, or route of administration used in their study. In contrast, the significant effects observed at all tested doses of CEAE ($p < 0.001$) highlight the plant's potential in relieving visceral pain, as demonstrated by the reduction in abdominal constrictions. This effect is likely linked to the antinociceptive and anti-inflammatory properties of the extract (Boiko et al., 2019; Jang et al., 2020; Karami et al., 2022; Okur and Şakul, 2021; Zhang et al., 2008). Despite the frequent citation of *Centaurium erythraea* in ethnobotanical literature for its traditional use as an analgesic, few pharmacological studies have focused on its analgesic activity. Moreover, limited research has investigated the phenolic profile of the aqueous extract and its possible contribution to pain relief. These findings underscore the need for further studies better to understand the bioactive compounds responsible for the observed effects.

3.6.2. Hot plate test

The hot plate test is a well-established model for assessing centrally acting analgesics, as it primarily stimulates nociceptors through sensory nerve pathways, with minimal involvement of endogenous mediators such as prostaglandins (Jang et al., 2020). The results demonstrating the central antinociceptive effects of CEAE are summarized in Table 5.

Treatment with CEAE at doses of 200 and 300 mg/kg body weight significantly increased the reaction time in rats compared to the

Table 5
Central analgesic effects of *C. erythraea* aqueous extract in the hot plate test in rats.

Groups	Dose (mg/kg)	Reaction time in seconds at various time intervals			
		0 min	30 min	60 min	90 min
Control	–	5.00 ± 0.10	5.00 ± 0.10	5.30 ± 0.50	6.00 ± 0.00
CEAE	100	5.66 ± 0.37	6.64 ± 0.08*	6.33 ± 0.52*	6.56 ± 0.57
	200	5.45 ± 0.25	6.00 ± 0.20*	9.33 ± 0.45**	11.00 ± 0.20***
	300	5.60 ± 0.28	9.66 ± 0.51**	12.33 ± 0.49***	12.66 ± 0.72***
Paracetamol	150	5.60 ± 0.00	6.50 ± 1.00*	10.0 0 ± 0.30***	11.30 ± 0.60***

Values are expressed as mean ± SD, $n = 5$.
*p < 0.05; **p < 0.01; ***p < 0.001 when treated groups are compared to the negative control group.

negative control group ($p < 0.01$). Paracetamol (150 mg/kg b.w.) also increased the basal reaction time at 30, 60, and 90 min; however, the 300 mg/kg dose of CEAE exhibited a stronger analgesic effect than the reference drug. The hot plate test is widely recognized for evaluating centrally acting analgesics, as it involves higher brain functions and responses to nociceptive stimuli organized at the supraspinal level. In our study, CEAE significantly prolonged the basal reaction time ($p < 0.001$), indicating a central antinociceptive effect. Only a few studies have specifically assessed the central analgesic effects of *C. erythraea* aqueous extracts. For instance, Berkan et al. (1991) did not report significant effects in mice using aqueous extracts in the hot plate test, which contrasts with our results. In contrast, recent work by Chabane et al. (2024) demonstrated that the ethanolic extract of *C. erythraea* possesses a strong analgesic activity, suggesting that the type of extract and its phytochemical composition may play a critical role in the observed pharmacological effects. This effect is likely linked to the antinociceptive and anti-inflammatory properties of phenolic acids, flavonoids, and other bioactive molecules present in the extract (Adrar et al., 2021; Boiko et al., 2019; Karami et al., 2022; Okur and Şakul, 2021; Zhang et al., 2008).

Table 6
The molecular docking interactions parametres of compounds with AChE.

Compounds	Binding affinity (kcal/mol)	Distance	Aminoacids	Interactions
trans-Ferulic acid	−6.7	3.36822	GLY122	Con. HB
		2.89422	TYR124	Con. HB
		2.97396	SER203	Con. HB
		2.95695	ASN87	Con. HB
		3.96388	GLY121	Pi-sigma
		5.11953	TRP86	Pi-Pi T-shaped
		5.2666	TRP86	Pi-Pi T-shaped
		4.96138	PHE297	Pi-Alkyl
		4.97404	PHE338	Pi-Alkyl
		5.19369	HIS447	Pi-Alkyl
Syringic acid	−6.7	3.10006	GLY122	Con. HB
		3.01027	SER203	Con. HB
		2.94976	TYR337	Con. HB
		2.28327	GLY120	Con. HB
		2.9351	TYR133	Con. HB
		2.50131	TYR124	Con. HB
		3.5337	GLY448	CHB
		3.63743	SER125	CHB
		3.86169	GLY121	Pi-sigma
		5.4133	TRP86	Pi-Pi T-shaped
		5.4677	TRP86	Pi-Pi T-shaped
		5.73444	TYR337	Pi-Pi T-shaped
		5.06262	TRP86	Pi-Alkyl
		4.86787	PHE297	Pi-Alkyl
		4.73882	PHE338	Pi-Alkyl
		4.94131	HIS447	Pi-Alkyl
trans-Ferulic/ Syringic acid	−7.1	3.11286	ARG247	Con. HB
		3.7062	ASN533	CHB
		3.53817	HIS405	CHB
		3.97706	ARG296	Pi-Cation; Pi-Donor HB
		3.80874	PRO537	
		4.38164	PRO537	Pi-sigma
		5.07528	VAL239	Alkyl
		4.59606	ARG296	Alkyl
		4.40605	HIS405	Alkyl
		5.12042	TRP532	Pi-Alkyl
		5.14457	TRP532	Pi-Alkyl
		5.31251	ARG296	Pi-Alkyl
				Pi-Alkyl

Abbreviations: Con. HB: Conventional Hydrogen Bond, CHB: Carbon Hydrogen Bond.

3.7. Synergistic binding interactions of phenolic compounds with AChE

The molecular docking results summarized in Table 6 revealed that *trans*-ferulic acid and syringic acid exhibited favorable binding affinities toward acetylcholinesterase (AChE), supporting the experimentally observed enzyme inhibition. The trans-ferulic/syringic acid adduct showed a stronger binding affinity (−7.1 kcal/mol) compared to the individual compounds (−6.7 kcal/mol), indicating a synergistic interaction at the AChE active site. This enhanced affinity may be attributed to the formation of additional hydrogen bonds and hydrophobic interactions with key amino acid residues within the active site of AChE (Fig. 3). Binding mode analysis demonstrated that the ligands were well accommodated within the active binding pocket, suggesting a stable and energetically favorable protein–ligand complex (Aktar et al., 2020; Yenigün et al., 2024). These *in silico* findings are consistent with the *in vitro* anticholinesterase activity of the extract and provide mechanistic insight into the observed bioactivity. The docking results highlight the contribution of phenolic compound synergy to the neuroprotective potential of *C. erythraea.*

4. Conclusions

This study presents a detailed investigation into the phenolic composition and the associated biological activities both *in vitro* and *in vivo* of the aqueous extract of *Centaurium erythraea*, a medicinal plant traditionally used in Algeria. The extract was prepared using a hot infusion method, aligning with traditional preparation practices, thereby enhancing the ethnopharmacological relevance of the findings. Through LC-MS/MS analysis, a diverse array of phenolic compounds and flavonoids was identified, including trans-ferulic acid, syringic acid, polydatin, vanillin, and quercetin, among others.

The biological evaluations demonstrated that the extract possesses moderate yet meaningful antioxidant activity, as evidenced by its performance in FRAP, $ABTS^{+}$, and DPPH assays. Additionally, CEAE exhibited significant inhibitory activity against both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), suggesting its potential utility in managing neurodegenerative disorders such as Alzheimer's disease. DNA protection assays further supported the extract's role in mitigating oxidative damage, while *in vivo* analgesic models confirmed its dose-dependent antinociceptive effects, surpassing conventional analgesics in some cases. Importantly, acute toxicity testing at a high dose (2000 mg/kg) revealed no signs of adverse effects, reinforcing the extract's safety profile for oral use.

To further support the observed anticholinesterase activity, molecular docking studies were performed against acetylcholinesterase

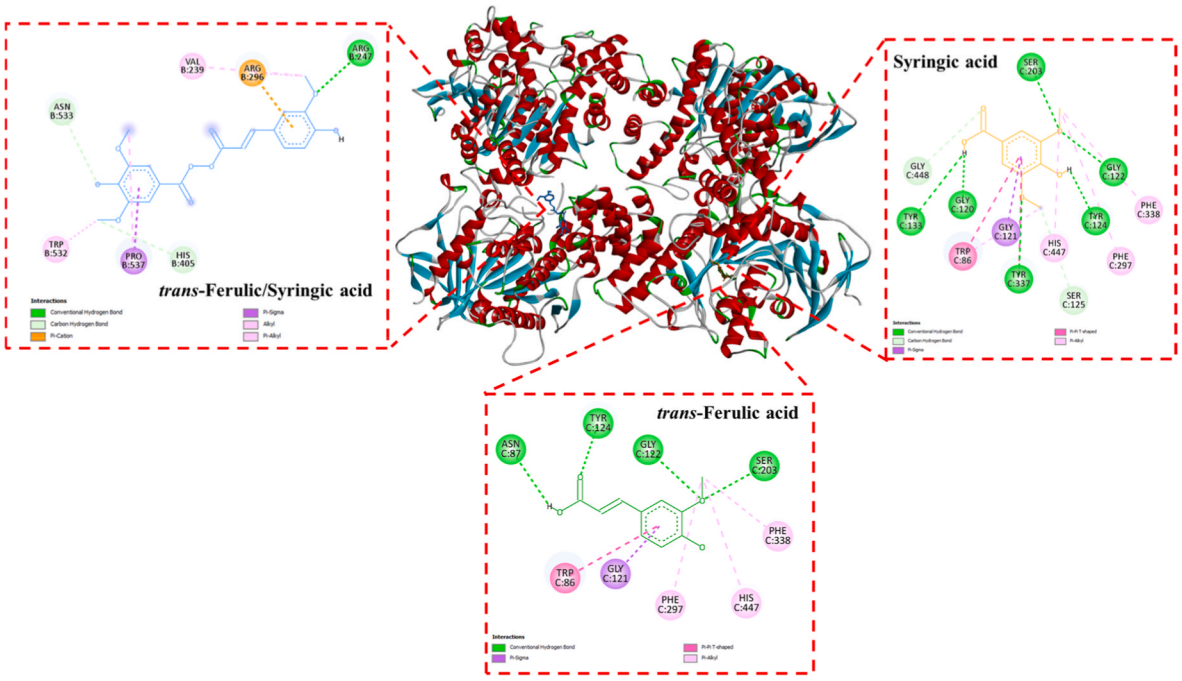

Fig. 3. The schematic diagram of molecular docking between *trans*-ferulic acid, syringic acid, and *trans*-ferulic/syringic acid adduct with AChE.

(AChE), revealing favorable binding affinities for major phenolic constituents of the extract. Docking analysis demonstrated that compounds such as *trans*-ferulic acid and syringic acid effectively interacted with key amino acid residues within the AChE active site through hydrogen bonding and hydrophobic interactions.

These *in silico* findings corroborate the *in vitro* enzyme inhibition results and suggest a synergistic contribution of the identified phenolics to the neuroprotective potential of the extract. Overall, the docking results provide mechanistic insight into the molecular basis of AChE inhibition and strengthen the pharmacological relevance of *C. erythraea*.

Collectively, these findings validate the traditional use of *C. erythraea* in the treatment of pain, inflammation, and related conditions, and underscore the pharmacological relevance of its phytochemical constituents. The demonstrated bioactivities highlight *C. erythraea* as a promising candidate for the development of novel therapeutic agents or nutraceutical formulations. Future studies should focus on isolating individual bioactive compounds, elucidating their mechanisms of action, and evaluating their efficacy in clinical models to fully harness the therapeutic potential of this underexplored medicinal plant.

CRediT authorship contribution statement

Sarra Chabane: Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Amel Boudjelal:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ibrahim Demirtas:** Writing – review & editing, Methodology, Conceptualization. **Souheila Bouchahdane:** Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ghania Tail:** Writing – review & editing, Validation, Supervision. **Semiha Yenigün:** Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Tevfik Ozen:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Ilyas Yıldız:** Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Abdeltif Amrane:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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