Research Article

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# Does *Erodium trifolium* (Cav.) Guitt exhibit medicinal properties? Response elements from phytochemical profiling, enzyme-inhibiting, and antioxidant and antimicrobial activities

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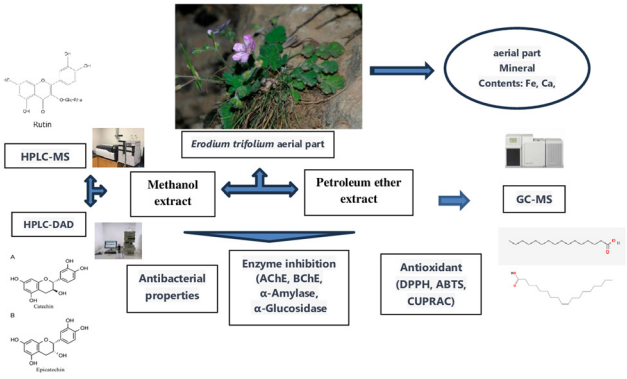
**Abstract:** Geraniaceae are typically used as diuretic, anti-diarrhoeal, stomachic, and anti-hemorrhagic drugs. This study examined the phytochemicals and bioactivities in methanolic extract (ME) and petroleum ether extract (PEE) of the *Erodium trifolium* aerial part. Inductively coupled plasma mass spectrometry was used to assess the mineral profiles, high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) and high-performance liquid chromatography with diode-array detection (HPLC-DAD) were used to assess the phenolic content of ME, and gas chromatography-mass spectrometry (GC-MS) was used to assess the fatty acid and volatile composition of the PEEs. In addition, the bioactivities of extracts were evaluated by using the 1,1-diphenyl-2-picrylhydrazyl, ABTS, and cupric reducing antioxidant capacity assays, including enzyme inhi-




**Graphical abstract**

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bition against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), α-amylase, α-glucosidase activities, and antibacterial properties. HPLC-MS identified eight compounds in ME: rutin, catechin, and caffeine were the major phenolic compounds detected. HPLC-DAD analysis showed that 11 compounds detected among epicatechin, catechin, chlorogenic acid, and gallic acid were predominant. GC-MS analysis revealed 30 fatty acids in PEE, with palmitic acid and oleic acid being predominant. The mineral content showed that Fe was the abundant microelement, and Ca and K were the abundant macroelements. The ME exhibited the highest activity compared to the PEE in all tests regarding antioxidant and antibacterial activities. Furthermore, all the extracts showed moderate inhibition against AChE, BChE, α-amylase, and α-glucosidase. In conclusion, *E. trifolium* may be employed to separate novel bioactive metabolites with potential pharmaceutical activities.

**Keywords:** *Erodium trifolium*, phenolic compounds, HPLC-DAD, fatty acid, minerals, biological activity

# 1 Introduction

The etymology of the *Erodium* genus is derived from the Greek word “Erodius,” which means heron (signified fruit with a long beak); for that reason, the genus is commonly known as “Heron’s Bill” [1]. This genus belongs to the Geraniaceae, a cosmopolitan family, but is mainly found in temperate zones [2]. The plants of this genus are annual, perennial (sometimes biennial), inerminous, or subshrubs [3]. A major center of *Erodium* diversity is found in the Mediterranean basin region (about 63 species), where it grows in mountainous and disturbed areas, while other regions have only a few native species: one in North America, one in South America, five in Australia, and four in Asia [4]. In North Africa (Algeria, Morocco, Tunisia, Libya, and Egypt), there are 134 taxa in the genus *Erodium*, the majority of which are recognized in Morocco (37 taxa) and Algeria (35 taxa). Among these taxa, three are endemic to Algeria [5]. *Erodium trifolium* (Cav.) Guitt. was first described as endemic to Algeria as *Geranium geifolium* Desf., then taken by Quézel and Santa [6] under *E. hymenodes* L’Her. It was subsequently retained for Morocco and Tunisia as *E. trifolium* endemic to these three countries (Algeria, Tunisia, and Morocco). In Algeria, the species is mainly found in the rocky areas of the high mountains of Oran, Algiers, and Constantine, going up to the highlands, including the mountains of Djelfa, where it becomes very rare [6]. *E. trifolium* is an annual plant, perennial herb, or subshrub. The leaves are simple or compound, arranged alternately or oppositely, divided palmately or pinnately, and divided up to 2 × 3 rounded crenelated lobes; flowers are dark pink, primarily bisexual, actinomorphic, solitary, or in inflorescences arranged in cymes and pseudoumbels [6]. The seeds are pendulous, mostly with little endosperm or exalbuminous. The schizocarpic fruit is formed from five carpels joined and attached to the capsule, enveloping the seed at the base of the fruit [7]. At maturity, the carpels separate elastically around a central axis (named the beak) [8].

*Erodium* is used in traditional medicine in many cultures. This cultural heritage was passed down from generation to generation long before modern medical practices took place and current drug development strategies were established [9]. The whole plant of *Erodium* has been used as an astringent, to stop the bleeding of uterine and other hemorrhages, and as an abortifacient [10]. It has also been used to restore various diseases (indigestion, urinary inflammation, diabetes, constipation, eczema, gastrointestinal, hemorrhage, skin disorders, carminatives, and anasarca) [11]. *E. trifolium* extracts were also used in traditional medicine as diuretic, anti-diarrhoeal, stomachic, and antihemorrhagic drugs [12]. In Algeria, few ethnobotanical studies reported traditional uses of *Erodium* species, Wang et al. [13] reported traditional uses of *E. glaucophyllum* L. in the Algerian desert against diarrhea, colds, influenza, and respiratory system disorders.

Nowadays, the exploration of bioactive substances from the genus of *Erodium* is considered a novel source of bioactive compounds and has been gaining attention over the last few decades [13]. The extraction is an important factor in the recovery of antioxidant compounds [14,15]. From a phytochemical point of view, Fecka and Cisowski [12] demonstrated that the main phenolic compounds present in *Erodium* were gallic acid, protocatechic acid, ellagic acid, corilagin, geraniin, isoquercetin, methyl gallate 3-*O*-β-D-glucopyranoside, and rutoside by using two analytical methods: mass spectrometry (MS) and nuclear magnetic resonance (NMR). Tannins, sugars (galactose, fructose, and glucose), catechins, amino acids (glutamic acid, alanine, tyrosine, proline, tryptophan, histidine, and glycine), and vitamins K and C were identified in the extracts of some *Erodium* species (*E. cicutarium*) [16,17].

However, the predominant phenolic compounds vary between species, and it is also mentioned that *Erodium* contains essential oils [11]. It is also relevant to mention that *Erodium* spp. contains essential oils. Lis-Bachlin and Hart [18] identified the following volatile compounds: methyl eugenol, geraniol, citronellol, isomenthone, and linalool. Generally, *Erodium* spp. is a rich source of natural antioxidants, phenolic compounds, and other secondary plant

metabolites. The information from the literature indicated, particularly for phenolic composition and antioxidant potential, that *Erodium* species and solvent composition are the relevant factors to be considered. At the moment, the phytochemical composition of *Erodium* spp. needs to be better investigated to find more data on polyphenols, antioxidant compounds, essential oils, and other compounds.

A recent study [19] suggested that *Erodium* spp. is a discernible source of antimicrobial and antioxidant components with significant usefulness for medicinal purposes. It seems, therefore, that the anti-inflammatory potential of the *Erodium* spp. extracts require more research because of the limited number of published *in vitro* and *in vivo* studies [20]. However, except for some works carried out on *E. glaucophyllum* [21], there are almost no studies exploring the composition and chemical properties of *Erodium* species native to Algeria, especially those endemics. The present study pointed to carrying out phytochemical profiling through a qualitative and quantitative analysis using gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography coupled with mass spectrometry (HPLC MS), and high-performance liquid chromatography with diode-array detection (HPLC-DAD), antioxidant, antimicrobial, and anti-enzymatic activities, and mineral analyses of *E. trifolium* extracts, endemic to North Africa and growing in Algeria.

# 2 Materials and methods

## 2.1 Chemicals and reagents

The measurements of bioactivity were performed on a 96-well microplate reader (SpectraMax 340PC384, Molecular Devices, USA). The results were then treated using Softmax PRO v5.2 software. GC-MS analyses were accomplished with the aid of Varian Saturn 2100T coupled with 3900GC (USA). Ammonium acetate, boron trifluoride–methanol complex, copper(II) chloride, ethanol, ferrous chloride, methanol, *n*-hexane, pyrocatechol, and quercetin were obtained from E. Merck (Darmstadt, Germany). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 3-(2pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt (Ferene), 4-*N*-nitrophenyl-a-D-glucopyranoside (PNPG), 5,5′-dithiobis (2 fatty acids and activities of 4 Serratula species, 88 nitrobenzoic) acetic acid, acetonitrile, acetylcholinesterase (AChe) from electric eel (Type-VI-S, EC 3.1.1.7, 425.84 U/mg; Sigma), acetylthiocholine iodide acid DTNB, amoxicillin (AMC), amphotericin B (AMB), ascorbic acid, butylatedhydroxyl anisole (BHA), butyrylcholinesterase (BChE) from horse serum (EC 3.1.1.8, 11.4 U/mg; Sigma), butyrylthiocholine chloride, caffein, catechin, cefazolin (CZ), cefoxitin (FOX), ceftriaxon (CRO), chloroform, chlorohydric acid, Dragendorff's reagent (potassium bismuth iodide composed of basic bismuth nitrate [$Bi(NO_3)_3$]), Folin–Ciocalteu's reagent, formic acid, galantamine, gallic acid, hydrochloric acid, hydrogen peroxide, iodine, isoamyl alcohol, kojic acid, 3,4-dihydroxy-D-phenylalanine, magnesium turnings, Mayer's reagent (composed of mercuric chloride, potassium iodide [KI]), Mueller–Hinton (MH) agar, neocupron, nitric acid, nicotinamide, petroleum ether, phosphate buffer, polytetrafluoroethylene, KI), potassium persulfate, rutin, sinapic acid, sodium acetate, sodium carbonate, sodium chloride, sodium hydroxide, sodium phosphate, Stiasny's reagent, sulfuric acid, tartaric acid, tyrosinase from mushroom (EC 232-653-4, 250 KU, ≥1,000 U/mg solid; Sigma), vanillin, α-amylase, α-glucosidase, and α-tocopherol were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, and *Staphylococcus aureus* (MRSA) ATCC 34300 were obtained from the Pasteur Institute of Msila (Algeria). All other chemicals and solvents were of analytical grade.

## 2.2 Plant materials

The stems, leaves, and flowers of the plant *E. trifolium* (Cav.) are its aerial parts. The Guitt. were collected in Bou Taleb, Setif province, Algeria, in April 2021, at an altitude of 680 m, with geolocation coordinates N 35.6686°, W 5.3243°. Professor Rebbas K. utilized the available literature and assistance from Algeria Flora to conduct botanical identification. An archived specimen (No KR0044) has been stored in K. Rebbas' personal herbarium at the Natural and Life Sciences Department, University of M'sila, Algeria (Figure 1).

## 2.3 Phytochemical screening

Phytochemical screening involves qualitative analysis utilizing colorimetric reactions and precipitation to identify major chemical groups, categorized as noticeable positive reactions (+++), positive reactions (++), moderate positive reactions (+), or adverse reactions (−). Tannin's presence was assessed using various tests: adding 1 mL of the methanolic extract (ME) to 1% $FeCl_3$ for green or dark blue coloration; boiling a 5 mL extract with concentrated hydrochloric acid for catechin tannins indicated by a red precipitate, and employing Stiasny's reagent followed by $FeCl_3$ solution (1%) for gallic tannins, revealed by a precipitate [22]. Flavonoid detection involved adding 1 mL of the extract to 1 mL of hydrochloric acid and then adding some magnesium turnings and


**Figure 1:** Illustration of *E. trifolium* plant: (a) and (b) photos taken by K. Rebbas (Zone of Bou Taleb, 21/4/2020), and (c) plant deposited on the herbarium board of Faurel (Zone of Bou Taleb, 23/5/1937).

1 mL of isoamyl alcohol, with red indicating flavonols, orangish pink indicating flavones, and purplish pink indicating flavonols [22]. Sterols were identified through a reddish-brown ring formed by adding 1 mL of sulfuric acid ($H_2SO_4$) to 1 mL of extract [23]. Alkaloids were detected using 1 mL of Dragendorff's reagent with 1 mL of each extract, with smoky orange coloration indicating alkaloids in Dragendorff's test and yellowish coloration in Mayer's test [24]. The presence of saponosides following vigorous shaking of 0.2 mL of the extract with 5 mL of distilled water indicated saponins [24], while a temporary red coloration after adding 1 mL of extract to a few drops of dilute iodine solution suggested phenolic compounds [24]. Coumarin was detected by the appearance of a yellow color after mixing 0.5 mL of extract volume with 0.2 mL of NaOH and chloroform [24].

## 2.4 Preparation of crude extracts

Before extraction, plant material was cleaned and dried in a well-ventilated, humidity-free environment at room temperature for 7 days, avoiding direct exposure to sunlight. The dried aerial parts thus obtained were crushed into a fine powder using an electric milling machine to be used thereafter to prepare the extracts. To extract polyphenols, the air-dried powdered material of the plant (20 g) was macerated at room temperature three times in 200 mL of methanol for 72 h [25]. The solvent used was commonly renewed at the start of all attempts upon evaporation under a vacuum [25]. In the case of fatty acids and volatile components, extraction was carried out using a Soxhlet apparatus using 1 L of petroleum ether (six cycles) (60°C), and the lipophilic compounds were obtained after removing the solvent by evaporation [25]. The filtrates were finally stored at 4°C until use.

## 2.5 HPLC-DAD analysis

The phenolic components present in the ME of *E. trifolium* were analyzed using a Shimadzu reversed-phase HPLC-DAD system (Shimadzu Cooperative, Japan) using a

validated method based on 27 standards [26]. Separation was performed on an Inertsil ODS-3 column (4 µm film thickness, Inertsil, 150 mm × 4.0 mm) and an Inertsil ODS-3 guard column; the column temperature was set at 40°C. A stock solution (8 mg/mL) of each extract was prepared in methanol/water (80/20, v/v). The stock solutions were pre-filtered using disposable LC disc filters (Agilent 0.45 µm). The 0.5% acetic acid in water (A) and methanol (B) were used as the mobile phase. The gradient elution was programmed and lasted for 40 min: 0–0.01 min (0–20% B); 0.01–2 min (20–60% B); 2–15 min (60–80% B); 15–30 min (100% B); 3–35 min (100–10% B); and 35–40 min (10–0% B). The flow rate was 1.5 mL/min, and 20 µl of the sample was introduced. Twenty microliters of the sample were injected. Identification was performed using a diode array detector (DAD) in the 230–350 nm range and referred to each commercial standard's UV data and retention times (RTs). All samples and standards were filtered using Agilent 0.45 µm filters. Three replicates were performed for every analysis. Phenolic products were recognized after comparison of their times of retention, with findings presented as mg/g dry weight of extract.

## 2.6 Analysis using HPLC-MS

HPLC system equipped with MS detection was utilized for chromatographic analysis of the methanolic extracted portion of *E. trifolium*. The HPLC YL-Clarity 9100 series has a C18 reversed-phase HPLC column made up of silicon dioxide particles with an apolar hydrocarbon item attached to them. Ten milligrams of methanolic extracted portion were mixed with 2 mL of methanol, treated with sonication for 60 min, and then passed through a microfilter (0.45 µm). Reverse-phase HPLC categorizes various phytochemical categories based on their polarity (ranging from moderate to apolar) within one chromatogram. Next, the ME was introduced inside a gradient pump that was linked to a photodiode detector. The flow rate was set at 1 mL/min, with a pressure of 245 bar, and operating at a temperature of 25°C. A C18 column (4.6 mm × 250 mm) was used for separation, with a gradient system of acidified water and 1% formic acid-acetonitrile. The solvent used was 1% formic acid mixed with water (v/v: "eluent A") and pure acetonitrile (v/v: "eluent B"), with a linear gradient starting at A 95% (B: 5%) at 0 min, A 60% (B 40%) at 50 min, and A 5% (B 95%) at 60 min. The extracts were analyzed at a wavelength of 254 nm. The compounds of the plant were identified and quantified, and each molecule was based on the RT, absorption spectrum, and chromatographic peak area by direct comparison with different concentrations of relatively pure standards [27].

## 2.7 Methylation and analysis of fatty acids, volatiles, and other apolars using GC-MS and GC-FID

About 25 mg of petroleum ether extract (PEE) of *E. trifolium* were thawed in 2 mL of 0.5 N NaOH in a 25 mL flask. The bottle was sealed and warmed in a water basin for 5 min, taken out, cooled, and reacted with 2 mL of boron tri-fluoride–methanol complex (BF3–MeOH). The blend was covered and heated again in a boiling aqueous bath at 80°C for 3 min, followed by cooling and the addition of 5 mL of a saturated solution of sodium chloride and shaken. In the end, it was subjected to two extractions using 20 mL of *n*-hexane each time. The analysis involved using a Shimadzu GC17 AAF with a DB-1 capillary column (ID: 0.25 mm, film: 0.25 µm, length: 30 m) for GC-FID analysis. Helium was the carrier gas flowing at 1.3 mL/min. The temperature program began at 100°C for 5 min, then ramped up to 238°C at a rate of 3°C min, and was maintained for 9 min. Injector and detector temperatures were set at 250 and 280°C, respectively. The concentration of fatty acid methyl esters (FAMEs) was determined using CLASS GC10 computer software.

An ion trap analyzer Varian Saturn 2100T with a non-polar DB-1 capillary column (ID: 0.25 mm, film: 0.25 µm; length: 30 m) was used for GC-MS analysis. The carrier gas, helium, flowed at 1.3 mL/min. The temperature program mirrored that of the GC-FID analysis. Electron ionization at 70 eV was employed. Injector, transfer line, and detector temperatures were set at 220, 290, and 240°C, respectively. FAMEs were identified through Nist/Wiley 2005 library searches and comparisons with fatty acid standards [28,29].

### 2.7.1 Mineral analyses

The samples were prepared using multiple steps and the method outlined by Cicero et al. [30] with a few modifications. First, the gathered samples were cleaned and cut, and then dried for 24 h at 105°C in a Nüve oven located in Istanbul, Turkey. After being dried, the samples were mixed with an IKA homogenizer from Staufen (Germany) and then sieved on a 10-mesh to obtain particles measuring 1,600 µm. The particles were later stored in sanitized polyethylene containers. Deionized water (conductivity of 18.2 MΩ $cm^{-1}$) from a Milli-Q® system (Human Power I Plus, Sejong-si, Korea) was used during the entire process. Every tool was carefully disinfected using a mixture of 10% nitric acid and deionized water. A CEM Mars 5 microwave-sealed system from Matthews (North Carolina, United States) was

used for the digestion step. About 0.5 g of the dried plant material was crushed and digested with a mixture of 6 mL of 65% nitric acid ($HNO_3$) and 2 mL of 30% hydrogen peroxide ($H_2O_2$). The Ethos 1 digester, manufactured by Milestone in Bergamo, Italy, was used for the mineralization with the following settings: 1,000 W power, temperature between 150 and 200°C, a 20-min ramp, and a 2-min hold at 100% power. Following cooling, the samples were filtered, mixed with 100 mL of ultra-pure water, and subsequently stored at 4°C. Mineral levels were analyzed utilizing inductively coupled plasma optical emission spectrometry (ICP-OES), with precision confirmed using SRM NIST-CRM-1203 Drinking Water, maintaining RSD% below 8% consistently. Additionally, mineral concentrations on a dry-weight basis were analyzed using an Agilent 7700x ICP-MS. Blank samples were included in the analysis to monitor for potential contamination. Detailed operating conditions for the inductively coupled plasma mass spectrometry (ICP-MS) analysis are provided in Table 1.

## 2.8 Biological activities

### 2.8.1 Antioxidant activity

#### 2.8.1.1 Free radical scavenging activity on DPPH assay

The Blois [31] assay was used to determine the free DPPH radical-scavenging capacity of the *E. trifolium* extracts. A 0.1 mM solution was made by dissolving 39.4 mg of DPPH in methanol, and 4 mL of this solution was combined with 1 mL of extracts in methanol at various concentrations. After stirring the resulting solution for 30 min at room temperature in the dark, its absorbance was measured at 517 nm using a 96-well microplate reader. Butylated hydroxyanisole (BHA) was used as the reference for further antioxidant activity comparisons. The ability to scavenge the DPPH was determined using the following formula:

Scavenging activity % = [Abs (control) – Abs (sample)]/Abs (control) × 100,

where Abs Sample is the absorbance of the remaining DPPH concentration in the presence of extract and positive control, and Abs Control is the original concentration of DPPH.

The results are reported in $IC_{50}$ (g/mL), indicating the amount of extract needed to achieve a 50% inhibition of DPPH.

**Table 1:** Operating conditions of the ICP-MS instrument

| Instrument | Agilent™ 7700x ICP-MS |
|---|---|
| RF power | 1,600 W |
| RF match | 2.10 V |
| Depth of sampling | 10.0 nm |
| Nebulizer gas | 0.57 L/min |
| S/C temperature | 2°C |
| Type of nebulizer | MicroMist |
| Spray chamber | Scott-type double-pass |
| Rate of flow | Plasma: 15 L/min; Auxiliary: 0.9 L/min; Nebulizer: 1.0–1.1 L/min |
| Rate of solution uptake | 1.8 mL/min |
| Vacuum interface | 4 torr, quadrupole: 2 105 torr |
| Acquiring data | Peak hopping; replicate time, 200 ms; dwell time, 200 ms; sweeps/reading, 3; readings/replicate, 3; number of replicates, 3 |

#### 2.8.1.2 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)

The assay of Re et al. [32] was used to assess the ABTS scavenging capacity of ME and PEE of *E. trifolium*. The ABTS was prepared by reacting 2.45 mM potassium persulfate with 7 mM potassium ABTS in water for 12 h at room temperature in the dark. Ethanol was used to dilute the ABTS solution before use, yielding an absorbance of 0.703–0.025 at 734 nm using a 96-well microplate reader. Ethanol was used as the control. The results are expressed as $IC_{50}$. A graph displaying the percentage of ABTS scavenging effect versus sample concentration was used to estimate the sample concentration that gave 50% ABTS scavenging effect ($IC_{50}$): ABTS scavenging effect % = [(A Control − A Sample)/A Control] × 100, where A Sample is the absorbance of the residual ABTS concentration in the presence of the extract and positive control, and A Control is the starting ABTS concentration. The antioxidant standard BHA was utilized to compare the activity.

#### 2.8.1.3 Cupric reducing antioxidant capacity (CUPRAC)

With some adjustments to Öztürk and Akgün [33], the CUPRAC test of ME and PEE of *E. trifolium* was conducted using the methodology of Apak et al. [34]. Following the addition of 60 µL of 1 M ammonium acetate buffer ($NH_4Ac$ buffer, pH 7.0), 40 µL of sample solutions at different concentrations was added along with 50 µL of 7.5 mM neocupron and 50 µL of 10 mM Cu(II) solution. The absorbance was measured at 450 nm using a 96-well microplate reader following an hour of incubation at room temperature. Each sample was measured three times. Results are presented as $A_{0.50}$ values (g/mL), where $A_{0.50}$ represents the sample concentration, and 0.50 represents the absorbance intensity. BHA was employed as the benchmark antioxidant to measure activity.

#### 2.8.2 Enzyme inhibition activity

##### 2.8.2.1 Anticholinesterase activity

The method of Ellman et al. [35] was used to assess the enzyme inhibitory activity of ME and PEE of *E. trifolium* to AChE and BChE. After mixing 150 µL of buffer with 10 µL of sample solutions in ethanol, 20 µL of either AChE ($5.32 \times 10^{-3}$ U) or BChE ($6.85 \times 10^{-3}$) was added to the enzyme solution and incubated at 25°C for 15 min. Finally, 10 µL of 0.5 mM DTNB was added. Ten microliters of either butyrylthiocholine chloride (0.2 mM) or iodide acetylthiocholine (0.71 mM) were added to start the reaction. The hydrolysis of these substrates was observed using a 96-well microplate reader every 5 min for 15 min at 412 nm. Each sample was measured three times. Galantamine was employed as the control, using an equal concentration of the samples under analysis. The formula to calculate the percentage of AChE and BChE inhibition is equal to ([enzyme activity with no extract − enzyme activity with extract]/enzyme activity with no extract) × 100.

##### 2.8.2.2 α-Amylase/α-glucosidase inhibitory activities

To test the α-amylase/α-glucosidase inhibitory activity of *E. trifolium*, ME and PEE were analyzed spectrophotometrically by using a 96-well microplate reader [36,37]. Softmax PRO v5.2 software (Molecular Devices, Silicon Valley) was utilized to calculate and measure the data related to bioactivity.

Using the method previously published by Quan et al. [37], the α-amylase inhibitory activity of *E. trifolium* ME and PEE was assessed. Slight adjustments were made to the incubation times, reagents, volumes of reagents, and samples used. About 50 µL of α-amylase solution (0.1 unit/mL) was mixed with 25 µL of sample solution in phosphate buffer (20 mM, pH = 6.9 phosphate buffer made with 6 mM NaCl). The mixture was incubated for 10 min at 37°C. Then, 50 µL of starch solution (0.05%) was added after pre-incubation and incubated at 37°C for 10 min. To stop the process, 100 µL of Lugol's solution and 25 µL HCl (0.1 M) were mixed. A microplate reader was used to determine the absorbance at 565 nm. The standard chosen was acarbose. Based on the graph showing the inhibitory activity of α-amylase versus sample concentration, the concentration of the sample was determined that result in 50% inhibitory activity ($IC_{50}$).

The method reported by Kim et al. [36] was used to assess the α-glucosidase inhibitory activity of *E. trifolium* ME and PEE, with a few minor modifications to incubation periods, reagents, volumes of reagents, and samples used. The following ingredients were combined: 25 µL of PNPG (4-*N*-nitrophenyl-a-D-glucopyranoside), 50 µL of phosphate buffer (0.01 M, pH = 6.9), 10 µL of sample solution in phosphate buffer (0.01 M pH = 6.9), and 25 µL of phosphate buffer (0.01 M, pH = 6.0) containing 0.1 unit/mL of α-glucosidase. For 20 min, the mixture was incubated at 37°C. In order to complete the procedure, 90 µL of $Na_2CO_3$ solution (0.1 M) was introduced to the microplate. A microplate reader was used to determine the absorbance at 400 nm. Acarbose was used as a standard. The $IC_{50}$ value was determined based on the graph showing the inhibitory activity of α-glucosidase against different sample concentrations.

#### 2.8.3 Determination of antibacterial activities

Following the recommendations of the Clinical and Laboratory Standards Institute [38], the disc diffusion test was used to investigate the antibacterial activity of MEs of *E. trifolium*. This method was reported by Nicoletti et al. [39]. All used equipment were autoclaved for 15 min at 121°C. Three reference strains of bacteria from the American Type Culture Collection (ATCC) were used as test subjects to determine the antibacterial activity: *Escherichia coli* ATCC 8739, *P. aeruginosa* ATCC 9027, and *S. aureus* (MRSA) ATCC 34300. To obtain young cultures and isolated colonies, bacterial strains were serially subcultured overnight at 37°C in nutrient agar, incubated for 24 h at 37°C to maximize their growth, and then streaked to ensure purity. Briefly, cells were resuspended in saline ($1–2 \times 10^8$ cells/mL for bacteria (0.5 McFarland) and $10^6$ cells/mL for Candida) and plated on MH agar in Petri dishes. Six-millimeter diameter sterile Whatman paper discs were positioned on the inoculation Petri dish surface with 10 µL of 50, 100, and 200 mg/mL methanolic extracted portion dissolved in dimethyl sulfoxide (DMSO). The Petri dishes containing MH agar were then incubated for 24 h at 35 (±1)°C. The growth inhibition zone (IZD) surrounding the paper disc was measured compared to the reference antibiotic diameter, and the activity was replicated three times. The diameter is restricted to 6–14 mm, on average ranging from 14 to 20 mm. DMSO-soaked Wattman discs with AMC (30 µg), CZ (30 µg), ceftriaxone (CRO, 30 µg), cefoxitin (FOX, 30 µg), and AMB (20 µg) were used to adjust negative controls.

### 2.9 Statistical examination

The results from three independent replicates were averaged for all biological tests. The information was presented as the average plus/minus standard deviation (SD). The results of all biological assessments represent the average of three independent replicates. One-way analysis of

variance (1-ANOVA) was initially applied to search for global differences. Tukey's HSD test was specifically used to determine the significance of the difference between standards and extracts after multiple comparisons between means. The results of each of the statistical analyses above were deemed significant at *p*-values less than 0.05.

# 3 Results and discussion

## 3.1 Phytochemical screening results

Table 2 lists the phytochemicals screening found in *E. trifolium* extracts, such as tannins, gallic tannins, catechin tannins, sterols, flavonoids, alkaloids, phenolic compounds, saponosides, and coumarin. The highlighting of different classes of phytochemicals allows us to have a good idea about the phytochemistry of the plant. According to the results of *E. trifolium*, ME and PEE contained alkaloids, flavonoids, phenolic compounds, tannins, and coumarins in varying concentrations. In trace amounts, traces of saponosides were found in the MEs. The findings from the screening of phytochemicals confirmed that the methanolic extracted portion of *E. trifolium* had a higher amount of phytochemical components compared to the PEE. According to Lis-Balchin and Hart [18], *E. cicutarium* contains alkaloids. Besides, Al-Shamm and Mitscher [40] confirmed that *E. acaule*, *E. pelargonijlorum*, and *E. laciniatum* contained all alkaloids. However, negative results were reported for *E. cicutarium* [41], *E. ciconium*, *E. glaucophyllum* [40], and *E. rupestre* [42], which confirms that alkaloid synthesis is variable within species of the genus *Erodium* and depends on environmental factors.

**Table 2:** Phytochemical screening results of ME and PEE of *Erodium trifolium*

| Assays | Extracts | |
|---|---|---|
| | Methanolic | Petroleum ether |
| Tannins | +++ | − |
| Gallic tannins | ++ | − |
| Catechin tannins | ++ | − |
| Sterols | − | + |
| Flavonoids | +++ | − |
| Alkaloids | ++ | − |
| Phenolic compounds | +++ | − |
| Saponosides | + | − |
| Coumarins | + | − |

## 3.2 Qualitative analysis of phenolic compounds by HPLC-MS

The results of the chemical composition of *E. trifolium* obtained by HPLC-MS show that only eight compounds were identified (Table 3 and Figure 2). Sinapinic acid, nicotinamide, ascorbic acid, gallic acid, vanillin, caffeine, rutine, and catechin were found in the ME of *E. trifolium*. To the best of our knowledge, no previous publications have addressed the chemical makeup of *E. trifolium*. Only a few studies attempted to identify and/or quantify phenolic chemicals from *Erodium* spp. Studies on the phytochemical composition of *Erodium* species have revealed the presence of phenolic substances such as flavonoids and tannins. It was claimed that the primary phenolic compounds in *Erodium petraeum*, *Erodium botrys*, and *Erodium gruinum* were ellagic acids; however, in *Erodium cicutarium* and *Erodium manescavi*, brevifolin predominated [11]. With the aid of

**Table 3:** Phenolic composition of *E. trifolium* MEs by HPLC-MS analysis

| No | RTmin | Area (mV s) | Height (mV) | Area (%) | Height (%) | W05 (min) | Compound |
|---|---|---|---|---|---|---|---|
| 1. | 3.183 | 95.150 | 10.873 | 0.8 | 2.7 | 0.11 | Cinapic acid |
| 2. | 3.913 | 245.792 | 10.505 | 2.1 | 2.6 | 0.27 | Nicotinamide |
| 3. | 4.513 | 536.915 | 17.501 | 4.5 | 4.4 | 0.65 | Ascorbic acid |
| 4. | 5.453 | 302.943 | 12.561 | 2.5 | 3.1 | 0.37 | / |
| 5. | 7.683 | 250.616 | 16.123 | 2.1 | 4.0 | 0.25 | Gallic acid |
| 6. | 11.103 | 73.335 | 5.429 | 0.6 | 1.4 | 0.19 | / |
| 7. | 12.327 | 864.639 | 48.525 | 7.3 | 12.1 | 0.53 | Vanilin |
| 8. | 13.283 | 1001.498 | 63.966 | 8.4 | 16.0 | 0.23 | Caffeine |
| 9. | 14.120 | 1486.119 | 70.164 | 12.5 | 17.5 | 0.52 | / |
| 10. | 15.220 | 1374.780 | 76.789 | 11.5 | 19.2 | 0.25 | Rutin |
| 11. | 28.867 | 5682.787 | 67.631 | 47.7 | 16.9 | 0.62 | Catechin |
| Total | | 11914.575 | 400.068 | 100.0 | 100.0 | | |

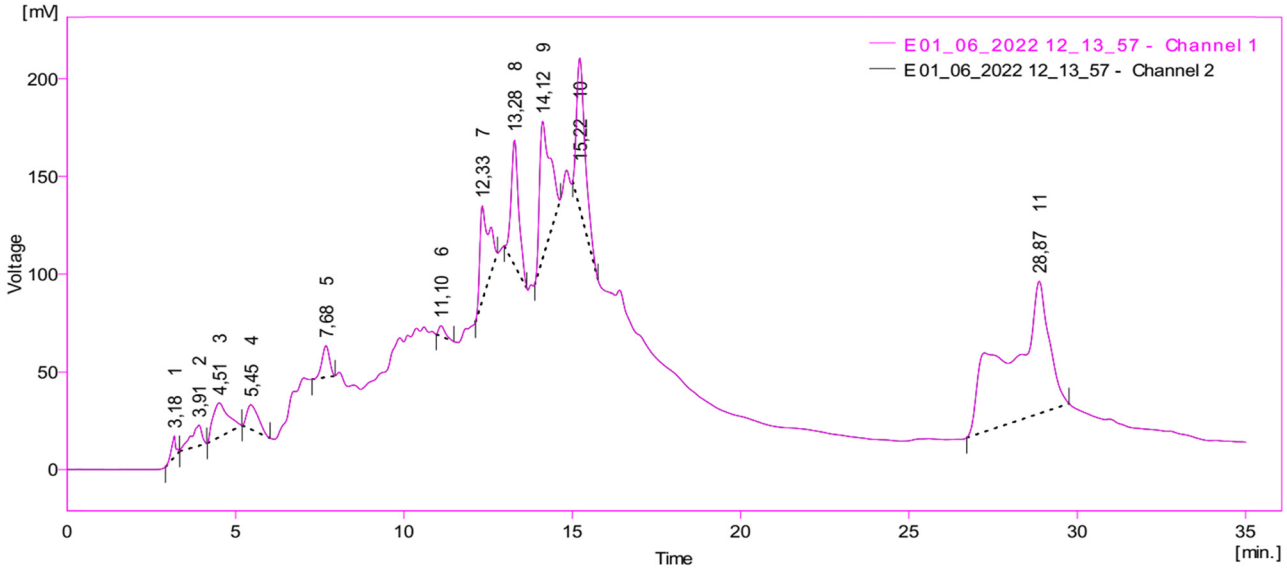


**Figure 2:** HPLC-MS chromatogram of *E. trifolium* MEs.

planar chromatography, Fecka and Cisowski [12] demonstrated the presence of geraniin, isoquercitrin, corilagin, and dehydrogeraniin in *Erodium* spp. In a different study, Fecka and Cisowski [12] used nuclear magnetic resonance and mass spectrometry to examine the structure of the main phenolic compounds of *E. cicutarium*, including 3-*O*-(6″-*O*-galloyl)-β-D-galactopyranoside, methyl gallate 3-*O*-β-D-glucopyranoside, rutin, gallic acid, 3-*O*-galloylshikimic acid, protocatechuic acid, corilagin, geraniin, didehydrogeraniin, isoquercitrin, and hyperin. Additionally, the analysis of the chemical profiles of *E. cicutarium* using UHPLC and MS methods revealed the presence of 85 phenolic compounds, the majority of which are derivatives of gallic acid with 24 compounds, several derivatives of ellagic acid, including ellagitannins with 22 compounds, flavonol glycosides with 19 compounds; derivatives of hydroxycinnamic acid with 8 compounds, and other hydroxybenzoic acid derivatives with 7 compounds, flavonol aglycones with 3 compounds, and procyanidins with 2 compounds [21].

## 3.3 Quantitative analysis of phenolic compounds by HPLC-DAD

A DAD detector-equipped HPLC was used to investigate the phenolic chemicals. The compounds were identified by contrasting the spectroscopic properties of compounds' and RTs with those of reference compounds (Table 4 and Figure 3). Eleven compounds were detected, among which epicatechin was the predominant (21.02 mg/g extracts), followed by catechin (10.78 mg/g), chlorogenic acid (6.09 mg/g), gallic acid (3.21 mg/g), and rutin (2.19 mg/g). Other constituents were protocatechuic acid (1.88 mg/g), fisetin (1.08 mg/g), *p*-hydroxybenzaldehyde (0.71 mg/g), propyl gallate (0.52 mg/g), *trans*-2-hydroxycinnamic acid, and apigenin in trace amounts. Epicatechin and catechin are flavonoid-class natural antioxidants present in grapes, fruits, and green tea [43,44]. These two elements are flavan-3-ol stereoisomers with identical antioxidant and free radical scavenging properties. Chlorogenic acid is one of the most common polyphenols found and is highly abundant in most foods and coffee, with antioxidant potential [45], anti-inflammatory bioeffects [46], anti-obesity, anti-diabetic properties, anti-hypertensive properties, and an antibacterial effect [47]. According to Farbood et al. [48], gallic acid has potent antioxidant, anti-

**Table 4:** Phenolic composition of *E. trifolium* MEs by HPLC-DAD analysis

| No | Compounds | RT | mg/g extract |
|---|---|---|---|
| 1. | Gallic acid | 15.225 | 3.21 |
| 2. | Protocatechuic acid | 24.625 | **1.88** |
| 3. | Catechin | 30.274 | **10.78** |
| 4. | *p*-Hydroxybenzaldehyde | 33.367 | **0.71** |
| 5. | Epicatechin | 35.278 | **21.02** |
| 6. | Chlorogenic acid | 40.116 | 6.09 |
| 7. | Prophylgallate | 46.984 | **0.52** |
| 8. | Rutin | 47.527 | 2.19 |
| 9. | *Trans*-2-Hydroxycinnamic acid | 48.243 | *tr* |
| 10. | Fisetin | 51.243 | **1.08** |
| 11. | Apigenin | 64.071 | *tr* |
| | **Total** | – | **47.48** |

*tr*: trace amount.

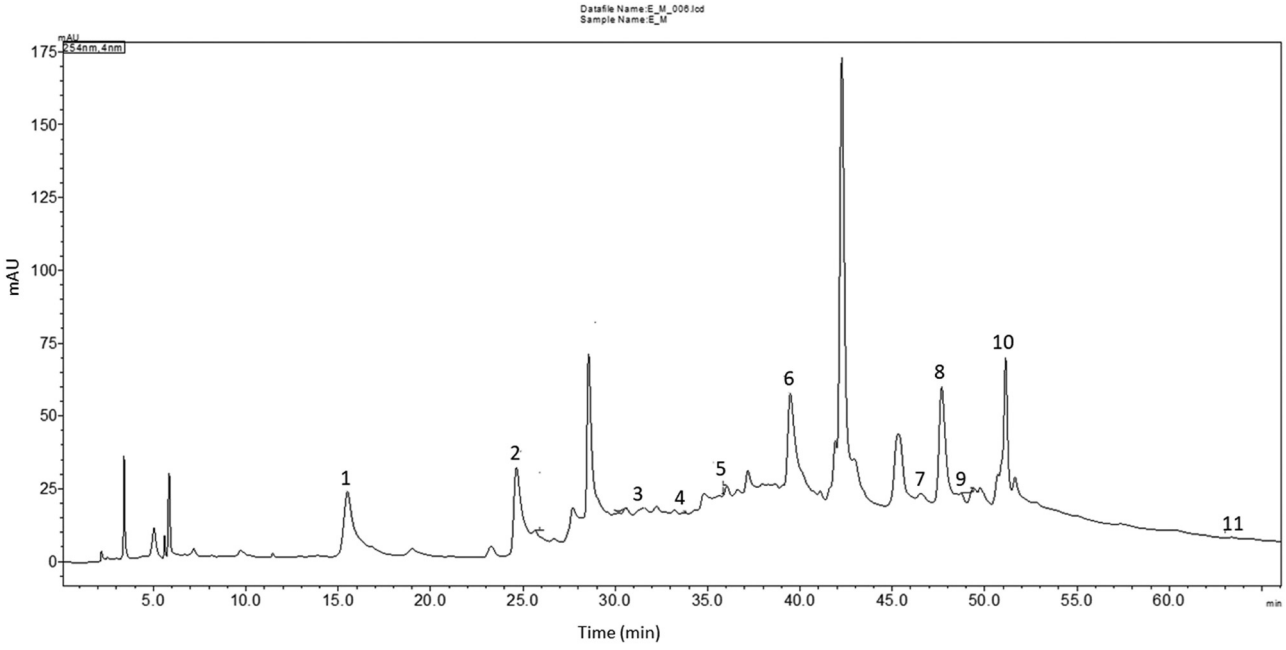


**Figure 3:** HPLC-DAD chromatogram of *E. trifolium* at 254 nm (Inertsil ODS-3 column (4 µm, 4 mm × 150 mm)). Mobile phase: 0.1% acetic acid–methanol (gradient elution). Flow rate: 1 mL/min. DAD (254 nm). (1) Gallic acid, (2) protocatechuic acid, (3) catechin, (4) *p*-hydroxybenzaldehyde, (5) epicatechin, (6) chlorogenic, (7) acid prophylgallate, (8) rutin, (9) *trans*-2-hydroxycinnamic acid, (10) fisetin, and (11) apigenin.

mutagenic, anti-carcinogenic, and neuroprotective properties. According to Harborne [49], rutin is an essential nutrient for plants, and its name comes from the plant *Ruta graveolens*, which also contains rutin. Chemically, it is a glycoside comprising the disaccharide rutinose and the flavonol aglycone quercetin [50]. Due to its numerous qualities, such as its antioxidant, cardiovascular, neuroprotective, anti-inflammatory, antidiabetic, and anti-carcinogenic actions, rutin has been demonstrated to have a wide range of therapeutic applications [51,52].

## 3.4 Fatty acid and volatile composition

The fatty acid and volatile composition of the *E. trifolium* PEE is given in Table 5, and the chromatogram is shown in Figure 4. Thirty fatty acids, volatile and other apolar, were detected by using GC and GC–MS in the *E. trifolium* PEE. The dominants were palmitic acid (29.49%) and oleic acid (21.48%). The total unsaturated and saturated fatty acid percentages were 34.95 and 44.08%, respectively. However, isobutyl methyl phthalate, myristic acid (C14:0), diisobutyl phthalate, linoleic acid (C18:2), stearic acid (C18:0), and eicosanoic acid (C20:0) were found in the extract tested in a small amount (between 1 and 7.5% in concentration). Other fatty acids such as *trans*-2-nonenal, nonanoic acid, methyl 9-oxodecanoate, dimethyl phthalate, buthylatedhydroxytoluene, nonanedioic acid (azaleic acid), tridecanol, methyl 10-oxo-8-decenoate,1-hexadecanol, pentadecanoic acid (C15:0), phytone, 1-heptadecanol, 14-methyl-hexadecanoic acid, margaric acid (C17:0), docosane,11-eicosenoic acid (C20:1), and docosanoic acid (C22:0) were detected in small quantities (less than 1% in concentration). The results obtained herein are different from those of Lis-Balchin and Hart [18], who showed that the volatile compounds from leaves of *E. cicutarium* comprise methyl eugenol (10.6%), geraniol (16.7%), citronellol (15.4%), isomenthone (11.2%), and linalool (3.1%).

## 3.5 Mineral content

To the best of our knowledge, no reports on mineral quantification on the *E. trifolium* plant have been published. Notably, this is the first report in our work on the elemental mineral content. The mineral composition was determined using ICP-OES. The results are shown in Table 6.

Our results show that the examined plant material contains high amounts of minerals, especially iron, manganese, zinc, boron, and copper. The most abundant trace element in the aerial part was iron at a concentration of 627.67 ± 12.55 ppm, followed by zinc, manganese, boron, and copper (45.27 ± 1.35; 44.22 ± 1.76; 26.54 ± 0.53; and 10.43 ± 0.5 ppm, respectively). Calcium had the highest concentration among the macro elements at 2.15 ± 0.01%, while

**Table 5:** Fatty acids, volatile composition, and other apolar compounds (%) of *E. trifolium* PEE

| No | RT | Compound name | RI | Area | Concentration (%) |
|---|---|---|---|---|---|
| 1. | 9.126 | *trans*-2-Nonenal (volatile) | 1161 | 100 | 0.00 |
| 2. | 11.773 | Nonanoic acid | 1226 | 100 | 0.00 |
| 3. | 15.827 | Methyl 9-oxo decanoate | 1429 | 505995 | 0.64 |
| 4. | 16.682 | Dimethyl phthalate (volatile) | 1431 | 769413 | 0.97 |
| 5. | 18.978 | Butylated hydroxytoluene (volatile) | 1443 | 478606 | 0.60 |
| 6. | 20.448 | Nonanedioic acid (azaleic acid) | 1540 | 530929 | 0.67 |
| 7. | 22.125 | Tridecanol (fatty alcohol) | 1577 | 100 | 0.00 |
| 8. | 22.232 | Methyl 10-oxo-8-decenoate | 1592 | 443912 | 0.56 |
| 9. | 25.23 | Isobutyl methyl phthalate (esters) | 1689 | $2.35 \times 10^6$ | 2.96 |
| 10. | 27.212 | Myristic acid (C14:0) | 1725 | $1.34 \times 10^6$ | 1.69 |
| 11. | 29.632 | 1-Hexadecanol | 1810 | 326157 | 0.41 |
| 12. | 30.872 | Pentadecanoic acid (C15:0) | 1815 | 367070 | 0.46 |
| 13. | 31.529 | Phytone | 1842 | 410266 | 0.52 |
| 14. | 32.392 | Diisobutyl phthalate (ester phthalate) | 1862 | 841678 | 1.06 |
| 15. | 34.425 | Palmitic acid (C16:0) | 1938 | $2.34 \times 10^7$ | **29.49** |
| 16. | 35.762 | Unidentified | | $1.34 \times 10^6$ | 1.69 |
| 17. | 36.562 | 1-Heptadecanol (fatty alcohol) | 1968 | 370982 | 0.47 |
| 18. | 36.774 | 14-Methyl-hexadecanoic acid | 1985 | 216867 | 0.27 |
| 19. | 37.717 | Margaric acid (C17:0) | 2028 | 475526 | 0.60 |
| 20. | 38.065 | Unidentified | | 547145 | 0.69 |
| 21. | 38.992 | Unidentified | | $2.57 \times 10^6$ | 3.23 |
| 22. | 39.893 | Linoleic acid (C18:2) | 2090 | $5.69 \times 10^6$ | 7.16 |
| 23. | 40.11 | Oleic acid (C18:1) | 2095 | $1.71 \times 10^7$ | **21.48** |
| 24. | 40.928 | Stearic acid (C18:0) | 2128 | $5.01 \times 10^6$ | 6.30 |
| 25. | 44.262 | Unidentified | | $1.47 \times 10^6$ | 1.85 |
| 26. | 46.044 | Docosane | 2200 | 92370 | 0.12 |
| 27. | 46.372 | 11-Eicosenoic acid (C20:1) | 2279 | 392164 | 0.49 |
| 28. | 46.951 | Eicosanoic acid (C20:0) | 2248 | $1.49 \times 10^6$ | 1.88 |
| 29. | 52.601 | Docosanoic acid (C22:0) | 2531 | 279207 | 0.35 |
| 30. | 57.854 | Unidentified | | $1.07 \times 10^7$ | 13.40 |
| | | **Total** | | **79462587** | **100.00** |
| | | Total saturation | | | **44.08** |
| | | Total unsaturation | | | **34.95** |
| | | Others | | | **20.86** |
| | | Saturation/unsaturation ratio | | | **1.26** |
| | | Linoleic acid/oleic acid ratio | | | **0.33** |

[a]RT: retention time; [b]RI: retention index on Rxi-5Sil MS (Restek) fused silica non-polar capillary column (30 mm × 0.25 mm ID, film thickness: 0.25 μm); t: trace, less than 0.1%.

potassium had a concentration of 1.46 ± 0.04%. However, the concentrations of phosphorus and magnesium in the aerial parts of the plants studied were lower than 0.4%. The minerals play essential roles in body function, making *E. trifolium* an excellent nutritional supplement. Phosphorus and magnesium are necessary for bone mineralization [53]. Iron plays a vital role in preventing anemia by synthesizing hemoglobin and myoglobin [53]. It is also involved in many metabolic processes, including respiration and DNA synthesis; it is also responsible for producing iron-containing enzymes and other types of enzymes [53].

Calcium is essential for the growth of teeth and bones, as well as the proper functioning of muscles and nerve fibers [54]. Potassium can lower blood pressure and enhance bone health. It is also a crucial nutrient in the diet for maintaining the acid/base and water balances in cells, where it accounts for ~70% of the total cations. Calcium is essential for the cardiac and smooth muscles and, therefore, for the normal course of digestion and muscle contraction [55].

In addition, magnesium is involved in glucose homeostasis and significantly affects diabetes control [29]. It is a cofactor for numerous metabolic processes and is crucial for the mineralization of bones and the relaxation of muscles [56]. Manganese, copper, and zinc play important roles in various functions within cells, tissues, and subcellular structures. These functions include immune regulation,

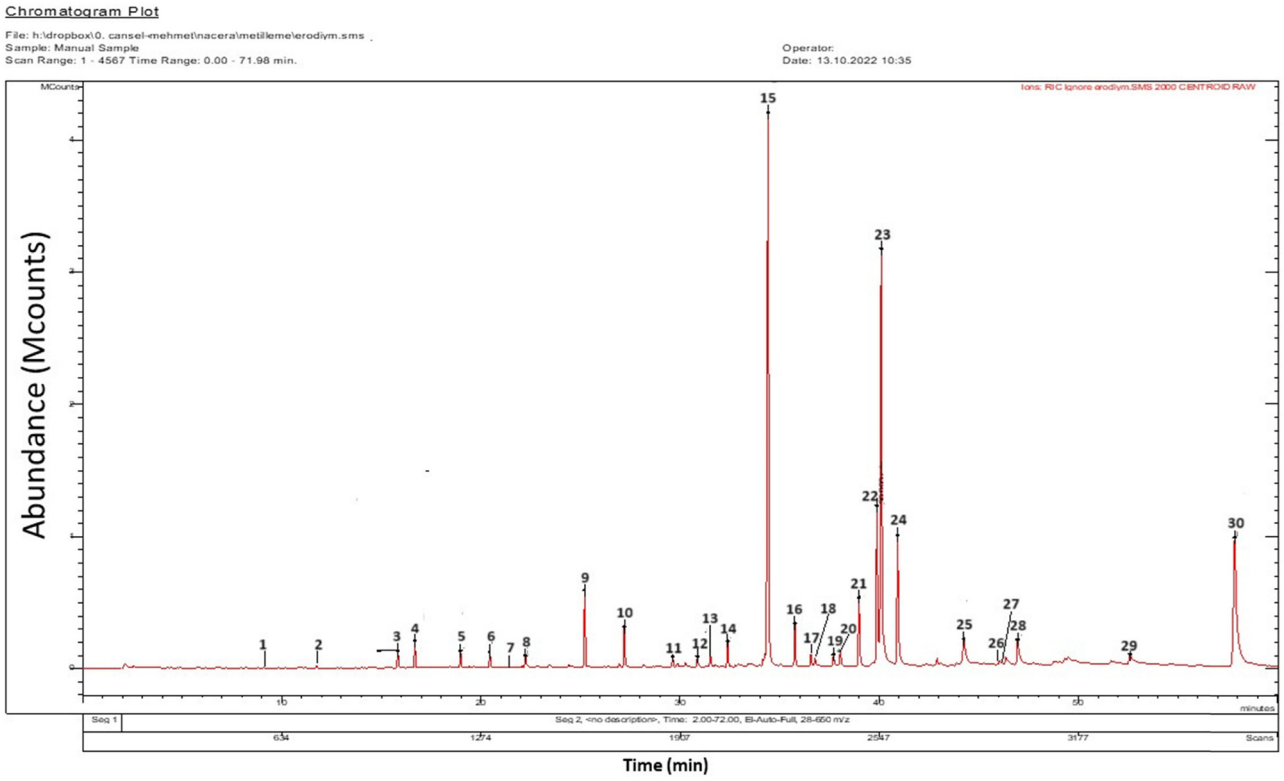


**Figure 4:** The chromatogram of the fatty acid and volatile composition of *E. trifolium* PEE. (1) *trans*-2-nonenal (volatile); (2) nonanoic acid; (3) methyl 9-oxo decanoate; (4) dimethyl phthalate (volatile); (5) butylated hydroxytoluene (volatile); (6) nonanedioic acid (azaleic acid); (7) tridecanol (fatty alcohol); (8) methyl 10-oxo-8-decenoate; (9) isobutyl methyl phthalate (esters); (10) myristic acid (C14:0); (11) 1-hexadecanol; (12) pentadecanoic acid (C15:0); (13) phytone; (14) diisobutyl phthalate (ester phthalate); (15) palmitic acid (C16:0); (16) unidentified; (17) 1-heptadecanol (fatty alcohol); (18) 14-methyl-hexadecanoic acid; (19) margaric acid (C17:0); (20) unidentified; (21) unidentified; (22) linoleic acid (C18:2); (23) oleic acid (c18:1); (24) stearic acid (C18:0); (25) unidentified; (26) docosane; (27) 11-eicosenoic acid (C20:1); (28) eicosanoic acid (C20:0); (29) docosanoic acid (C22:0); (30) unidentified.

**Table 6:** Mineral contents of the aerial parts of *E. trifolium*

| Mineral contents | | Mineral contents |
|---|---|---|
| Macro element | Phosphorus (%) | 0.15 ± 00.001 |
| | Potassium (%) | 1.46 ± 0.04 |
| | Calcium (%) | 2.15 ± 0.01 |
| | Magnesium (%) | 0.21 ± 0.01 |
| Microelement | Iron (ppm) | 627.67 ± 12.55 |
| | Copper (ppm) | 10.43 ± 0.5 |
| | Manganese (ppm) | 44.22 ± 1.76 |
| | Zinc (ppm) | 45.27 ± 1.35 |
| | Boron (ppm) | 26.54 ± 0.53 |

Values are mean ± SD of three parallel measurements. PPM: part-per-million.

muscle contraction, nerve transmission, membrane potential regulation, enzymatic reactions, and mitochondrial activity. Zinc and copper play vital roles as antioxidant defenses [57,58]. Every mineral and trace element that were tested are essential micronutrients needed for proper bodily function because of their beneficial impact on physiological processes [59]. These components have been involved in numerous reactions involving biochemistry; they serve as steadfast constituents of proteins and enzymes, playing a role as cofactors. for various enzymes [60].

## 3.6 Biological activities

### 3.6.1 Antioxidant properties

The antioxidant characteristics of methanol and petroleum ether extracted portions from the aerial parts of *E. trifolium* were assessed using the DPPH, ABTS, and CUPRAC methods. These tests are commonly utilized to assess the capacity of natural antioxidants in transferring protons to free radicals from hydrogen. The antiradical activity was assessed in this study by measuring the $IC_{50}$ values; the findings are displayed in Table 7 in comparison to the standard BHA.

According to the results obtained (Table 7), we can say that ME shows better antiradical activity compared to the

**Table 7:** Antioxidant activities of the ME and PEE of *E. trifolium*

| Extracts and compounds | Antioxidant activity $IC_{50}$ (µg/mL) | | |
|---|---|---|---|
| | DPPH assay | $ABTS^{+}$ assay | CUPRAC assay |
| ME | 4.76 ± 0.68 | 2.06 ± 0.08 | 13.23 ± 0.06 (*) |
| PEE | 282.2 ± 79.4 (****) | 224.9 ± 7.88 (****) | 193.7 ± 5.05 (****) |
| BHA | 3.44 ± 0.09 | 1.88 ± 0.06 | 5.62 ± 0.08 |

BHA: 2-*tert*-Butyl-4-hydroxyanisole. Values are mean ± SD of three parallel measurements. Stars indicate significant differences with BHA (Tukey's HSD test): $p < 0.05$ (*); $p < 0.0001$ (****).

PEE in all tests, and is thus closely related to the standard activity (BHA). The highest activity for ME was exhibited by the ABTS+ assay and the DPPH assay ($IC_{50}$: 2.06 ± 0.08 and 4.76 ± 0.68 µg/mL, respectively), followed by the CUPRAC assay ($IC_{50}$: 13.23 ± 0.06 µg/mL). Indeed, only a significant difference was detected for the latter assay between the antioxidant activities recorded in the ME extract and the standard BHA (Tukey's HSD *post-hoc* test, $p < 0.05$). Simultaneously, PEE exhibited weak activity in all tests (193.7 ± 5.05, 224.9 ± 7.88, and 282.2 ± 79.4 for the CUPRAC, ABTS, and DPPH assays, respectively). Such a pattern was supported by highly significant differences with BHA (Tukey's HSD test, $p < 0.0001$).

According to the literature, no study has been conducted on the antioxidant activity in *E. trifolium* species. Still, in similar studies, the $IC_{50}$ value was 20.29 ± 2.64 µg/mL in *E. glaucophyllum*, 49.1 ± 3.6 µg/mL in *E. hirtum*, and 56.9 ± 3.3 µg/mL for *E. guttatum* with the DPPH test [9], which has high antioxidant activity (DPPH) and is matchless with our extract. Compared to the study of Alali et al. [61], the ME of *E. bryoniifolium* presented a lower activity (25.4 µmol TE/g) than that found in our samples for the APTS test. Similarly, compared with the work of Sarikurkcu et al. [62] on *E. cicutarium* using the CUPRAC test, the result of the reducing power was 130.44 ± 4.26 mg ETs/g. It was weaker than our results obtained for the ME.

The results obtained in our previous tests can be explained by the fact that there is a significant correlation between the antioxidant activities and the phenolic content of *E. trifolium*. Analysis using HPLC-DAD showed the richness of the ME of *E. trifolium* in polyphenols, such as epicatechin and catechin, which have been identified as antioxidants [63–65] and could scavenge hydroxyl, peroxyl, and DPPH radicals and chelate iron ions [66]. The same assumption is valid for chlorogenic acid, which is considered an antioxidant and is present in extracts of various plants [48]. At the same time, gallic acid is a potent antioxidant with anti-mutagenic and anti-cancer properties [48]. These results suggest that phenolic compounds in plant extracts contribute significantly to their antioxidant potential [67]. Even though PEE is an apolar extract, it is for sure less rich in polyphenols.

### 3.6.2 Anticholinesterase activity

The anticholinesterase activity of *E. trifolium* ME and PEE is shown in Table 8. Plants are considered an important natural source of cholinesterase inhibitors, useful in treating neurodegenerative diseases [68]. The anticholinesterase activity of ME and PEE of *E. trifolium* was determined using galantamine as a standard compound to validate these methods. Anticholinesterase assays were performed against two enzymes: AChE and BChE. The $IC_{50}$ results of the inhibitory activity of AChE showed that the activity of ME and PEE was weak (>200 µg/mL) compared to the standard value of 5.01 µg/mL. The $IC_{50}$ results of BChE showed that the methanol extract had weak inhibitory activity (>200 µg/mL) and the PEE had moderate inhibitory activity (105.1 µg/mL), significantly higher than the standard (53.9 µg/mL) (Tukey's HSD test: $p < 0.0001$). Compounds exhibiting anticholinesterase activity also involve

**Table 8:** Anticholinesterase and anti-diabetic activities ($IC_{50}$ values) of the ME and PEE of *E. trifolium*

| Extracts and compounds | Anticholinesterase activity $IC_{50}$ (µg/mL) | | Antidiabetic activity $IC_{50}$ (µg/mL) | |
|---|---|---|---|---|
| | AChE assay | BChE assay | α-Amylase inhibitory assay | α-Glucosidase inhibitory assay |
| Methanol extract | >200 (NA) | >200 (NA) | >400 (NA) | >400 (NA) |
| PEE | >200 (NA) | 105.1 ± 1.12 (***) | >400 (NA) | >400 (NA) |
| Galantamine | 5.01 ± 0.10 | 53.9 ± 0.56 | Nd | Nd |
| Acarbose | Nd | Nd | 0.26 ± 0.02 | 0.28 ± 0.0 |

AChE: acetylcholinesterase; BChE: butyrylcholinesterase. The values expressed herein are mean ± SD of three parallel measurements. Stars indicate significant differences with references (Tukey's HSD test): $p < 0.0001$ (***); Nd: not determined; NA: statistical comparison not applicable with the standard; ψ: reference compound (standard).

antiradical or antioxidant activity [69]. *E. trifolium* is rich in phenolic compounds (Table 4), such as chlorogenic acid, which is abundant in the human diet and has attracted great interest due to its antioxidant activity associated with neurological benefits [70].

The neuroprotective effects of polyphenolic gallic acid have been attributed to its ability to penetrate the blood–brain barrier [48]. Endurance exercise, chronic gallic acid administration, and simultaneous treatment with exercise and gallic acid consumption had adequate neurotrophic and immunomodulatory effects, especially in male Alzheimer's disease rats [48]. Rutin has shown its efficacy in remedying many diseases through pharmacological investigations. Its therapeutic potential has also sparked a lot of interest in numerous models of neurodegenerative disorders [71]. Several studies suggest that rutin is potentially protective against neurodegenerative diseases due to its powerful antioxidant benefits [72,73]. This is also because it can reduce and reverse Aβ25-35 fibril formation and probably in relation to its free radical scavenging activity and subdued neurotoxicity [74].

### 3.6.3 Antimicrobial activity

The antimicrobial activities of *E. trifolium* ME and PEE on *S. aureus*, *P. aeruginosa,* and *E. coli* are shown in Table 9 and Figure 5. The disc approach allowed for the determination of the effects of the plant extracts dissolved in DMSO on the various strains, according to the data acquired. This test showed that the ME had a significant antibacterial impact on all examined microorganisms. Generally, values of the inhibition zone diameter (IZD, mm) ranged between 14 and 15 mm. While the petroleum ether had moderate activity against all strains tested (7 and 8 mm), these results indicated that ME from *E. trifolium* possess the greatest inhibitory activity. A stronger antimicrobial activity was thus shown for ME, whereas the PEE from *E. trifolium* possess less clear activity. These results also confirm that ethanol extracts contain a relatively higher antimicrobial activity than PEE at the same concentration.

**Table 9:** Antimicrobial activity of ME and PEE of *E. trifolium*

| Microorganism | Extract | |
|---|---|---|
| | Methanolic | Petroleum ether |
| *S. aureus* | 15 mm | 8 mm |
| *P. aeruginosa* | 15 mm | 8 mm |
| *E. coli* | 14 mm | 7 mm |

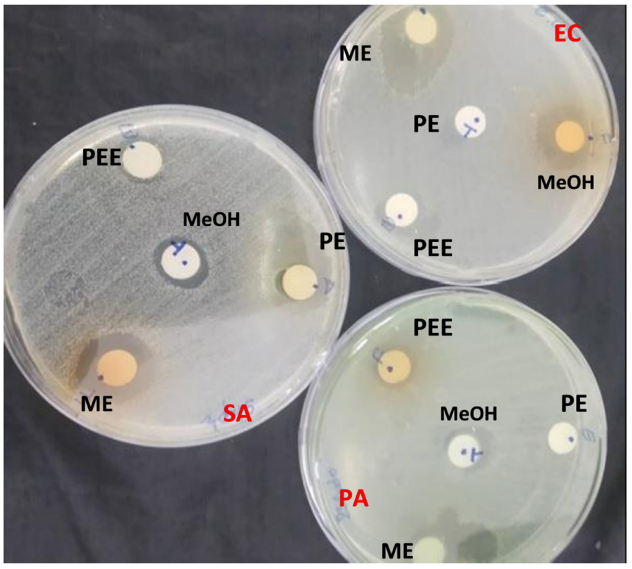


**Figure 5:** Antibacterial effectiveness of ME and PEE of *E. trifolium* using the agar disk-diffusion method. SA: *Staphylococcus aureus*; PA: *Pseudomonas aeruginosa*; EC: *Escherichia coli*; ME: methanolic extract; PEE: petroleum ether extract; MeOH: methanol; PE: petroleum ether.

This is consistent with the results of Al-Hadid et al. [75], who observed the antibacterial properties of Jordanian *E. gruinum* ethanol extract against *P. aeruginosa* and *S. aureus*. Furthermore, Abdelkebir et al. [17] showed that the extracts of *E. glaucophyllum* obtained with high extract solvent showed zone diameters of the extracts up to 14 mm for Gram-negative bacteria and up to 15 mm for Gram-positive bacteria. Besides, Bakari et al. [21] demonstrated that methanolic/water extracts of the *E. glaucophyllum* were considered a powerful antimicrobial agent against all strains of bacteria including *Bacillus cereus* JN 934390, *B. subtilis* JN 934392, *Staphylococcus aureus* ATCC 6538, *Micrococcus luteus*, *Enterococcus faecalis*, *Salmonella* enteric serotype *enteritidis* ATCC43972, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae*.

In this study, *E. trifolium* extract was shown to have promising antibacterial activity against *S. aureus*, *P. aeruginosa*, and *E. coli*. The inhibition of these strains by the ME of *E. gruinum* may also be attributed to phenolic compounds, which have recently attracted significant attention due to their diverse practical, pharmacological, and biological proprieties, such as protocatechuic acid, catechin, and epicatechin, which are known to have antibacterial activity against several pathogenic bacteria [76].

## 4 Conclusions

The current study investigated the chemical composition of ME and PEE of *E. trifolium* (Cav.) Guitt. The HPLC-MS was

used to identify and quantify eight compounds in the ME: rutin (19.2%), catechin (16.9%), caffeine (16%), and vanillin (12.1%) were the major phenolic compounds detected. The HPLC-DAD was used to identify and quantify 11 compounds in the ME, where the major compounds detected are epicatechin (21.02 mg/g extracts), catechin (10.78 mg/g), chlorogenic acid (6.09 mg/g), gallic acid (3.21 mg/g), and rutin (2.19 mg/g). The GC-MS was used to detect 30 fatty acids and volatile in PEE, where the dominant compounds were palmitic acid (29.49%), oleic acid (21.48%), and linoleic acid (7.16%). We also determined the mineral contents and the results indicated that the highest values of macro-elements were calcium (2.15%) and microelements were iron (627.67 ppm). Furthermore, the antioxidant activities showed that the extract of methanolic was significative with all assays (4.76 µg/mL for DPPH, 20.6 µg/mL for ABTS, and 13.23 µg/mL for CUPRAC) compared with petroleum ether. In addition, anticholinesterase (AChE and BChE) activity, α-amylase, and α-glucosidase were evaluated in both extracts and showed moderate inhibition for all extracts and activities. Based on the antibacterial activities observed, it was clear that ME showed the highest antimicrobial activity, with values of 15 mm for *S. aureus* and *P. aeruginosa* for each, and 14 mm for *E. coli*. Overall, our findings suggest that *E. trifolium* exhibits interesting properties and may be used as a potential source of natural phenolic compounds for feeding and pharmaceutical purposes. More studies are currently needed to investigate the presence of medicinal compounds in different solvent extracts and their bioactivities.

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