

LC-MS/MS characterization of phenolic compounds and *in vitro* assessment of antioxidant, antidiabetic, antimicrobial, and anti-Alzheimer activities of *Echium creticum* L.

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ABSTRACT: The present work provides the first comprehensive LC–MS/MS phytochemical profiling and pharmacological investigation of *Echium creticum* L., Boraginaceae, native to the Mediterranean region, used in folk medicine. The aerial parts were sequentially extracted using petroleum ether (PEEEC), chloroform (CEEC), ethyl acetate (EAEEC), and *n*-butanol (BEEC). LC-MS/MS profiling of the polar extracts identified 25, 22, and 24 bioactive compounds, with rosmarinic acid, ferulic acid, salicylic acid, and gentisic acid as major phenolics. Several flavonoid glycosides (cosmosiin, nicotiflorin, genistin) are reported for the first time in the *Echium* genus. Antioxidant activity was evaluated using DPPH, ABTS FRAP, and Fe³⁺-phenanthroline assays, was strongest in the ethyl acetate fraction. Antidiabetic potential was confirmed via α -amylase inhibition, where EAEEC demonstrated effective inhibition ($IC_{50} = 207.20 \pm 2.58 \mu\text{g/mL}$), comparable to quercetin and Acarbose. The antibacterial activity was also evaluated through the disc diffusion methods and MIC tests against a panel of 10 gram-positive (+) and gram-negative (–) bacterial strains (references μ -drug resistant bacteria) (MIC 32–80 $\mu\text{g/mL}$). The *n*-butanol extract (BEEC) displayed the strongest anticholinesterase activity ($IC_{50} = 68.98 \pm 1.33 \mu\text{g/mL}$), consistent with its flavonoid glycoside enrichment. Altogether, these results highlight *E. creticum* as an underexplored source of phenolic compounds with multi-target *in vitro* bioactivities and expand the phytochemical diversity of the *Echium* genus.

Keywords: *Echium creticum*, LC–MS/MS, phenolic compounds, Antioxidant, Anti-diabetic, Anticholinesterase

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1 Introduction

For hundreds of years, medicinal and aromatic plants have been known to be essential sources of therapeutic chemicals (Ceylan et al., 2016). They are used in both ancient healing systems and modern ones like medications and functional foods. Their wide range of pharmacological effects comes from a large number of secondary metabolites, including as flavonoids, phenolic acids, alkaloids, and terpenoids (Inci et al., 2023). These compounds have a variety of

biological effects, such as being antioxidants, anti-inflammatory, antibacterial, anticancer, and enzyme-inhibitory (Zengin et al., 2017).

Echium creticum L. (Boraginaceae) is an herbaceous plant, commonly known as Cretan viper's bugloss (locally called Leçane el assilis) is an annual to biennial herbaceous species of the Boraginaceae family. Said plant is on the western and central Mediterranean regions; along with Algeria and Morocco, Tunisia, Spain, Portugal, France, and Italy (POWO). In Algeria, it is found throughout the Tell region between Algiers and Constantine but it is rare in most parts of the country. It grows in fields, near grasslands, dry and uncultivated soils, and is often found among rocks and rubble. The plant is an erect herbaceous plant, which can be distinguished

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Figure 1. Image of *E. creticum* original photo (from the collection site)

by the coarseness and hairiness of its surface (Figure 1). The flowers emerge in funnel-like form, and can be pale purple to blue in color and hairy on the outer portion, grouped in the distinctive appearances of scorpioid cymes, appearing as if it were scorpion tails. The lower leaves are petiolate and often lanceolate, while the upper leaves are elongated and sessile (Guide illustré de la flore algérienne).

The genus *Echium* was demonstrated to possess medicinal properties such as depurative, diaphoretic, diuretic, and mood-enhancing across the Mediterranean area have been used for some time (Shafaghi et al., 2002; Heidari et al., 2006). Of the *Echium* species, *Echium amoenum* has been researched most extensively as the Romans and Iranians previously used it to treat fever, respiratory infections, and mood disorders. Similarly, *E. vulgare* has been traditionally used in Türkiye and Germany for treating wounds, bruises, and even snakebites, though detailed preparation methods are often undocumented (Eruygur et al., 2016). The biological activities of this genus are linked to both the antioxidant activity and possible therapeutic effects, and *Echium* is also acknowledged as because of their hepatotoxic and genotoxic qualities, a valuable source of flavonoids. Luteolin-7-O-glucosides, myricetin, quercetin and myricitrin in *E. arenarium* (Kefi et al., 2018), and phenolic acids as important bioactive compounds: rosmarinic acid was reported as the main compound in *E. amoenum* (Mehrabani et al., 2005) and chlorogenic acid and hydrocaffeic acid in extracts from *E. vulgare* (Dresler et al., 2017).

Different research has also investigated the capacity of *Echium* species extracts as natural antioxidant agents. Multiple pure compounds may be responsible for the anti-oxidant activity of *Echium* species. Although the role of polyphenols has been widely recognized, new research indicates that naphthoquinones may also play a major role in these extracts' antioxidant potential (Jin et al., 2020). The antioxidant activities of extracts from different species of *Echium* using different solvents have been assessed by many different assays (DPPH, ABTS, FRAP). Hydroalcoholic extracts always had the most antioxidant activity due to the higher level of phenolics (Jin et al., 2020).

Species of the genus *Echium*, in particularly *E. vulgare* and *E. amoenum* might have significant antimicrobial activity.

E. vulgare, for example, have described moderate antimicrobial activity specifically using disk diffusion method against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus megaterium*, *Klebsiella pneumoniae* and *Candida albicans* microorganisms (Arslan Ateşşahin et al., 2023). Further to this, Patocka reported that *E. amoenum* produced an aqueous extract with considerable antibacterial activity toward *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Patocka & Navratilova, 2019).

Although *E. creticum* has been occasionally mentioned in ecological or preliminary pharmacological studies, detailed phytochemical and multi-target biological evaluations remain scarce. To the best of our knowledge, the present work is the first to integrate LC-MS/MS phenolic profiling and pharmacological potential of aerial parts of *E. creticum* L., a species of *Echium* which is a medicinal plant used in folklore medicine from the Mediterranean region. The aboveground part of the plant will be exhaustively extracted with petroleum ether, chloroform, ethyl acetate and *n*-butanol to determine the bioactive components. LC-MS/MS will be used to identify some significant phenolic compounds to assess the biological potential of the extracts, while we will evaluate antioxidant activity using DPPH, ABTS scavenging, reducing power and phenanthroline, as well as α -amylase inhibition to explore possible antidiabetic effect. The study ultimately intends to assess antibacterial and anticholinesterase activities to better understand the plant's potential in relation to microbial infections and neurodegenerative diseases. Ultimately the objective of the study is to provide evidence that support a future use of the extracts derived from *E. creticum* in the pharmaceutical formulation of therapeutic agents with multiple action profile.

2 Material and Methods

2.1 Chemicals

BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole), α -tocopherol, acetylcholinesterase, butylcholinesterase, α -amylase enzymes, acetylcholine iodide, solvent and standard phenolics LC-MS/MS and other chemicals were purchased from Merck or Sigma-Aldrich.

2.2 Microorganisms

The Pasteur Institute in Algiers provided the reference bacterial strains, which were *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 43300. while, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Morganella morganii*, and *Salmonella heidelberg* are among the multi-drug-resistant bacteria. The clinical bacterial isolates were obtained from the Bacteriology Laboratory hospital of Constantine (CHUC)-Algeria. These isolates were part of the routine diagnostic workflow and were anonymized before use. Bacterial isolates (*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Morganella morganii*, *Enterobacter aerogenes*, and *Salmonella heidelberg*) were collected from clinical samples under sterile conditions. Primary identification was performed by a combination of conventional microbiological tests (colony morphology, Gram staining, biochemical assays), commercial API galleries (bioMérieux, France), and confirmed with the automated **MicroScan WalkAway system** (Beckman Coulter, USA). The visible zone of the inhibition bacterial colony formation served as the basis for determining antibacterial activity. The lowest extract concentrations that successfully inhibited bacterial growth were known as minimum inhibitory concentrations, or MICs. As antibiotics of control, Ampicilline and Gentamycine were employed (Clinical and Laboratory Standards Institute (CLSI), 2015; Limbago, 2001).

2.3 Plant Materials

Fresh aerial parts of *E. creticum* were collected from Djebel El Ouahch Constantine, Algeria (36.39447°N, 6.79213°E, alt. $\approx$ 1280 m) at the flowering stage on May 2022. The plant was authenticated by Pr. Gérard De Bélair (University Badji Mokhtar-Annaba). The voucher specimen was deposited at the herbarium of the Laboratory of Therapeutic Substances, University of Constantine 1, Algeria with the voucher code LOST ZK EC05/22. The botanical identification of the plant was performed using descriptions provided by various sources (POWO; Guide illustré de la flore algérienne) and botanical references (Vipérine de Crète; Tela Botanica).

2.4 Plant Extraction

The aerial parts of *E. creticum* (950 g) were air-dried at room temperature and powdered then macerated in a mixture of methanol-water 80:20 (v/v). for 48 hours at room temperature (the process was repeated three times), then filtrated, the filtrate was concentrated under low pressure at 37 °C then extracted with petroleum ether, chloroform, ethyl acetate and *n*-BuOH. The evaporation of solvents in vacuum led to the respective dried extracts yielding: 25 g of *n*-Butanol (BEEC), 8 g ethyl acetate (EAEEC), 6 g chloroform (CEEC), 0.6 g petroleum ether (PEEEC).

2.5 Phytochemical Analysis

2.5.1 Determination of Total Phenolics Contents (TPC)

Total phenolics in *E. creticum* extracts were quantified by the Folin–Ciocalteu method (Singleton & Rossi, 1965). Briefly,

0.5 mL of each extract solution was mixed with 1.0 mL of Folin Ciocalteu reagent. and sodium carbonate solution, incubated in the dark at room temperature for 2 hours. Absorbance was then measured at 765 nm against a blank sample prepared with methanol instead of extract. A standard calibration curve was prepared using gallic acid at different dilution using methanol as solvent. Gallic acid was used for calibration.

The standard curve of gallic acid for total phenolic contents (r^2 : 0,9972) is presented in Figure 2.

2.5.2 Determination of Total Flavonoid Contents (TFC)

The total flavonoid content (TFC) of *E. creticum* BEEC, EAEEC, and CEEC extracts were determined according to a validated colorimetric method (Yilmaz et al., 2023; Goudjil et al., 2024).

The coloration procedure specified adding 0.5 mL of extract to 1.5 mL of 95% methanol. A 0.5 mL aliquot of 1M potassium acetate solution was added followed by 1.5 mL of 10% aluminum nitrite solution. Deionized H₂O was added to the line-up to a total of (2.3 mL). incubated at room temperature (25 °C) in the dark for 40 minutes before measuring the absorbance (415 nm) with a reagent blank. The calibration curve was constructed using quercetin, and the results calculated as milligrams quercetin equivalents (mg QE) per gram dry extract is presented in Figure 3.

2.5.3 Determination of Total Flavonol Contents (TFOC)

The total flavonol content of *E. creticum* BEEC, EAEEC, and CEEC extracts was determined using the aluminum chloride (AlCl₃) colorimetric method, according to Kumaran and Karunakaran (Kumaran & Joel Karunakaran, 2007). Briefly, 50 μ L of each extract solution (1 mg/mL in methanol or distilled water) was mixed with 50 μ L of AlCl₃ solution and 150 μ L of sodium acetate solution in a 96-well microplate. After incubation in the dark for 2.5 h at room temperature, absorbance was measured at 440 nm. A calibration curve was prepared with quercetin standard solutions (25–200 μ g/mL), and results were expressed as milligrams of quercetin equivalents per gram of dry extract (mg QE/g), as shown in Figure 4. All assays were performed in triplicate and reported as mean $\pm$ SD.

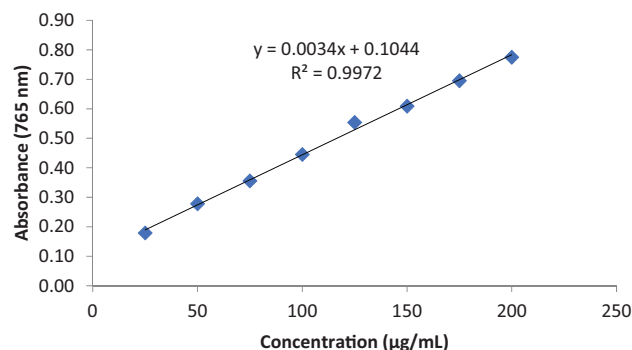


Figure 2. The standard curve of gallic acid for total phenolic contents (R^2 : 0,9972)

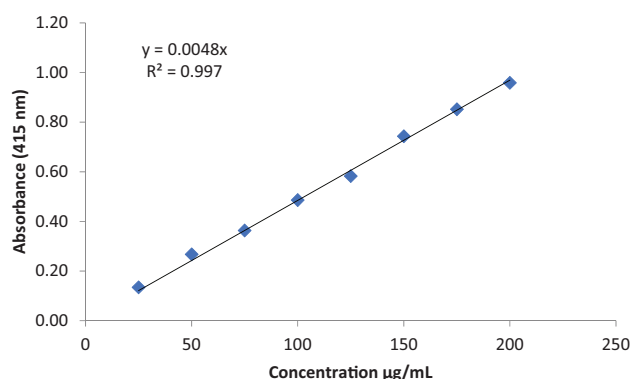


Figure 3. The standard curve of quercetin for total flavonoid contents (R^2 : 0.9994)

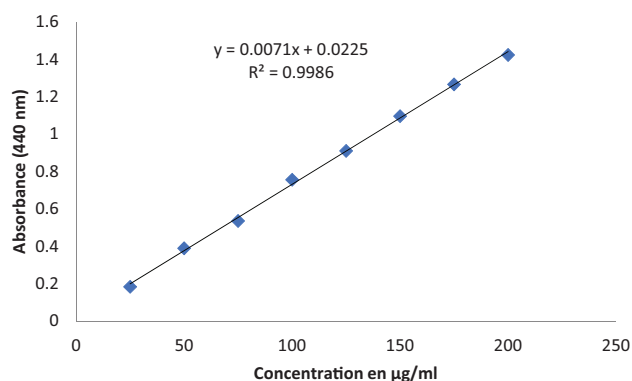


Figure 4. The standard curve of Total flavonol contents (R^2 : 0.9986)

2.5.4 Determination of Total Hydrolysable Tannin Contents (HTC)

The total hydrolysable tannin content (THTC) of *E. creticum* BEEC, EAEEC, and CEEC extracts was determined using a colorimetric method adapted to microplate format, based on potassium carbonate (K_2CO_3), following the procedure described by Makkar et al. (1995), with slight modifications. In each well of a 96-well microplate, 50 μ L of the plant extract was mixed with 150 μ L of freshly prepared 2.5% (w/v) potassium carbonate solution. The reaction mixture was incubated in the dark at room temperature for 15 minutes. Absorbance was then measured at 550 nm using a microplate reader. A standard calibration curve was established using tannic acid (Figure 5), and the results were expressed as micrograms of tannic acid equivalents per milligram of extract (μ g TAE/mg extract).

2.5.5 Determination of Total Condensed Tannin Contents (CTC)

The total condensed tannin Content of *E. creticum* BEEC, EAEEC, and CEEC extracts was quantified using the vanillin-HCl colorimetric method (Hagerman, 2002). Briefly, 1 mL of each extract solution (prepared in methanol) was mixed with 5 mL of reagent (2.5 mL of 1% vanillin in methanol + 2.5 mL of 8% HCl in methanol). then add to 5 mL of HCl (4%), the mixture was incubated at 30 °C

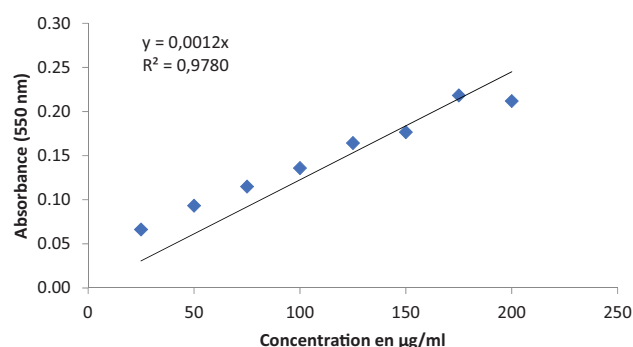


Figure 5. The standard curve of hydrolysable tannin contents (R^2 : 0.9780)

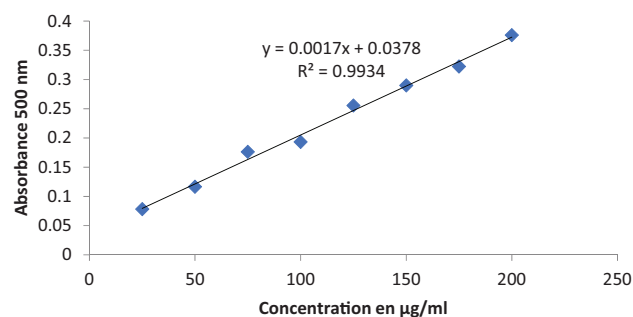


Figure 6. The standard curve of hydrolysable tannin contents (R^2 : 0.9934).

for 20 min. Absorbance was then measured at 500 nm. A standard calibration curve was prepared using Catechin (Figure 6), and results were expressed as milligrams catechin equivalents per 100 g of dry extract (mg CE/100 g). All assays were performed in triplicate and results expressed as mean $\pm$ SD.

2.6 Analysis of Polyphenolic Composition by LC-MS/MS

2.6.1 Preparation of Extracts for LC-MS/MS Analysis

Using a previously developed and validated analytical method (Yilmaz, 2020), the phenolics of the *E. creticum* extracts: BEEC, EAEEC, and CEEC fractions were identified by LC-MS/MS. In a volumetric flask, 100 mg of each extract was weighed and dissolved in 5 mL of a 1:1, v/v ethanolic-water solution. In order to adjust the concentration of the stock initial solution for dilution purposes in the injection into the LC-MS/MS instrument, a 1 mL aliquot of the solution was taken using a micropipette and transferred to a second 5 mL volumetric flask. The original stock solution was used as the diluent.

Programmatically, 1.5 mL of each sample was filtered through a 0.22 μ m syringe filter to imply no particles were passed on for LC-MS/MS injection, and with this filtered volume, each was transferred into sealed LC vials. Each sample volume of 5 μ L was injected into the LC-MS/MS instrument. All of the samples were kept cool (15 °C) in the autosampler during the analysis. This modernized method allows for complete profiling of polyphenolic compounds in

complex plant extracts and has been used in previous studies with propolis and other botanicals (Karageçili et al., 2023).

2.6.2 LC-MS/MS Instrumentation and Conditions

A Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) system with an electrospray ionization (ESI) source capable of operating in both positive and negative ionization modes was used for polyphenolic analysis. Chromatographic separation involved a C18 reverse-phase column maintained at an optimum temperature. Solvents A (water with 0.1% formic acid) and B (acetonitrile with 0.1 mg/g formic acid) made up the mobile phase, which was supplied at a gradient flow rate of 0.5 mL/min. Each sample had an injection volume of 5 μ L (Yilmaz, 2020).

Mass spectrometric detection was performed using the multiple reaction monitoring (MRM) mode, enabling accurate quantification and identification of target phenolic compounds based on their retention times, precursor ion (m/z), and characteristic fragment ions. The calibration and identification were achieved through comparisons of chromatographic and spectral data with 53 phenolic compounds which were also used as references for the standardization of *E. creticum* extracts (Yilmaz, 2020; Karageçili et al., 2023; Aydin et al., 2021).

The system was fine-tuned and optimized for maximal sensitivity and reproducibility, and all analyses were performed with controlled temperature and pressure to maintain analytical reproducibility and precision. Method variability was assessed through relative standard deviation (RSD%) of intraday and intraday measurements, providing a standard measure of repeatability for the LC–MS/MS procedure. A total of 56 phenolic standards were used for identification and quantification; standards were selected based on their availability, chemotaxonomic relevance to the Boraginaceae family (including the genus *Echium*), and their representation of major phenolic subclasses, thereby ensuring accurate profiling and quantification of the target compounds.

2.7 Biological Assays

2.7.1 Antioxidant Assays

2.7.1.1 DPPH Radical Scavenging Assay

The antioxidant potential of *E. creticum* extracts BEEC, EAEEC, and CEEC was evaluated using (DPPH•) radical scavenging assay. The method was based on Blois's original claims (Blois, 1958) and modified slightly for the later phytochemical studies (Yilmaz, 2020; Labed-Zouad et al., 2017).

Different concentrations of extracts were mixed with a methanolic DPPH solution (0.1 mM), incubated in the dark for 30 min at room temperature, and absorbance was measured at 517 nm. Results were expressed as IC₅₀ values (μ g/mL) which was calculated from the dose-response curve and used as an indicator of antioxidant strength. Each experiment was conducted in triplicate.

2.7.1.2 ABTS^{•+} Radical Scavenging Assay

Evaluating the antioxidant capacities of *E. creticum* extracts (BEEC, EAEEC, and CEEC) we determined percentage of discoloration of stable ABTS radical cation ABTS^{•+} following the protocol of Re et al. (1999) and adapted as reported in phenolic phytochemical assessments (Altay et al., 2022).

The preparation of the ABTS^{•+} solution consisted of mixing the same volume of 2 mM ABTS with 2.45 mM potassium persulfate and incubating for 6 hours in the dark then diluted to a final concentration with phosphate buffered saline (PBS, pH 7.4), with an absorbance value of 0.700 ± 0.020 at 734 nm.

For each assay, 3 mL of *E. creticum* extract at various concentrations [10–30 μ g/mL] was added to 1 mL of ABTS^{•+} solution. Radical scavenging activity IC₅₀ values were calculated from the plotted dose-response curves. Each experiment was conducted in triplicate.

2.7.2 Ferric Reducing Antioxidant Power (FRAP) Assay

The reduced ferric Fe³⁺ capacity of (BEEC, EAEEC and CEEC) extracts of *E. creticum* were assessed by using a modified procedure from the original method of Oyaizu (Oyaizu, 1986). with modifications typical of natural product investigations (Altay et al., 2022; Kızıldaş et al., 2021). All extracts were dissolved and mixed with phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide, incubated for 20 minutes at 50 °C, the reaction was stopped by trichloroacetic acid and centrifuging the supernatant was combined with FeCl₃ solution the absorbance was measured at 700 nm. Ascorbic acid and α -tocopherol served as reference antioxidants. Results were expressed as mg equivalents/g extract, and all analyses were performed in triplicate.

2.7.3 Ferric Ion (Fe³⁺) Reducing Power Assay Using 1,10-Phenanthroline

The Fe³⁺-reducing capacity of *E. creticum* extracts (BEEC, EAEEC, and CEEC) was determined using the 1,10-phenanthroline method with minor modification (Altay et al., 2022; Szydlowska-Czerniaka et al., 2008; Güven et al., 2024). In brief, 1 mL of each extract was added to a test tube (10–30 μ g/mL) with 1 mL of FeCl₃ solution (200 μ M) and 1 mL of 0.05% (w/v) 1,10-phenanthroline in ethanol. incubated in the dark for 30 minutes at 37 °C. The absorbance was read using a UV-Vis spectrophotometer at 510 nm.

BHT and BHA were used as standard antioxidants for comparison.

All assays were performed in triplicate, and results were expressed as mean $\pm$ standard deviation.

2.7.4 Enzyme Inhibition Studies

2.7.4.1 In Vitro Antidiabetic Activity α -Amylase Inhibition Assay

The α -amylase inhibitory activity of *E. creticum* L. extracts (BEEC, EAEEC and CEEC) was evaluated using a modified starch–iodine colorimetric method (Tel et al., 2013),

and minor adaptation of phytochemical studies (Altay et al., 2022; Güven et al., 2024).

Briefly, α -amylase solution (2 U/mL in phosphate buffer, pH 6.9) was pre-incubated at 37 °C with the same volume of each extract at different concentrations (10–100 μ g/mL) flowed by the addition of 1% soluble starch solution was added as the substrate the reaction was stopped with HCl. The absorbance of the resulting solution was measured at 630 nm against a blank containing all reagents except the extract.

Quercetin and acarbose served as positive controls. All experiments were conducted in triplicate and results were determined from inhibition curves and expressed as IC₅₀ values (μ g/mL).

2.8 In Vitro Anti-Alzheimer activity AChE (acetylcholinesterase) and BChE (butyrylcholinesterase) inhibition tests

The anti-Alzheimer potential of *E. creticum* extracts was evaluated by measuring their inhibition activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). This assay followed a modified version of Ellman's spectrophotometric method (Ellman et al., 1961; Labed et al., 2016), according to minor changes by Karageçili (Durling et al., 2007; Sheydaei et al., 2025). Briefly, extracts (10–100 μ g/mL) were incubated with either AChE (0.03 U/mL) or BChE (0.01 U/mL) in phosphate buffer (pH 8.0). DTNB (0.5 mM) and acetylthiocholine or butyrylthiocholine (0.75 mM) were then added as substrates, and the absorbance was monitored at 412 nm for 15 min. This method has been widely used in screening for anti-Alzheimer agents from natural products (Labed et al., 2016; Yilmaz et al., 2012). Notably, similar approaches have been applied in studies investigating enzyme inhibition by plant phenolics, including research by Kabouche and co-authors, which demonstrated the relevance of polyphenolic constituents in neuroprotective activity (Kabouche et al., 2021).

2.8.1 Antibacterial Assays: Disc Diffusion and MIC Determination Against Bacterial

The antibacterial activity of *E. creticum* extracts BEEC, EAEEC, and CEEC was evaluated using disc diffusion and minimum inhibitory concentration methods (MIC) against Gram-positive (+) and Gram-negative (–) bacterial strains, as well as reference strains of *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 supplied by Pasteur Institute (Algiers, Algeria). Moreover, clinical multidrug-resistant isolates, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Morganella morganii* and *Salmonella heidelberg* were provided by Bacteriology Laboratory from University Hospital of Constantine according to standard microbiology procedures (Clinical and Laboratory Standards Institute (CLSI), 2018).

The disc diffusion test was performed using 6 mm sterile paper disc impregnated with 50 μ L of each extract

(128 μ g/mL) in Mueller-Hinton agar plate with previously inoculated bacterial suspensions (0.8 McFarland standard) by placing disc on agar surface. Plates were incubated in 37 °C for 24 hours. The diameters of inhibition zones (millimeters) were measured. Ampicillin (10 μ g/disc) and gentamicin (10 μ g/disc) were used as reference antibiotics (Djahdou et al., 2020; Labed-Zouad et al., 2015).

Minimum inhibitory concentrations (MICs) were assessed using agar dilution method where serial dilutions of each extract were incorporated in Mueller-Hinton agar. After spotting the bacterial inoculum on the agar surface, the plates were incubated for 24 h at 37 °C. The MIC was recorded as the lowest extract concentration that completely inhibited visible microbial growth (Clinical and Laboratory Standards Institute (CLSI), 2015; Limbago, 2001). Development of this method was based on previously established protocols utilized for phytochemical antibacterial research (Djahdou et al., 2020; Labed-Zouad et al., 2015).

2.9 Statistical Analysis

All experiments were performed in triplicate, and results are expressed as mean $\pm$ standard deviation (SD). Statistical differences among extracts were assessed using one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test ($p < 0.05$). Pearson's correlation coefficients were calculated to explore relationships between phytochemical contents and biological activities. Principal Component Analysis (PCA) was also conducted to visualize the clustering of extracts based on their chemical composition and bioactivity profiles.

3 Results and Discussion

3.1 Total Phenolic, Flavonoid, Flavonol, Hydrolysable Tannin and Condensed Tannin Contents

The phytochemical screening of the extracts of *E. creticum* L. showed a wide variation in total phenolic and flavonoids contents in the three solvent fractions: butanolic (BEEC), ethyl acetate (EAEEC) and chloroformic (CEEC) (Table 1). The highest total polyphenol content was found in the BEEC extract with (350.37 $\pm$ 1.19 μ g GAE/mL), followed by EAEEC (161.84 $\pm$ 0.68 μ g GAE/mL) and a much lower value recorded in the CEEC (2.14 $\pm$ 0.17 μ g GAE/mL). From these results, it can be concluded that polar solvents, such as *n*-butanol, are more effective for the extraction of phenolic compounds, a trend consistent with previous studies on the *Echium* genus (Bošković et al., 2017; Heidari et al., 2006).

In flavonoids content, BEEC was also the highest containing 50.00 $\pm$ 2.06 μ g QE/mL, followed by EAEEC and CEEC 38.05 $\pm$ 0.15 and 29.37 $\pm$ 0.29 μ g QE/mL, respectively. Flavonol was found in highest amount in the EAEEC fraction (81.52 $\pm$ 0.41 μ g QE/mL); this could be related to its higher capacity to extract medium polarity compounds like ferulic acid derivatives. Additionally, EAEEC has also the highest content of hydrolysable tannins (714.44 $\pm$ 2.41 μ g TAE/mL), showing the possible richness in ellagitannins as well. compounds known for their significant biological effects (Gülçin et al., 2011).

Table 1. Total phenolic, flavonoid, flavonol, and tannin contents of *E. creticum* extract

Phytochemical Parameters	Contents of <i>E. creticum</i> extracts $\pm$ SD		
	BEEC	EAEEC	CEEC
Total Polyphenols (μg GAE/mL)	350.37 $\pm$ 1.19	161.84 $\pm$ 0.68	2.14 $\pm$ 0.17
Total Flavonoids (μg QE/mL)	50.00 $\pm$ 2.06	38.05 $\pm$ 0.15	29.37 $\pm$ 0.29
Total Flavonols (μg QE/mL)	21.01 $\pm$ 0.08	81.52 $\pm$ 0.41	42.60 $\pm$ 0.73
Hydrolysable Tannins (μg TAE/mL)	122.50 $\pm$ 1.44	714.44 $\pm$ 2.41	219.17 $\pm$ 2.89
Condensed Tannins (μg CE/mL)	33.65 $\pm$ 3.06	41.69 $\pm$ 0.68	74.63 $\pm$ 1.36

Values were expressed as means $\pm$ SD (n=3).

Total phenolic compounds were expressed as μg gallic acid equivalent/ml (μg GAE/ml)

Total flavonoids contents were expressed as μg quercetin equivalent/ml (μg QE/ml)

Total flavonols contents were expressed as μg quercetin equivalent/ml (μg QE/ml)

Total hydrolysable tannins content was expressed as μg tannic acid equivalent/ml (μg TAE/ml)

Total condensed tannins content was expressed as μg catechin equivalent/ml (μg CE/ml).

The significant condensed tannins content in the CEEC extract (74.63 $\pm$ 1.36 μg CE/mL) despite relatively low polyphenol level, indicates a selective solubility, with the possibility of enrichment in some proanthocyanidins. These results are in line with the phytochemical profiles reported in other *Echium* species like *Echium amoenum* and *Echium italicum* that contain high amounts of polyphenols, including rosmarinic, protocatechuic and gentisic acids, played a major role on these compounds their known antioxidant and therapeutic activities (Bošković et al., 2017).

ANOVA followed by Tukey's HSD test revealed highly significant differences ($p < 0.001$) among the extracts. BEEC contained the highest total phenolic content (350.3 $\pm$ 0.7 mg GAE/g DW), significantly greater than both EAEEC (161.7 $\pm$ 0.3 mg GAE/g DW) and CEEC (2.14 $\pm$ 0.16 mg GAE/g DW). By contrast, EAEEC presented the highest flavonol concentration (81.6 $\pm$ 0.2 mg QE/g DW), while BEEC was richer in total flavonoids (50.0 $\pm$ 1.5 mg QE/g DW). Hydrolysable tannins were particularly abundant in EAEEC (714.4 $\pm$ 1.7 mg TAE/g DW), followed by CEEC (219.2 $\pm$ 2.9 mg TAE/g DW) and BEEC (122.8 $\pm$ 0.9 mg TAE/g DW). CEEC was distinguished by its elevated condensed tannins (74.6 $\pm$ 1.3 mg CE/g DW), significantly higher than EAEEC (41.5 $\pm$ 0.3 mg CE/g DW) and BEEC (33.6 $\pm$ 2.3 mg CE/g DW).

3.2 LC-MS/MS Profiling of *E. creticum* Extracts

Secondary metabolite profiling LC-MS/MS analysis of the *E. creticum* extracts displayed a high diversity featured rich with secondary metabolites with 24, 22 and 25 compounds in BEEC, EAEEC and CEEC, respectively (Table 2, Figure 7).

Butanolic Extract (BEEC)

The most abundant compound in BEEC was rosmarinic acid (21.658 mg/g), a well-known ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. Rosmarinic acid has been extensively reported for its strong antioxidant, anti-inflammatory, and neuroprotective effects, making it a crucial contributor to the antioxidant and anti-Alzheimer properties of this extract (Topçu & Kuşman, 2014). Moreover,

Table 2. Number of LC-MS/MS identified compounds in *E. creticum* extracts

Extract type	Number of compounds identified (LC-MS/MS)
Butanolic Extract (BEEC)	24
Ethyl Acetate Extract (EAEEC)	22
Chloroform Extract (CEEC)	25

its capacity to inhibit acetylcholinesterase and scavenge reactive oxygen species (ROS) has been demonstrated in both *in vitro* and *in vivo* models (Bursal et al., 2020).

Protocatechuic acid (3.185 mg/g) and gentisic acid (1.403 mg/g), two dihydroxybenzoic acid derivatives, were also identified. These compounds are well documented for their free radical scavenging, anti-inflammatory, and enzyme inhibitory capacities, including α -amylase and cholinesterase inhibition, thus potentially contributing to the extract's antidiabetic and neuroprotective activities (Zhang et al., 2021b).

The identification of salicylic acid (2.474 mg/g), known for its anti-inflammatory and antimicrobial roles, aligns with the biological outcomes observed in BEEC. The presence of cymaroside (1.3 mg/g), a flavonoid glycoside with reported antioxidant and enzyme inhibitory properties (Güven et al., 2023), further supports its multipotent bioactivity.

Ethyl Acetate Extract (EAEEC)

The predominant compound in EAEEC was ferulic acid (6.752 mg/g), a hydroxycinnamic acid derivative recognized for its antioxidant, antidiabetic, and anti-aging properties (Zduńska et al., 2018). Ferulic acid has shown promising AChE and BChE inhibition, as well as capacity to modulate glucose metabolism pathways, which may explain the favorable neuroprotective and antidiabetic effects recorded in this extract.

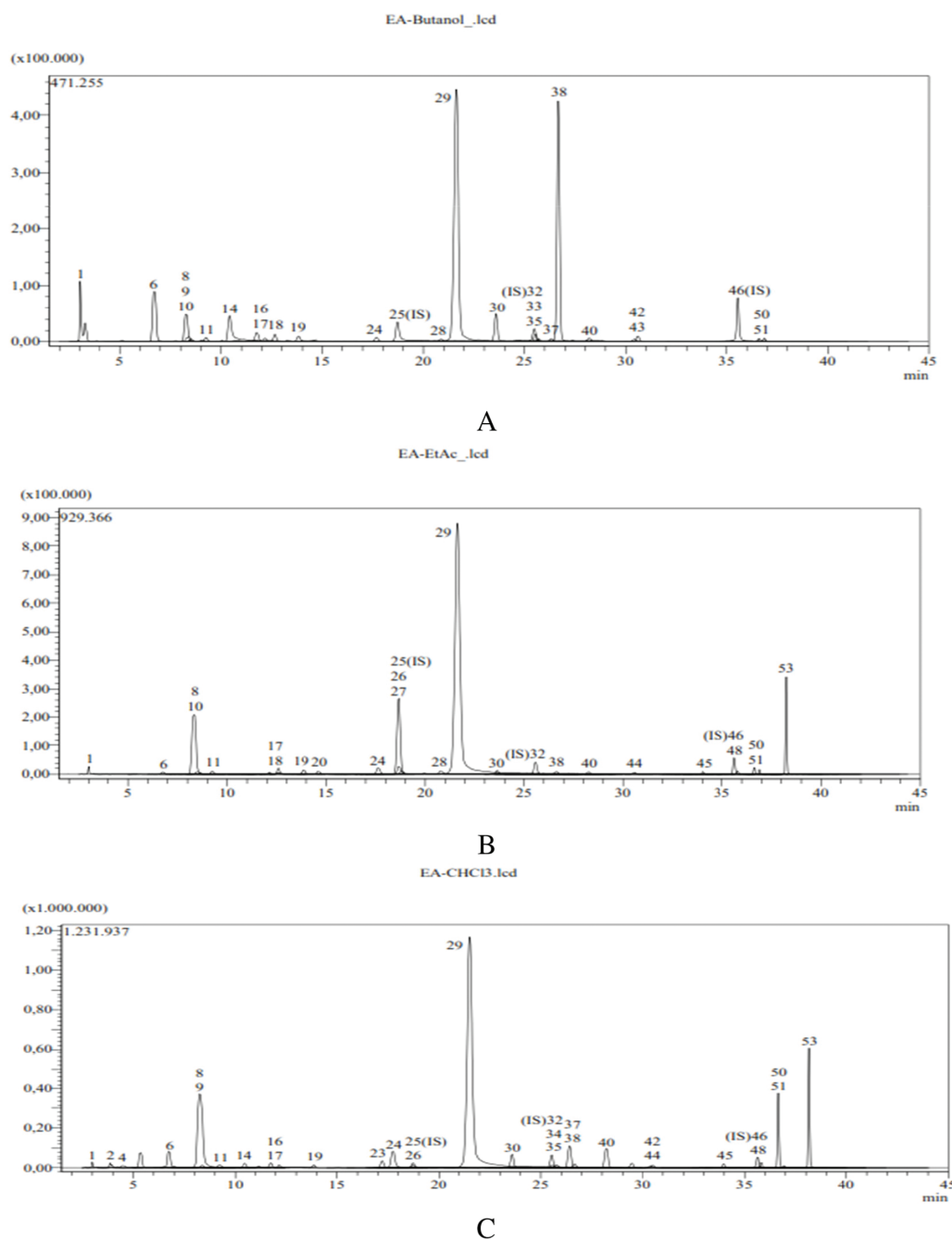


Figure 7. LC-MS/MS total ion chromatograms of **A.** Butanolic extract of *E. creticum* species **B.** Ethyl acetate extract of *E. creticum* species **C.** Chloroformic extract of *E. creticum* species

Salicylic acid (2.867 mg/g), also present in EAEEC, reinforces the antimicrobial and anti-inflammatory potential of this extract.

Chloroform Extract (CEEC)

The major compound in the CEEC was salicylic acid (7.814 mg/g), which, along with *p*-coumaric acid (3.29 mg/g), contributes to the extract's antimicrobial and antioxidant effects and known for their antimicrobial and antioxidant effects, particularly through microbial cell membrane disruption and oxidative stress mitigation. *p*-Coumaric acid is known to modulate oxidative stress and has also shown inhibitory

activity against AChE and α -amylase enzymes (Khan et al., 2022).

Additional compounds in CEEC included protocatechuic acid (2.744 mg/g), gentisic acid (2.47 mg/g), and cosmosiin (2.87 mg/g). Cosmosiin (also known as apigenin-7-O-glucoside) is a flavonoid glycoside known for its neuroprotective, anti-inflammatory, and enzyme inhibitory activities (Mohammad Khanizadeh et al., 2025). The presence of cynaroside (1.899 mg/g) and 4-hydroxybenzoic acid (1.812 mg/g) further supports the observed bioactivities.

Interestingly, quinic acid (1.078 mg/g) was also detected in CEEC. Quinic acid is a precursor of several phenolic acids and has demonstrated significant antioxidant and antidiabetic potential in various studies (Arya et al., 2014). Its presence may explain the residual biological activity in CEEC despite its lower polyphenol content.

The distribution of phenolic acids across solvent fractions was largely consistent with polarity expectations, as major compounds such as rosmarinic and ferulic acids accumulated in the ethyl acetate fraction (EAEEC), while flavonoid glycosides were more enriched in the *n*-butanol fraction (BEEC). Minor amounts of phenolic acids were also detected in the chloroform extract (CEEC), which can be attributed to their intermediate polarity and partial co-extraction during sequential fractionation. Such cross-distribution of phenolics into less polar fractions has been previously reported in phytochemical studies, reflecting solvent affinity and matrix effects inherent to complex plant extracts (Durling et al., 2007).

The identification of phenolic compounds in the extracts was based on comparison with 56 authenticated reference standards covering major phenolic subclasses, ensuring accurate confirmation of the LC–MS/MS results (see Table 3 for details).

To better contextualize our LC–MS/MS results, we compared the major compounds identified in *E. creticum* with previous reports from other *Echium* species. As shown in Table 4, several phenolic acids such as rosmarinic, ferulic, *p*-coumaric, salicylic acids, and quercetin derivatives have already been reported in *E. amoenum*, *E. vulgare*, and *E. italicum* (Mehrabani et al., 2005; Dresler et al., 2017; Jin et al., 2020). Their occurrence in *E. creticum* is therefore confirmatory. In contrast, cynaroside (luteolin-7-O-glucoside), although newly detected in *E. creticum*, has previously been documented in *E. arenarium* (Kefi et al., 2018); thus, it represents a species-level novelty rather than a genus-level one. Importantly, our profiling also revealed several flavonoid glycosides, including cosmosiin (apigenin-7-O-glucoside), nicotiflorin (kaempferol-3-O-rutinoside), and genistin, which, to the best of our knowledge, have not been reported previously in the *Echium* genus (Sheydaei et al., 2025). These findings expand the phytochemical diversity of *Echium* and reinforce the novelty of our results at both the species (first LC–MS/MS profile of *E. creticum*) and genus levels (new glycoside records).

The biological activities in many ways could be attributed to such a complex phytochemical formula, and also demonstrate the polarity-based extraction efficiency of each solvent.

Rosmarinic acid (21.66 mg/g) was the most abundant compound in BEEC, among the main compounds found, followed by 4-hydroxybenzoic acid (7.53 mg/g), protocatechuic acid (3.18 mg/g), and salicylic acid (2.47 mg/g). Rosmarinic acid is a potent polyphenol known for its antioxidant, anti-inflammatory, and neuroprotective properties (Asghari et al., 2019). Its majority composition present in BEEC probably gives total strong antioxidant activity comparing along with DPPH, ABTS and phenanthroline assays (see 3.3). Analogous to related species such as *E. plantagineum*, rosmarinic

acid has also been described there as the main bioactive marker with strong antioxidant power (Sousa et al., 2020). The total phenolic content of the EAEEC extract was less than for BEEC, however it was featured by significant content of ferulic acid (6.75 mg/g) and salicylic acid (2.87 mg/g). Ferulic acid and its derivatives have showed antioxidant, anticancer and anti-inflammatory efforts among others, both antioxidant and enzyme inhibitory activities, particularly in relation to α -amylase and cholinesterase enzymes (Zheng et al., 2020). This might shed light on the moderate antidiabetic and anti-Alzheimer effects of EAEEC when compared to BEEC (see Sections 3.3.2 and 3.3.3). Ferulic acid has also been identified in extracts of *Echium vulgare*, playing a role in its noted biological effects (Bouzaiene et al., 2015).

CEEC showcased a richer phytochemical profile, featuring salicylic acid (7.81 mg/g), *p*-coumaric acid (3.29 mg/g), and cosmosiin (2.87 mg/g) as its key components. Although this extract had the lowest overall polyphenol content, the presence of these medium polarity phenolics hints at possible antimicrobial and neuroprotective benefits. Cosmosiin, a glycosylated flavonoid has been known to inhibit acetylcholinesterase which fits with its limited anti-Alzheimer potential (Olennikov et al., 2017). Also, protocatechuic acid and gentisic acid, which are both in CEEC, are recognized to scavenge radicals and enhance antioxidant mechanisms by donating hydrogen (Skoza et al., 2022). It's interesting to note that cynaroside was found in all three extracts, though in varying amounts (1.3 mg/g in BEEC and 1.899 mg/g in CEEC). This flavonoid glycoside has been previously identified in the *Echium* genus and is known to help with antioxidant and hypoglycemic effects by influencing glucose uptake and reactive oxygen species (ROS) scavenging pathways (Jlizi et al., 2022). The dominance of rosmarinic acid in the BEEC extract likely underlies its strong antioxidant performance across DPPH, ABTS, and phenanthroline assays. Rosmarinic acid has consistently been identified as a major antioxidant marker in related *Echium* species such as *E. amoenum*, *E. plantagineum*, and *E. russicum* (Asghari et al., 2019; Olennikov et al., 2017) presence of bioactive polyphenols like rosmarinic acid, ferulic acid, cosmosiin, and salicylic acid seem to be key factors defining the antioxidant, antidiabetic, and anti-Alzheimer potential of *E. creticum* extracts. These findings also confirm previous studies in the *Echium* genus pertaining a potential therapeutic value to the phenolic richness (Sousa et al., 2020). Most common phenolic compounds found in the extracts of *E. creticum* are presented in Figure 8.

3.3 Biological Activities

3.3.1 Antioxidant Activity

The antioxidant potential of the *E. creticum* extracts was evaluated using four different *in vitro* assays: DPPH, ABTS, FRAP, and Fe^{3+} -phenanthroline methods. These methods assess the free radical scavenging ability and reducing power of the samples, which represent distinct antioxidant mechanisms. The findings are summarized in Table 5.

Table 3. LC–MS/MS profiling of phenolic compounds in *E. creticum* extracts (BEEC, EAEEC, CEEC)

No	Analytes	RT ^a	M.I. (m/z) ^b	F.I. (m/z) ^c	Ion. mode	r ^{2d}	RSD% ^e	U ^g	BEEC	EAEEC	CEEC
Quantification (mg analyte/g extract)											
1	Quinic acid	3.0	190.8	93.0	Neg	0.996	0.69	0.0372	0,048	0,714	1,078
2	Fumaric acid	3.9	115.2	40.9	Neg	0.995	1.05	0.0091	N.D.	N.D.	0,432
3	Aconitic acid	4.0	172.8	129.0	Neg	0.971	2.07	0.0247	N.D.	N.D.	N.D.
4	Gallic acid	4.4	168.8	79.0	Neg	0.999	1.60	0.0112	N.D.	N.D.	0,037
5	Epigallocatechin	6.7	304.8	219.0	Neg	0.998	1.22	0.0184	N.D.	N.D.	N.D.
6	Protocatechuic acid	6.8	152.8	108.0	Neg	0.957	1.43	0.0350	3,185	0,087	2,744
7	Catechin	7.4	288.8	203.1	Neg	0.999	2.14	0.0221	N.D.	N.D.	N.D.
8	Gentisic acid	8.3	152.8	109.0	Neg	0.997	1.81	0.0167	1,403	0,232	2,470
9	Chlorogenic acid	8.4	353.0	85.0	Neg	0.995	2.15	0.0213	0,238	N.D.	0,042
10	Protocatechuic aldehyde	8.5	137.2	92.0	Neg	0.996	2.08	0.0396	0,080	0,020	N.D.
11	Tannic acid	9.2	182.8	78.0	Neg	0.999	2.40	0.0190	0,047	0,030	0,185
12	Epigallocatechin gallate	9.4	457.0	305.1	Neg	0.999	1.30	0.0147	N.D.	N.D.	N.D.
13	Cynarin	9.8	515.0	191.0	Neg	0.999	2.42	0.0306	N.D.	N.D.	N.D.
14	4-OH Benzoic acid	10.5	137.2	65.0	Neg	0.999	1.24	0.0237	7,535	N.D.	1,812
15	Epicatechin	11.6	289.0	203.0	Neg	0.996	1.47	0.0221	N.D.	N.D.	N.D.
16	Vanillic acid	11.8	166.8	108.0	Neg	0.999	1.92	0.0145	0,607	N.D.	0,524
17	Caffeic acid	12.1	179.0	134.0	Neg	0.999	1.11	0.0152	0,103	0,016	0,150
18	Syringic acid	12.6	196.8	166.9	Neg	0.998	1.18	0.0129	0,795	0,393	N.D.
19	Vanillin	13.9	153.1	125.0	Poz	0.996	1.10	0.0122	0,042	0,148	0,094
20	Syringic aldehyde	14.6	181.0	151.1	Neg	0.999	2.51	0.0215	N.D.	0,045	N.D.
21	Daidzin	15.2	417.1	199.0	Poz	0.996	2.25	0.0202	N.D.	N.D.	N.D.
22	Epicatechin gallate	15.5	441.0	289.0	Neg	0.997	1.63	0.0229	N.D.	N.D.	N.D.
23	Piceid	17.2	391.0	135/106.9	Poz	0.999	1.94	0.0199	N.D.	N.D.	0,015
24	p-Coumaric acid	17.8	163.0	93.0	Neg	0.999	1.92	0.0194	0,158	0,854	3,290
25	Ferulic acid-D3-ISH	18.8	196.2	152.1	Neg	N.A.	N.A.	0.0170	N.A.	N.A.	N.A.
26	Ferulic acid	18.8	192.8	149.0	Neg	0.999	1.44	0.0181	N.D.	6,752	0,996
27	Sinapic acid	18.9	222.8	193.0	Neg	0.999	1.45	0.0317	N.D.	0,500	N.D.
28	Coumarin	20.9	146.9	103.1	Poz	0.999	2.11	0.0383	0,025	0,058	N.D.
29	Salicylic acid	21.8	137.2	65.0	Neg	0.999	1.48	0.0158	2,474	5,867	7,814
30	Cynaroside	23.7	447.0	284.0	Neg	0.997	1.56	0.0366	1,300	0,036	1,899
31	Miquelianin	24.1	477.0	150.9	Neg	0.999	1.31	0.0220	N.D.	N.D.	N.D.
32	Rutin-D3-IS	25.5	612.2	304.1	Neg	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
33	Rutin	25.6	608.9	301.0	Neg	0.999	1.38	0.0247	0,174	N.D.	N.D.
34	isoquercitrin	25.6	463.0	271.0	Neg	0.998	2.13	0.0220	N.D.	N.D.	0,017
35	Hesperidin	25.8	611.2	449.0	Poz	0.999	1.84	0.0335	0,247	N.D.	0,011
36	o-Coumaric acid	26.1	162.8	93.0	Neg	0.999	2.11	0.0147	N.D.	N.D.	N.D.
37	Genistin	26.3	431.0	239.0	Neg	0.991	2.01	0.0083	0,008	N.D.	0,147
38	Rosmarinic acid	26.6	359.0	197.0	Neg	0.999	1.24	0.0130	21,658	0,012	0,057
39	Ellagic acid	27.6	301.0	284.0	Neg	0.999	1.57	0.0364	N.D.	N.D.	N.D.
40	Cosmosiin	28.2	431.0	269.0	Neg	0.998	1.65	0.0083	0,108	0,066	2,870
41	Quercitrin	29.8	447.0	301.0	Neg	0.999	2.24	0.0268	N.D.	N.D.	N.D.
42	Astragalin	30.4	447.0	255.0	Neg	0.999	2.08	0.0114	0,024	N.D.	0,496
43	Nicotiflorin	30.6	592.9	255.0/284.0	Neg	0.999	1.48	0.0108	0,416	N.D.	N.D.
44	Fisetin	30.6	285.0	163.0	Neg	0.999	1.75	0.0231	N.D.	0,062	0,040
45	Daidzein	34.0	253.0	223.0	Neg	0.999	2.18	0.0370	N.D.	0,041	0,031
46	Quercetin-D3-IS	35.6	304.0	275.9	Neg	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
47	Quercetin	35.7	301.0	272.9	Neg	0.999	1.89	0.0175	N.D.	N.D.	N.D.
48	Naringenin	35.9	270.9	119.0	Neg	0.999	2.34	0.0392	N.D.	0,044	0,016
49	Hesperetin	36.7	301.0	136.0/286.0	Neg	0.999	2.47	0.0321	N.D.	N.D.	N.D.
50	Luteolin	36.7	284.8	151.0/175.0	Neg	0.999	1.67	0.0313	0,005	0,041	0,966
51	Genistein	36.9	269.0	135.0	Neg	0.999	1.48	0.0337	0,003	0,012	0,049
52	Kaempferol	37.9	285.0	239.0	Neg	0.999	1.49	0.0212	N.D.	N.D.	N.D.
53	Apigenin	38.2	268.8	151.0/149.0	Neg	0.998	1.17	0.0178	N.D.	0,552	0,986
54	Amentoflavone	39.7	537.0	417.0	Neg	0.992	1.35	0.0340	N.D.	N.D.	N.D.
55	Chrysin	40.5	252.8	145.0/119.0	Neg	0.999	1.46	0.0323	N.D.	N.D.	N.D.
56	Acacetin	40.7	283.0	239.0	Neg	0.997	1.67	0.0363	N.D.	N.D.	N.D.

N.D.: Not detected, N.A.: Not Applicable ^aR.T.: Retention time, ^bMI (m/z): Molecular ions of the standard analytes (m/z ratio), ^cFI (m/z): Fragment ions ^dr²: Coefficient of determination, ^eRSD: Relative standard deviation, ^gU (%): percent relative uncertainty at 95% confidence level ($k = 2$).

The ethyl acetate extract (EAEEC) showed the highest DPPH and ABTS scavenging activities with IC₅₀ = 31.56 ± 0.53 µg/mL and 20.52 ± 1.77 µg/mL, respectively.

Antioxidant assays confirmed these compositional differences. EAEEC displayed the strongest radical scavenging activities with the lowest IC₅₀ values for DPPH (31.6 ±

0.5 µg/mL) and ABTS (20.5 ± 1.8 µg/mL), significantly outperforming CEEC and BEEC ($p < 0.001$). BEEC demonstrated the highest ferric reducing antioxidant power (115.6 ± 1.4 µmol Fe²⁺/g DW), while CEEC showed poor activity (>200 µmol Fe²⁺/g DW). For the Fe³⁺-phenanthroline assay, CEEC exhibited the strongest reducing power (36.5

Table 4. Comparative occurrence of selected phenolic compounds in *Echium* species

Compound	Previously reported in <i>Echium</i> species	References	Reported for <i>Echium</i> genus
Rosmarinic acid	<i>E. amoenum</i> (major), <i>E. vulgare</i>	Mehrabani et al. (2005), Dresler et al. (2017)	Confirmatory
Ferulic acid	<i>E. vulgare</i> , <i>E. italicum</i>	Mehrabani et al. (2005), Albrecht et al. (2009)	Confirmatory
<i>p</i> -Coumaric acid	<i>E. vulgare</i> , <i>E. arenarium</i>	Mehrabani et al. (2005), Kefi et al. (2018)	Confirmatory
Salicylic acid	<i>E. vulgare</i> (minor)	Dresler et al. (2017)	Confirmatory
Rutin	<i>E. vulgare</i> , <i>E. italicum</i>	Mehrabani et al. (2005), Albrecht et al. (2009)	Confirmatory
Gentisic acid	Minor phenolic in <i>E. vulgare</i>	Dresler et al. (2017)	Confirmatory
Vanillic acid	<i>E. italicum</i>	Albrecht et al. (2009)	Confirmatory
Cynaroside (Luteolin-7- <i>O</i> -glucoside)	<i>E. arenarium</i> (species-level novelty)	Kefi et al. (2018)	Confirmatory
Quinic acid	Not previously reported in <i>Echium</i>	Sheydaei et al. (2025)	Genus-level novelty
Cosmosiin (Apigenin-7- <i>O</i> -glucoside)	Rare in Boraginaceae, not reported in <i>Echium</i>	Sheydaei et al. (2025)	Genus-level novelty
Genistin	Not previously reported in <i>Echium</i>	Sheydaei et al. (2025)	Genus-level novelty
Nicotiflorin (Kaempferol-3- <i>O</i> -rutinoside)	Not previously reported in <i>Echium</i>	Sheydaei et al. (2025)	Genus-level novelty

Table 5. Antioxidant activities of *Echium creticum* extracts (Mean $\pm$ SD, n = 3)

Sample	IC ₅₀ /A _{0.5} $\pm$ SD (μ g/mL) values			
	DPPH ^c	ABTS ^c	FRAP ^d	Phenanthroline ^d
BEEC	41.54 $\pm$ 1.17	23.62 $\pm$ 1.53	115.57 $\pm$ 1.36	11.89 $\pm$ 1.94
EAEEC	31.56 $\pm$ 0.53	20.52 $\pm$ 1.77	92.38 $\pm$ 1.44	3.78 $\pm$ 0.74
CEEC	42.92 $\pm$ 1.12	62.78 $\pm$ 1.53	>200	36.51 $\pm$ 3.40
BHT ^e	1.29 $\pm$ 0.30	6.14 $\pm$ 0.41	–	2.24 $\pm$ 0.17
BHA ^e	1.81 $\pm$ 0.10	1.29 $\pm$ 0.41	–	0.93 $\pm$ 0.07
α -Tocopherol ^e	–	13.02 $\pm$ 5.17	34.93 $\pm$ 2.38	–
Ascorbic Acid ^e	–	–	6.77 $\pm$ 1.15	–

Values expressed are means $\pm$ S.D. of three parallel measurements

^cValues were given as IC₅₀ for DPPH free and ABTS cation radical scavenging activities

^dValues were given as A_{0.5} for Reducing power and Phenanthroline activity

^eStandard compounds: BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene, α -Tocopherol, Ascorbic acid.

$\pm$ 3.4 μ mol Fe²⁺/g DW), followed by BEEC (11.9 $\pm$ 1.9 μ mol Fe²⁺/g DW) and EAEEC (3.8 $\pm$ 0.7 μ mol Fe²⁺/g DW). All differences were statistically significant (ANOVA, p < 0.001).

The butanolic extract (BEEC) and chloroform extract (CEEC) were outperformed by the ethyl acetate extract (EAEEC). This aligns with the notable amounts of ferulic

acid (6.75 mg/g) and salicylic acid (2.87 mg/g) found in the EAEEC. Ferulic acid is a well-regarded antioxidant that helps stabilize free radicals and boosts the activity of our body's own antioxidant enzymes (Kikuzaki et al., 2002). Salicylic acid also Likely decreases oxidative stress and will likely work together with ferulic acid to enhance it ability as a radical scavenger (Skrypnik et al., 2022).

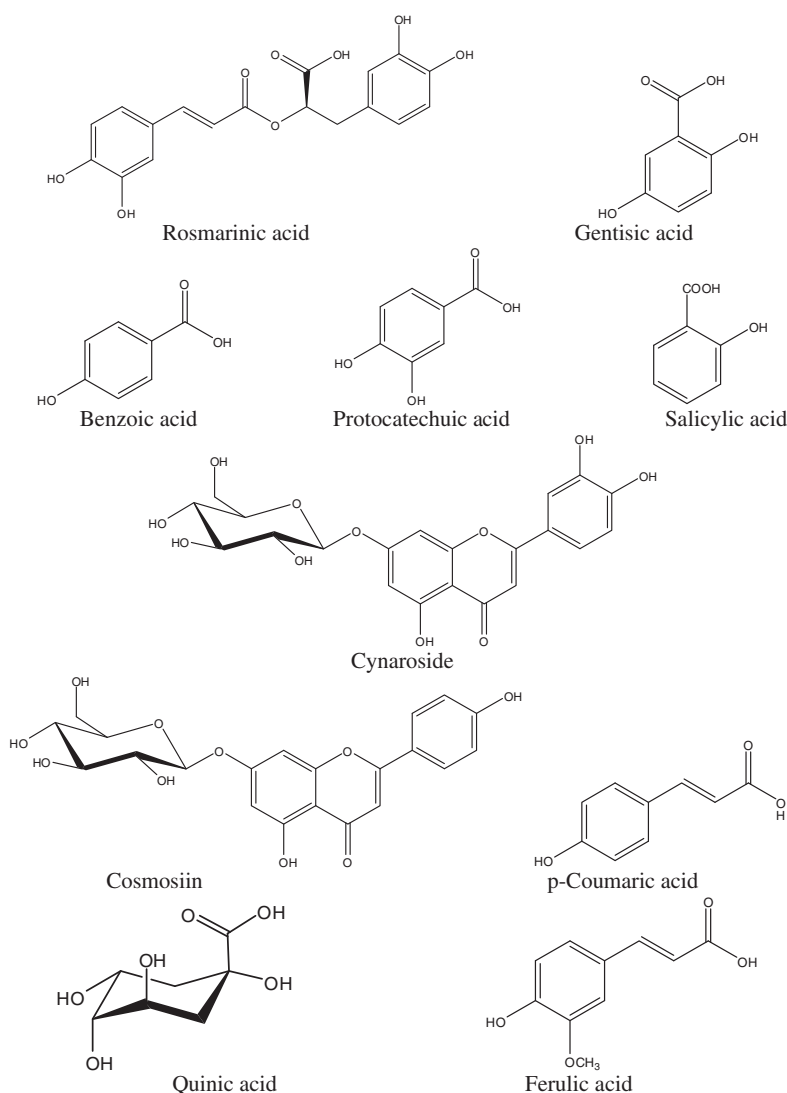


Figure 8. Most common phenolic compounds found in the extracts of *E. creticum*

The butanolic extract could possibly have an exceedingly high total polyphenol content ($350.37 \pm 1.19 \mu\text{g GAE/mL}$), but when tested, had only moderate antioxidant activity (DPPH $\text{IC}_{50} = 41.54 \pm 1.17 \mu\text{g/mL}$, ABTS $\text{IC}_{50} = 23.62 \pm 1.53 \mu\text{g/mL}$). But the moderate performance could be a result of the high presence of rosmarinic acid (21.66 mg/g) and protocatechuic acid (3.18 mg/g) which are both noted radical scavengers. Rosmarinic acid is especially renowned for its substantial antioxidant action, blocking lipid peroxidation and reducing the ability of reactive oxygen species (ROS) (Asghari et al., 2019). Protocatechuic acid has also been demonstrated to successfully scavenge hydroxyl and superoxide radicals (Li et al., 2011).

In a surprising twist, the chloroform extract (CEEC) stands out for having the highest number of identified compounds 25 in total but it actually showed the weakest DPPH ($\text{IC}_{50} = 42.92 \pm 1.12 \mu\text{g/mL}$) and ABTS ($\text{IC}_{50} = 62.78 \pm 1.53 \mu\text{g/mL}$) activities. This might be explained by the extract low total phenolic content ($2.14 \pm 0.17 \mu\text{g GAE/mL}$), hinting that the antioxidant activity is likely

driven by specific compounds like salicylic acid (7.81 mg/g), *p*-coumaric acid (3.29 mg/g), and cosmosiin (2.87 mg/g), which all have moderate antioxidant properties. Despite the relatively high compound numbers in CEEC, this did not lead to high antioxidant activity as a result of low total phenolic content. What seems more likely is that medium-polarity phenolics i.e. (salicylic acid, *p*-coumaric acid, and cosmosiin) contributed to the associated weak DPPH and ABTS activity, although these compounds exhibited moderate radical-scavenging and cholinesterase-inhibitory activity. It was not unexpected that CEEC had weak DPPH and ABTS activity given this previous literature that showed individual phenolics had more modest effects, while strong activity was more likely when total polyphenols were at higher levels (Lacko-Bartošová et al., 2023; Koyuncu et al., 2018).

The FRAP assay established the reducing capacity of EAEEC at $A_{0.5} = 92.38 \pm 1.44 \mu\text{g/mL}$, which was significantly higher than BEEC ($115.57 \pm 1.36 \mu\text{g/mL}$), while CEEC was again not an effective reducer, having an $A_{0.5} > 200 \mu\text{g/mL}$.

Overall, the trends from FRAP were the same in the phenanthroline assays, where the $A_{0.5}$ value achieved in EAEEC was again the lowest at $3.78 \pm 0.74 \mu\text{g/mL}$, further verifying its excellent electron-donating capacity. The exceptional performance of EAEEC in both reducing power assays was positively correlates with its flavonol and hydrolysable tannin content (Ali et al., 2006).

These results align well with results found in other species belonging to the genus *Echium*. For example, secreted rosmarinic acid and chlorogenic acid, in addition to antioxidants compounds, were assessed as the major contributors of antioxidant effects in *Echium vulgare* (Alsanie et al., 2018; Bošković et al., 2022). Likewise, a higher overall phenolic content in *Echium amoenum* was associated with an increased radical scavenging and reducing activity (Asghari et al., 2019).

In conclusion, the antioxidant ability of the extracts of *E. creticum* is likely due to the presence of rosmarinic acid, ferulic acid, protocatechuic acid, and tannins working together through hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms to scavenge free radicals and reduce oxidative state. The overall characteristics of each extract were clarified with the superior activity of the ethyl acetate extract in all assays which highlighted the importance of compound struggle with viability and separation, as well as extracts content, to a reduced total phenolic load alone.

3.3.2 Antidiabetic Activity α -Amylase Inhibition Assay

The α -amylase inhibitory activity of the extracts of *E. creticum* was assessed to gauge their antidiabetic ability. Out of the three tested fractions, ethyl acetate extract (EAEEC) had the highest activity inhibition potential with an IC_{50} value of $207.20 \pm 2.58 \mu\text{g/mL}$, while the chloroform extract (CEEC) was next with an IC_{50} of $213.18 \pm 1.37 \mu\text{g/mL}$. The butanolic extract (BEEC) did have inhibition, but was very poor, with an IC_{50} of $>400 \mu\text{g/mL}$. This showed that both the EAEEC and CEEC fractions contain bioactive compounds which can potentially alter carbohydrate metabolism via inhibition of α -amylase.

The α -amylase observed activity especially with the EAEEC extract has likely to be an effect not solely due to one component but even with the LC-MS/MS profile, one of the main components ferulic acid was isolated. Ferulic acid has been found to have inhibitory actions on α -amylase and may act to control glycemic levels in a variety of ways, namely through antioxidant properties and enzyme related interactions (Babich et al., 2023). Compared to the standards quercetin and Acarbose, which had the IC_{50} values of $239.54 \pm 5.21 \mu\text{g/mL}$ and IC_{50} of $260.32 \pm 1.15 \mu\text{g/mL}$ respectively for both extract fractions. The results support the potential medicinal use of *E. creticum* and polyphenolic analysis for antidiabetic use (Babich et al., 2023; Meng et al., 2016).

The variation in α -amylase inhibition among the extracts can be attributed to their distinct phytochemical profiles. LC-MS/MS analysis indicated that ferulic acid (6.75 mg/g) and salicylic acid (2.74 mg/g), was high in EAEEC while CEEC contained protocatechuic acid, *p*-coumaric acid (3.29 mg/g), which have shown α -amylase inhibitory effects according to

the literature (Khan et al., 2022). Mainly, ferulic acid has been shown to alter postprandial glucose concentration by inhibition of α -amylase and α -glucosidase enzyme agonists. In addition, protocatechuic acid and *p*-coumaric acid exert antidiabetic effects by regulating glucose metabolism and oxidative stress (Li et al., 2022). Due to this, rosmarinic acid could still provide systemic effects of antioxidants. The synergistic interaction of polyphenols such as flavonoids, phenolic acids and tannins, could also have an effect on the inhibitory activity. The amount of bioactivity with the EAEEC extract may be due to the high levels of hydrolysable tannins ($714.44 \pm 2.41 \mu\text{g TAE/mL}$) which could produce complexes either by enzyme or with substrate that prevented the hydrolytic process (Kim et al., 2018).

Overall, these results highlight the promising antidiabetic potential of EAEEC and CEEC extracts, attributable to their rich phenolic content especially ferulic, *p*-coumaric, and protocatechuic acids which collectively modulate α -amylase activity (Table 6).

Enzyme inhibition assays also showed significant differences between extracts ($p < 0.001$). CEEC and EAEEC displayed comparable α -amylase inhibitory activities (213.2 ± 1.4 and $207.2 \pm 2.6 \mu\text{g/mL}$, respectively), while BEEC was inactive at the tested concentrations ($>400 \mu\text{g/mL}$).

Table 6. Inhibitory effects of *E. creticum* extracts and standard compounds on α -amylase activity (IC_{50} , $\mu\text{g/mL}$)

Sample/Standard	Alpha amylase ^a IC_{50} ($\mu\text{g/mL}$) $\pm$ SD
BEEC (<i>n</i> -Butanol)	>400
EAEEC (Ethyl Acetate)	207.20 ± 2.58
CEEC (Chloroform)	213.18 ± 1.37
Quercetin ^e	239.54 ± 5.21
Acarbose ^e	260 ± 1.15

^aValues were given as IC_{50} for alpha amylase inhibition assay

^eStandard compounds: Quercetin, Galantamine.

Table 7. AChE and BChE inhibitory activities of *E. creticum* extracts (IC_{50} $\mu\text{g/mL}$).

Sample	Inhibition (IC_{50} $\mu\text{g/mL}$) $\pm$ SD	
	AChE ^b	BChE ^f
Butanolic (BEEC)	20.72 ± 2.01	41.22 ± 3.41
Ethyl Acetate (EAEEC)	32.40 ± 1.18	87.36 ± 4.32
Chloroform (CEEC)	48.33 ± 3.66	91.59 ± 2.32
Galantamine ^e	5.27 ± 1.15	50.80 ± 2.21

^bValues were given as IC_{50} for acetylcholinesterase inhibition assay

^fValues were given as IC_{50} for butylcholinesterase inhibition assay

^eStandard compound: Galantamine.

3.3.3 Anti-Alzheimer Activity AChE (Acetylcholinesterase) and BChE (Butyrylcholinesterase) Inhibition Tests

Alzheimer disease (AD) is primarily associated with cholinergic dysfunction, particularly the degradation of acetylcholine by acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Inhibiting those enzymes would be an interesting therapeutic way to effectively manage alzheimer (Orhan et al., 2011). For this study all *E. creticum* extracts inhibited AChE and BChE at variable levels (Table 7).

For cholinesterase inhibition, BEEC exhibited the strongest AChE inhibition (20.7 ± 2.0 $\mu\text{g/mL}$), significantly more potent than CEEC (48.3 ± 3.7 $\mu\text{g/mL}$) and EAEEC (32.4 ± 1.2 $\mu\text{g/mL}$). Conversely, BChE inhibition was more pronounced in CEEC (91.6 ± 2.3 $\mu\text{g/mL}$) and EAEEC (87.4 ± 4.3 $\mu\text{g/mL}$) compared to BEEC (41.2 ± 3.5 $\mu\text{g/mL}$).

In establishing AChE inhibition both the butanol extract (BEEC), $\text{IC}_{50} = 20.72 \pm 2.01$ $\mu\text{g/mL}$, and ethyl acetate extract (EAEEC), $\text{IC}_{50} = 32.40 \pm 1.18$ $\mu\text{g/mL}$, demonstrated much greater potency than the chloroform extract (CEEC), $\text{IC}_{50} = 48.33 \pm 3.66$ $\mu\text{g/mL}$.

A similar trend was observed for BChE inhibition, as the BEEC had substantially greater potency than the EAEEC and CEEC, (41.22 ± 3.41 $\mu\text{g/mL}$; 87.36 ± 4.32 $\mu\text{g/mL}$; and 91.59 ± 2.32 $\mu\text{g/mL}$, respectively).

The relative AChE and BChE inhibitory activity of the BEEC could be explained by its phytochemical composition. The LC-MS/MS phenolic profile of BEEC Nature revealed rosmarinic acid (21.66 mg/g), 4-hydroxybenzoic acid (7.53 mg/g), protocatechuic acid (3.18 mg/g), and finally salicylic acid (2.47 mg/g) as the major phenolics. Rosmarinic acid has been widely reported to demonstrate neuroprotective mechanisms including AChE inhibition, antioxidant activity, and protection in neuronal injury following oxidative stress (Yilmaz et al., 2017; Topal et al., 2020).

In the ethyl acetate extract (EAEEC), the predominant compounds were ferulic acid-D3 (6.75 mg/g) and salicylic acid (2.87 mg/g). Ferulic acid is a well-known neuroprotective that reduces neuroinflammation and modifies cholinergic neurotransmission (Lan et al., 2020). This perhaps partially contributes to the moderate enzyme inhibition potential of EAEEC. Salicylic acid, which was present in all three extracts, has been associated with neuroprotective effects from anti-inflammatory mechanisms, likely supports enzyme inhibition potentiation (Yeasmin & Choi, 2020).

Chloroform extract (CEEC) has the greatest number of total identified compounds (25), and, given that CEEC had the weakest inhibition, it does have cosmosiin (2.87 mg/g), cynaroside (1.89 mg/g) and *p*-coumaric acid (3.29 mg/g), all of which have been indicated to have potential to modulate targets relative to AD. More specifically, cosmosiin and cynaroside have been implicated in cholinesterase (Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE)) inhibition, but both also have some of their own antioxidant effects. At least they suggested these could act as multi-agent treatments. These compounds are known contributors to antioxidant, antimicrobial, and cholinesterase-inhibitory effects (Zhang et al., 2021a).

In summary, it appears that CEEC had at least moderate inhibition, and this could be due to having a relatively close amount of highly active individuals (for example, rosmarinic or ferulic acid). However, CEEC also appears to have an array of multiple potential combinatory or synergistic effects due to the multitude of flavonoids and phenolics in the CEEC that will need to be explored.

In conclusion, it appears that the neuroprotective potential of *E. creticum* extracts appears to be highly dependent on their phenolic composition. The cholinesterase inhibition appears to be contributed primarily by rosmarinic acid, ferulic acid, and cosmosiin. Parallel AChE and BChE inhibition activities by the butanolic and ethyl acetate extracts infer therapeutic relevance with managing symptoms related to AD by exposing additional cholinergic transmission and diminishing oxidative stress.

3.3.4 Antibacterial Activity

E. creticum L. extracts (BEEC, EAEEC and CEEC) were used to assess antibacterial activity against standard strains including *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 43300, and multidrug-resistant clinical isolates using the disc diffusion method at 128 g/mL and minimum inhibitory concentration. (Tables 8 and 9)

Chloroform extract (CEEC) exhibits a potent antibacterial effect against Gram-negative pathogens like *Pseudomonas analogues* such as *Pseudomonas aeruginosa* and *Salmonella heidelberg*, according to the disc diffusing method.

This activity is probably due to the high levels of salicylic acid (7.814 mg/g), *p*-coumaric acid (3.29 mg/g), and gentisic acids (2.47 mg/g) according to LC-MS/MS profiling. Many reports have shown phenolic acids to possess antimicrobial properties and have been linked to membrane disruption, nucleosynthesis inhibition, and the suppression of mbiotic enzyme systems (Takó et al., 2020; Borges et al., 2013).

BEEC, present a higher concentration of polyphenols (350.37 1.19 g GAE/mL), demonstrated moderate antibacterial activity. The presence of rosmarinic acid (21.658 mg/g) and protocatechuic acid (3.185 mg/g) as antimicrobial and antioxidant properties is likely to explain this (Ghasemzadeh Rahbardoar & Hosseinzadeh, 2020). Ethyl acetate extract (EAEEC) was found to possess mild amounts of ferulic acid (6.752 mg/g) and salicylic acid, which led to its selective antibacterial activity against *Staphylococcus aureus* isolates.

The MIC values further support these observations. CEEC showed the lowest MICs across most strains (≤ 64 $\mu\text{g/mL}$), while BEEC and EAEEC presented higher MIC values (>128 $\mu\text{g/mL}$ for some isolates), confirming the higher potency of CEEC.

The antibacterial profiles also correlated with the phytochemical distribution across extracts. Phenolic acids such as rosmarinic and ferulic acids, abundant in the EAEEC fraction, are likely contributors to the inhibition of Gram-negative strains (*E. coli*, *S. heidelberg*), while flavonoid glycosides present in BEEC may underlie its stronger activity against *S. aureus*. This pattern is consistent with earlier

Table 8. Inhibition zones (mm) of *E. creticum* extracts and reference antibiotics at 128 µg/mL

Microorganism	Inhibition zone (mm)				
	Antibiotics (10 µg /mL)		<i>E. creticum</i> Extracts (128 µg /mL)		
	Gentamicin ^a	Ampicillin ^a	BEEC	EAEEC	CEEC
Gram positive (+)					
<i>Staphylococcus aureus</i> ATCC 43300 ^b	22	28	30	22	18
<i>Staphylococcus aureus</i> ^c	20	24	26	16	–
Gram negative (–)					
<i>Escherichia coli</i> ATCC 25922 ^b	28	30	30	28	25
<i>Escherichia coli</i> ^c	14	26	20	22	–
<i>Pseudomonas aeruginosa</i> ATCC 27853 ^b	26	16	28	20	20
<i>Pseudomonas aeruginosa</i> ^c	14	12	18	–	–
<i>Klebsiella pneumoniae</i> ^c	26	–	20	–	–
<i>Morganella morganii</i> ^c	–	–	–	18	16
<i>Enterobacter aerogenes</i> ^c	12	–	17	–	–
<i>Salmonella heidelberg</i> ^c	–	28	26	18	–

^aControl, ^bObtained from the Pasteur Institute (Algiers); ^cClinical isolates from the laboratory of bacteriology (CHU Constantine, Algeria)

Table 9. Minimum inhibitory concentrations (MICs) of *E. creticum* Extracts (µg/mL)

Microorganism	MIC ^d (µg /mL)				
	Antibiotics		<i>E. creticum</i> Extracts		
	Gentamicin ^a	Ampicillin ^a	BEEC	EAEEC	CEEC
Gram positive (+)					
<i>Staphylococcus aureus</i> ATCC 43300 ^b	4	8	32	64	128
<i>Staphylococcus aureus</i> ^c	4	8	64	128	–
Gram negative (–)					
<i>Escherichia coli</i> ATCC 25922 ^b	4	8	32	32	64
<i>Escherichia coli</i> ^c	8	8	64	64	–
<i>Pseudomonas aeruginosa</i> ATCC 27853 ^b	4	16	64	128	128
<i>Pseudomonas aeruginosa</i> ^c	8	32	128	–	–
<i>Klebsiella pneumoniae</i> ^c	4	–	128	–	–
<i>Morganella morganii</i> ^c	–	–	–	128	128
<i>Enterobacter aerogenes</i> ^c	16	–	128	–	–
<i>Salmonella heidelberg</i> ^c	–	8	64	128	–

^aControl, ^bObtained from the Pasteur Institute (Algiers); ^cClinical isolates from the laboratory of bacteriology (CHU Constantine, Algeria)

reports linking phenolic acids and flavonoids to antibacterial action in *Echium* and related Boraginaceae species.

These findings are in agreement with earlier studies on the genus *Echium*. For instance, *Echium amoenum* extracts have demonstrated notable antibacterial effects linked to their rich phenolic composition, particularly rosmarinic and *p*-coumaric acids (Sabour et al., 2015). Moreover, salicylic acid, a major constituent in CEEC, has been repeatedly associated with antimicrobial activity against both Gram-positive and Gram-negative with antimicrobial activity (MIC 250–500 µg/mL) against Gram-positive and Gram-negative bacteria, highlighting its strong inherent bioactivity, supporting the results of the current study (Azizi et al., 2018).

These relationships between chemical composition and antibacterial efficacy were further explored through correlation and multivariate analyses, which confirmed distinct clustering of extracts according to their phytochemical content, antioxidant capacity, enzyme inhibitory potential, and antibacterial activity.

3.3.5 Correlation and PCA Analysis

Correlation analysis demonstrated strong associations between phytochemical composition and biological effects. Total phenolic content correlated negatively with IC₅₀ values of antioxidant assays, particularly ABTS ($r = -0.92$, $p < 0.001$) and DPPH ($r = -0.88$, $p < 0.001$), while flavonoid levels

showed a positive correlation with FRAP values ($r = 0.85$, $p < 0.001$). Interestingly, salicylic acid content correlated with antibacterial activity (MIC values), consistent with its known antimicrobial role. PCA further supported these findings, explaining 84.3% of the total variance with the first two components. PC1 (62.7%) was mainly driven by phenolics and antioxidant assays (DPPH, ABTS), whereas PC2 (21.6%) reflected enzyme inhibitory and antibacterial activities. The score plot clearly separated EAEEC (associated with high phenolics and antioxidant effects), BEEC (linked to AChE inhibition), and CEEC (characterized by condensed tannins, Fe^{3+} reducing power, and antibacterial/BChE activity). This multivariate approach highlights the distinct bioactive profiles of the three extracts.

In summary, correlation and PCA analyses confirmed that phenolic acids were the main contributors to antioxidant and antidiabetic effects, flavonoid glycosides were linked to cholinesterase inhibition, and tannins to both reducing power and antibacterial activity, thereby reinforcing the chemical basis of the observed bioactivities.

4 Conclusion

The current study offers the first integrated phytochemical and biological investigation of *Echium creticum* L. Sequential extraction and LC–MS/MS profiling revealed a diverse phenolic composition, with rosmarinic acid, ferulic acid, gentisic acid, and salicylic acid as dominant constituents, alongside several flavonoid glycosides newly reported in the *Echium* genus, which are well recognized for their antioxidant, enzyme inhibitory, and antimicrobial properties and correlate with the biological activities observed in the present work.

The ethyl acetate fraction (EAEEC) exhibited the highest radical scavenging and ferric-reducing power and displayed strong α -amylase inhibition in agreement with its richness in rosmarinic and ferulic acids, supporting a contribution of phenolic acids to the observed antidiabetic effect. The *n*-butanol fraction (BEEC), enriched in flavonoid glycosides, showed the most effective acetylcholinesterase inhibitor, suggesting a role for these compounds in the measured neuroprotective activity. Furthermore, all fractions exhibited measurable antibacterial effects, with MIC values (32 to 80 $\mu\text{g/mL}$), indicating that phenolic metabolites may also contribute to antimicrobial action. Altogether, these findings provide new phytochemical knowledge for *E. creticum* and establish, for the first time, direct associations between its chemical profile and multiple *in vitro* biological effects.

Multivariate analyses further reinforced these findings, showing that phenolic acids were the main drivers of antioxidant and α -amylase inhibition, flavonoid glycosides contributed to cholinesterase inhibition, and tannins were linked to both reducing power and antibacterial effects. These associations highlight the chemical basis of the distinct bioactivity patterns observed across the extracts.

In summary, this study not only confirms the occurrence of common phenolic acids such as rosmarinic, ferulic, and

salicylic acids in *E. creticum* but also introduces new records. Quinic acid, cosmosiin, nicotiflorin, and genistin represent novel findings at the genus level. These discoveries broaden the known phytochemical diversity of *Echium* and reinforce the originality of our work. However, limited to laboratory assays, this work represents an important step to understanding the phytochemical diversity and biological potential of this Mediterranean species, providing a basis for future isolation and *in vivo* validation studies.

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Author Contributions

I.L.: performed the experimental work, including extraction, biological assays, statistical evaluation, and manuscript preparation and revision. Z.K.: designed and supervised the study and reviewed the draft and final versions of the manuscript. N.B.: contributed to antioxidant and antidiabetic activity assays. A.L.: assisted in antibacterial and anti-Alzheimer activity assays. O.Ç.: conducted LC–MS/MS analyses and contributed to data validation and draft revision. M.A.Y.: participated in LC–MS/MS analyses, data interpretation, and the revision of the draft and final versions of the manuscript. All authors read and approved the final version of the manuscript.

Availability of Data and Materials

The authors declare that should any raw data files be needed about the further data of the study, they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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