

Article

Potential Natural Antioxidant and Anti-Inflammatory Properties of *Carthamus caeruleus* L. Root Aqueous Extract: An In Vitro Evaluation

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Abstract: *Carthamus caeruleus* L. is traditionally used in Algerian medicine, particularly for burn treatment, but its therapeutic potential remains insufficiently studied. This study aimed to evaluate the antioxidant and anti-inflammatory properties of the root aqueous extract, and to perform phytochemical characterization to identify its bioactive compounds. Phytochemical analysis was conducted using spectrophotometry and reverse-phase high-performance liquid chromatography (RP-HPLC). The antioxidant potential was assessed through various assays, including ferric reducing antioxidant power (FRAP), total antioxidant capacity (TAC), DPPH radical scavenging, hydroxyl radical scavenging, ferrous ion chelation, and hydrogen peroxide decomposition. Anti-inflammatory activity was evaluated using membrane stabilization, protein denaturation, and membrane peroxidation assays. The extract exhibited moderate levels of polyphenols, flavonoids, and condensed tannins, quantified as 21.19 ± 0.37 mg GAE/g, 0.72 ± 0.013 mg QE/g, and 27.28 ± 1.04 mg TAE/g of dry extract, respectively. RP-HPLC analysis identified 22 phytochemical compounds, primarily phenolic acids, flavonoids, and tannins, with orientin and vanillin as the major constituents. The extract demonstrated significant antioxidant activity, with moderate efficacy in TAC and FRAP assays (IC_{50} values of 5405.1 ± 4.42 and 1132.35 ± 4.97 μ g/mL, respectively). Notable activities included DPPH and hydroxyl radical scavenging (34.43 ± 4.83 and 512.81 ± 9.46 μ g/mL, respectively), ferrous ion chelation (2462.76 ± 1.38 μ g/mL), lipid peroxidation inhibition ($22.32 \pm 3.31\%$), and hydrogen peroxide decomposition (263.93 ± 7.87 μ g/mL). Additionally, the extract stabilized erythrocyte membranes under osmotic, thermal, and oxidative stress conditions ($98.13 \pm 0.15\%$, $70 \pm 1.27\%$, and $89 \pm 0.87\%$, respectively), inhibited ovalbumin denaturation ($81.05 \pm 2.2\%$), and protected against lipid peroxidation in brain homogenates ($69.25 \pm 0.89\%$). These findings support the traditional therapeutic applications of *C. caeruleus* and highlight its potential as a source of antioxidant and anti-inflammatory agents.

Keywords: *Carthamus caeruleus* L.; polyphenols; flavonoids; tannins; HPLC; oxidative stress; inflammatory



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1. Introduction

The Global Burden of Disease report [1] highlights a concerning rise in both chronic and acute diseases worldwide, posing a substantial challenge to public health systems. Major non-communicable diseases, including cardiovascular disorders, malignancies, chronic respiratory conditions, diabetes mellitus, and severe infectious diseases, have emerged as significant global health concerns, impacting both individual well-being and healthcare infrastructure [2,3]. Current evidence indicates that oxidative stress and inflammatory processes are fundamental, interrelated pathophysiological mechanisms underlying these disease states [4,5]. These interconnected pathways serve as critical targets for therapeutic intervention in the management of chronic diseases.

Oxidative stress is a pathological condition characterized by an excessive accumulation of free radicals—highly reactive molecular species generated through normal cellular metabolism. These reactive molecules, distinguished by unpaired electrons in their outer orbitals, exhibit high chemical reactivity, seeking electron transfer to achieve molecular stability. Under physiological conditions, cellular homeostasis is maintained through a delicate balance between reactive oxygen species (ROS) and endogenous antioxidant defense mechanisms that neutralize their activity. However, disruption of this equilibrium, either due to excessive free radical production or impaired antioxidant capacity, results in oxidative stress. This imbalance leads to structural and functional alterations in essential cellular macromolecules, including proteins, lipids, carbohydrates, and nucleic acids [6].

Oxidative stress-induced cellular damage acts as a potent activator of inflammatory responses [7]. While inflammation is a fundamental protective mechanism, its chronic persistence—independent of the initial stimulus—can lead to detrimental pathological consequences [8]. The sustained interplay between oxidative stress and chronic inflammation fosters a pathogenic environment that contributes to the progression of various disease states [5].

Current therapeutic strategies for managing oxidative stress and inflammation primarily rely on synthetic pharmaceutical interventions [9]. Despite their efficacy, these conventional treatments present notable limitations, particularly adverse effects that may compromise their long-term clinical utility [8,10]. These challenges underscore the urgent need for alternative therapeutic strategies with improved efficacy and safety profiles to address these complex pathophysiological conditions.

Phytotherapeutic agents, which have been utilized throughout human history, represent a vast reservoir of bioactive metabolites with significant therapeutic potential, warranting extensive scientific investigation [4,11]. Numerous pharmacological studies have validated the therapeutic efficacy of plant-derived compounds, particularly their potent antioxidant and anti-inflammatory properties [12]. Compared to synthetic pharmaceuticals, natural compounds from botanical sources generally exhibit superior safety profiles, providing a significant advantage in therapeutic applications [13]. The historical precedence of ethnopharmacological applications, along with widespread patient acceptance, further enhances their therapeutic value [14]. Recognizing this potential, contemporary global health initiatives, including those led by the World Health Organization (WHO), are allocating substantial resources to explore and optimize the medicinal applications of plant-derived compounds [15].

Burn injuries induce an inflammatory response that is essential for the healing process. However, excessive inflammation during the early stages of wound healing can lead to disfiguring and problematic scars, often necessitating surgical intervention. Therefore, effective inflammation management is crucial to prevent such complications [16]. Additionally, burns trigger an inflammatory cascade mediated by various chemical molecules,

including free radicals generated by heat exposure. The administration of antioxidants has been shown to mitigate this response, thereby promoting the healing process [17].

Given the detrimental effects of excessive inflammation and oxidative stress on burn injuries, natural remedies with antioxidant and anti-inflammatory properties—such as *Carthamus tinctorius* [18], *Carthamus oxyacantha* [19], and particularly *Carthamus caeruleus* [11]—are receiving increasing scientific interest. *C. caeruleus* L., a plant of significant relevance in Algerian traditional medicine, has long been utilized for the treatment of various dermatological conditions, particularly burn injuries [11,20]. Despite its established ethnopharmacological applications, scientific investigations into its therapeutic properties remain limited. This study provides new insights into the in vitro antioxidant and anti-inflammatory potential of *C. caeruleus* L. root aqueous extract, evaluating these properties for the first time through in vitro assays. Furthermore, phytochemical characterization will be conducted to elucidate the molecular basis underlying its traditional therapeutic applications.

2. Materiel and Methods

2.1. Chemicals and Instruments

The chemical products utilized in this study are: Folin–Ciocalteu reagent, sodium bicarbonate (Na_2CO_3), gallic acid, aluminum trichloride (AlCl_3), quercetin, Folin–Denis, tannic acid, sulfuric acid, sodium phosphate, ammonium molybdate, ascorbic acid, potassium ferricyanide, trichloroacetic acid (TCA), iron chloride (FeCl_3), 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), thiobarbituric acid, linoleic acid, ethylenediamine tetraacetic acid (EDTA), ferrous chloride (FeCl_2), ferrozine, iron sulfate (FeSO_4), hydrogen peroxide, saponin, aspirin, phosphate-buffered saline (PBS), sodium chloride (NaCl), hypochlorous acid (HClO), ovalbumin, sodium dodecyl sulfate (SDS), and acetic acid. The absorbances in this study were measured using a MEDLINE MD 2000 UV-visible spectrophotometer (Medline Industries, Inc., Northfield, IL, USA).

2.2. Plant Collection

Roots of *C. caeruleus* L. were collected in May 2022 from Freha, Tizi-Ouzou, Algeria ($36^\circ 45' 8.42''$ N; $4^\circ 18' 55.80''$ E). Botanical identification was carried out by Dr. Benghanem Nabil, a botanist at the National Superior School of Agronomy (ENSA), Algiers, Algeria. A sample was placed in the herbarium of Mouloud Mammeri University (UMMTO) under the reference number 2023/UMMTO/21. After cleaning, the roots were air-dried in the dark for 15 days, and then ground into a fine powder using an electric grinder (Siyo Lux Electro). The resulting powder was stored in opaque, hermetically sealed containers, and stored in a dry place at room temperature for later use.

2.3. Aqueous Extraction

Aqueous extraction was prepared by macerating 10 g of the powdered roots in 100 mL of distilled water (10%, w/v) under magnetic stirring at room temperature for 24 h. The macerate underwent coarse filtration through a sieve, followed by fine filtration using Whatman No. 1 paper. The filtrate was then lyophilized and stored in the dark.

2.4. Phytochemical Studies

2.4.1. Total Phenolic Content

Total polyphenol content was estimated using the Folin–Ciocalteu method, as described by Haddouchi et al. [21]. This technique relies on the oxidation of phenolic compounds by phosphomolybdic and phosphotungstic acids present in the Folin–Ciocalteu reagent, which results in a blue color upon reduction. In brief, 0.2 mL of the extract

(1 mg/mL) was mixed with 1 mL of freshly diluted Folin–Ciocalteu reagent (1:10, *v/v*). After a 4 min incubation at room temperature in the dark, 0.8 mL of sodium carbonate solution (Na_2CO_3) (7.5%, *w/v*) was added. The mixture was then stirred and incubated under the same conditions for 45 min. Absorbance was measured at 760 nm, and results were expressed as milligrams of gallic acid equivalent per gram of dry extract (mg GAE/g) based on a gallic acid calibration curve.

2.4.2. Total Flavonoid Content

Total flavonoid content was quantified according to Akrou et al. [22] using aluminum trichloride (AlCl_3), which forms a complex with flavonoids. A volume of 1 mL of the extract (10 mg/mL) was mixed with 1 mL of a 2% aluminum trichloride methanolic solution. Absorbance was measured after incubating for 15 min at 430 nm against a blank, where the extract was substituted with 1 mL of distilled water. Results were expressed as milligrams of quercetin equivalent per gram of dry extract (mg QE/g).

2.4.3. Condensed Tannins

The quantification of condensed tannins was performed using the Folin–Denis reagent, as described by Hmid et al. [23]. This method is based on the reduction of a mixture containing phosphomolybdic acid, sodium tungstate, and phosphoric acid in the presence of a saturated sodium carbonate solution, resulting in the formation of tungsten and molybdenum oxides, which impart a blue color.

A volume of 0.1 mL of the sample (10 mg/mL) was mixed with 0.75 mL of distilled water, 1 mL of Folin–Denis reagent, and 2 mL of saturated Na_2CO_3 solution. After incubating the mixture at room temperature for 30 min, the absorbance was measured at 760 nm. Results were reported in terms of milligrams of tannic acid equivalent per gram of dry extract (mg TAE/g).

2.4.4. RP-HPLC Analysis

Chromatographic analysis utilizing reverse-phase high-performance liquid chromatography (RP-HPLC) was conducted for the separation and identification of polyphenolic compounds in *C. caeruleus* L. root aqueous extract. This analysis employed an AGILENT 1100 series system equipped with a diode array detector (DAD G1315B), with varying wavelengths selected based on the specific molecules under investigation. The stationary phase of the C18 reverse-phase column (dimensions: 4.6×250 mm, particle size: 5 μm), was used for the separation. The mobile phase was a gradient composed of two solvents: acidified water (0.2% acetic acid, pH 3.1) and acetonitrile. The flow rate was set at 1.5 mL/min for 30 min at room temperature, beginning with 95% of acidified water and gradually increasing to 100% acetonitrile. Extract solutions were prepared in methanol at a concentration of 100 mg/mL, and were filtered through a 0.45 μm membrane filter before injection. The identification of phytochemical compounds in the extract was achieved by comparing their retention times with those of known reference molecules [24,25].

2.5. Antioxidant Activity

2.5.1. Total Antioxidant Capacity (TAC)

The total antioxidant capacity of the extract was assessed using the method outlined by Rao et al. [26]. This approach relies on the reduction of the molybdate ion Mo (VI) to Mo (V) by plant antioxidants, leading to the formation of a blue-green color.

To conduct the assay, 0.1 mL of the extract or a reference antioxidant (vitamin C) at concentrations ranging from 100 to 1000 $\mu\text{g/mL}$ was mixed with 1 mL of a reagent solution consisting of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The resulting mixture was incubated at 95 °C for 90 min, and absorbance was

measured at a wavelength of 695 nm. The total antioxidant capacity was calculated by determining the median inhibitory concentration (IC_{50}) from the linear regression curve plotted from the results graph.

2.5.2. Ferric Reducing Antioxidant Power (FRAP)

This test was conducted following the method of Moualek et al. [27], based on the reduction of ferric ions (Fe^{3+}) from potassium ferricyanide to ferrous ions (Fe^{2+}), resulting in the formation of a Prussian blue color. Consequently, the quantity of Fe^{2+} is determined by the formation of a Prussian blue complex.

For the test, 0.4 mL of the extract (100 and 1000 $\mu g/mL$) was combined with 0.4 mL of phosphate buffer (0.2 M, pH 6.6) and 0.4 mL of a 1% potassium ferricyanide solution. The mixture was incubated at 50 °C for 20 min, then 0.4 mL of 10% trichloroacetic acid was added to each tube. After centrifugation at $950 \times g$ for 10 min, 0.4 mL of the supernatant was transferred and mixed with 0.4 mL of distilled water and 0.16 mL of 0.1% $FeCl_3$. Absorbance was measured at 700 nm, and the results were expressed as the IC_{50} . Vitamin C was used as a positive control (10 to 100 $\mu g/mL$).

2.5.3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Test

The extract antiradical activity was assessed using a DPPH radical, as described by Toubane et al. [11]. DPPH is a stable purple radical that changes to yellow upon reduction by the extract.

To perform the assay, 1.5 mL of the extract (100 to 1000 $\mu g/mL$) or vitamin C (1 to 10 $\mu g/mL$) was combined with 0.1 mL of a DPPH methanolic solution (0.8 mM). The control tube contained methanol, replacing the extract. The mixture was incubated for 30 min, and the absorbance was measured at 517 nm. The scavenging activity was calculated using the following equation. The IC_{50} was determined graphically.

$$\text{Scavenging activity (\%)} = [(A_c - A_s) / A_c] \times 100 \quad (1)$$

A_c : absorbance of the control (methanol); A_s : absorbance of the sample.

2.5.4. Inhibition of Lipid Peroxidation: Thiobarbituric Acid (TBA) Method

Lipid peroxidation inhibition was assessed according to Kizil et al. [28] using linoleic acid. At elevated temperatures (100 °C) and low pH, thiobarbituric acid forms a red complex with malondialdehyde (MDA), which is generated during the oxidation of linoleic acid. In brief, 2 mL of the extract (1 mg/mL) or ethanol (control) was combined with 2 mL of linoleic acid (2.5%) dissolved in ethanol, 4 mL of phosphate buffer (0.05 M, pH 7), and 2 mL of distilled water. After incubating at 40 °C for 30 min in the dark, 1 mL of this mixture was mixed with 2 mL of trichloroacetic acid (20%) and 2 mL of thiobarbituric acid aqueous solution (0.67%). The tubes were then incubated for 10 min at 100 °C, centrifuged at $950 \times g$ for 20 min, and cooled. The absorbance of the supernatant was recorded at 532 nm, and the lipid peroxidation inhibition percentage was determined using the following calculation:

$$\text{Protection(\%)} = (A_c - A_s) / A_c \times 100 \quad (2)$$

“ A_c ” is the absorbance of the control (ethanol) and “ A_s ” is the absorbance of the sample. Butyl hydroxy toluene (BHT) served as the positive control of lipid peroxidation inhibition (1 mg/mL).

2.5.5. Ferrous Ion-Chelating Activity

The method described by Nabavi et al. [29] assesses the ion-chelating capacity of *C. caeruleus* L. roots extract. In this technique, a complex is formed between ferrozine and free iron from ferrous chloride (ferrozine-Fe²⁺). Consequently, the inhibition of this complex formation indicates the chelation of ferrous ions by the extract, reflecting its antioxidant potential.

Briefly, in each tube, 0.1 mL of the extract or EDTA (ethylenediamine tetra-acetic acid) (as a standard) at various concentrations was combined with 0.05 mL of ferrous chloride (FeCl₂, 2 mM) and 0.2 mL of ferrozine (5 mM). The mixture was incubated for 10 min at room temperature. A control tube was prepared by substituting distilled water for the extract. The absorbance was recorded at 562 nm, and the results were presented as a percentage of chelation using Equation (3). Finally, the IC₅₀ was determined to evaluate the concentration at which half of the chelating activity is achieved.

$$\text{Chelation}(\%) = \left[\frac{(A_c - A_s)}{A_c} \right] \times 100 \quad (3)$$

A_c: absorbance of the control (distilled water); A_s: absorbance of the sample.

2.5.6. Hydroxyl Radical Scavenging Assay

To assess the extract's ability to scavenge hydroxyl radicals (OH•), the method of Rajamanikandan et al. [30] was applied, which involves generating these radicals through the Fenton reaction. A total of 0.5 mL of the extract or vitamin C (200 to 2000 µg/mL) was added to 1 mL of FeSO₄ (1.5 mM), 0.7 mL of hydrogen peroxide (6 mM), and 0.3 mL of sodium salicylate (20 mM). The mixture was incubated for 1 h at 37 °C, after which the absorbance was read at 560 nm. Radical scavenging percentage was determined using Equation (4), and the IC₅₀ was determined graphically.

$$\text{Scavenging}(\%) = \left[\frac{(A_c - A_s)}{A_c} \right] \times 100 \quad (4)$$

A_c: absorbance of the control (distilled water); A_s: absorbance of the sample.

2.5.7. Hydrogen Peroxide (H₂O₂) Decomposition

C. caeruleus L. root extract capacity to decompose hydrogen peroxide (H₂O₂) was evaluated according to the method of Serteser et al. [31]. In each tube, 3.4 mL of either the extract or vitamin C, dissolved in 0.1 M phosphate buffer (pH 7.4), was combined with 0.6 mL of a 40 mM hydrogen peroxide solution in the same buffer. In the control tube, the extract was substituted with phosphate buffer. After incubating for 10 min in the dark, the absorbance was read at 230 nm. H₂O₂ decomposition was calculated according to Equation (5), and the IC₅₀ was graphically determined.

$$\text{Decomposition of H}_2\text{O}_2(\%) = \left[\frac{(A_c - A_s)}{A_c} \right] \times 100 \quad (5)$$

A_c: absorbance of the control (distilled water); A_s: absorbance of the sample.

2.6. Erythrocyte Suspension Preparation

Venous blood collected from healthy human donors in heparinized tubes was used to prepare an erythrocyte suspension. The University Ethics Committee approved the experimental protocol for this study under approval number 2023/EC/UMMTO-01, in accordance with the Algerian legislation (NoEthi/UMMTO/02/QPR-2022). Tubes containing blood were centrifuged at 700 × g/r 5 min/4 °C. The pellet obtained was washed three

times with phosphate-buffered saline (PBS), which consisted of NaCl (150 mM), NaH₂PO₄ (1.9 mM), and Na₂HPO₄ (8.1 mM), adjusted to pH 7.4. After washing, the pellet was re-dispersed in PBS and kept at 4 °C for later analysis.

2.7. Analysis of the Extract's Toxicity

The toxicity of the extract was assessed *in vitro*, as described by Haddouchi et al. [21]. An amount of 0.1 mL of the extract or saponine (1 to 8 mg/mL) was mixed with 1.9 mL of 5% erythrocyte suspension. Tubes were incubated for 1 h at 37 °C, and then centrifuged at 700× g/5 min/4 °C. Distilled water was employed as a positive control for hemolysis, with PBS serving as the negative control. The supernatant's absorbance was read at 540 nm, and the hemolysis percentage was calculated using the following equation:

$$\text{Hemolysis (\%)} = [(A_s - A_{c-}) / A_{c+}] \times 100 \quad (6)$$

A_s: absorbance of extract/saponin; A_{c−}: absorbance of negative control (PBS); A_{c+}: absorbance of the positive control (distilled water).

2.8. Anti-Inflammatory Activity

2.8.1. Protection Against Hypotonic Stress-Induced Hemolysis

The extract's anti-inflammatory activity was assessed, *in vitro*, on erythrocytes, following the procedure outlined by Henneh et al. [32]. This test aims to determine the extract's ability to stabilize erythrocyte membranes by preventing hemolysis induced by hypotonic stress. An amount of 1 mL of the extract (250 to 2000 µg/mL) was prepared in various concentrations of sodium chloride (NaCl: 0.9%, 0.7%, 0.5%, 0.3%, 0.1%), and 0.025 mL of the erythrocyte suspension was added. Then, the tubes were incubated for 30 min at 37 °C while stirring. A control containing distilled water instead of the extract was used. After incubation, tubes were centrifuged at 700× g/5 min/4 °C, and the supernatant was analyzed by measuring its absorbance at 540 nm. The protection percentage was determined using the following Equation (7):

$$\text{Protection (\%)} = \left[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \right] \times 100 \quad (7)$$

A_{sample}: absorbance of extract at different concentrations of NaCl; A_{control}: absorbance of negative control (distilled water).

2.8.2. Protection Against Heat Stress-Induced Hemolysis

The extract's protective effect against heat-induced lysis was tested as described by Henneh et al. [32]. An amount of 1 mL of the extract or aspirin (a reference anti-inflammatory) dissolved in PBS (at concentrations ranging from 100 to 3000 µg/mL) was combined with 1 mL of a 2% erythrocyte suspension, then incubated at 56 °C for 30 min. A control was included, with PBS replacing the extract. The tubes were centrifuged after cooling at 700× g/5 min/4 °C, and then the supernatant's absorbance was read at 560 nm. The percentage of protection against thermal denaturation was calculated according to Formula (7).

2.8.3. Protection Against Oxidative Stress-Induced Hemolysis

This study follows the protocol described by Suwalsky et al. [33] to test the extract's ability to protect erythrocytes against oxidative stress induced by hypochlorous acid (HClO). An amount of 1 mL erythrocyte suspension (at 10%) was mixed with 1 mL of the extract or aspirin dissolved in PBS (100 to 2000 µg/mL). After incubation at 37 °C for 15 min, 0.05 mL of HClO (0.5 mM) was added, followed by a second incubation at the same temperature

for 10 min. The mixture was centrifuged at $700 \times g$ for 5 min/4 °C, and the supernatant's absorbance was read at 540 nm. The percentage of protection was calculated according to Formula (7).

2.8.4. Inhibition of Ovalbumin Denaturation

The ability of the extract to prevent protein denaturation, such as in ovalbumin, is tested in this study, according to the protocol described by Ullah et al. [34]. A 5 mL reaction mixture was prepared, containing 0.2 mL ovalbumin (0.2%, *w/v*), 2.8 mL PBS (pH 6.4), and 2 mL extract or aspirin (from 20 to 2000 µg/mL). The control contained PBS instead of the extract. The mixture was heated to 72 °C for 5 min, then the absorbance was measured after cooling at 660 nm. The protection's percentage against ovalbumin denaturation was calculated using Formula (7).

2.8.5. Assessment of Lipid Peroxidation in Animal Tissues Using the TBARS Method (Thiobarbituric Acid Reactive Substance)

The inhibition of lipid peroxidation was assessed as described by Sakat et al. [35], adapted for the analysis of thiobarbituric acid reactive substances (TBARS) in brain homogenate prepared according to Anoopkumar-Dukie et al. [36]. Wistar rats (130–160 g), provided by the Pasteur Institute of Algeria, were sacrificed in accordance with Algerian legislation (Law No. 12-235/2012) to prepare the brain homogenate. The collected brains were homogenized at a 10% (*w/v*) ratio with PBS (0.1 M, pH 7.4).

In each assay, 1 mL of the homogenate was added to 1 mL of the extract or butylhydroxytoluene (BHT) as a standard (200 to 4000 µg/mL), followed by 0.1 mL of FeSO₄ (15 mM). After incubating for 30 min at 37 °C, 0.1 mL of the mixture (homogenate + extract/BHT) was mixed with 0.1 mL of SDS (8.1%, *w/v*), 0.75 mL of acetic acid (20%), and 0.75 mL of thiobarbituric acid (TBA, 0.8%). The final volume was adjusted to 2 mL with distilled water, and then heated 1 h at 95 °C. After cooling and centrifugation at $700 \times g$ for 10 min, the supernatant's absorbance was recorded at 532 nm, and the protection percentage against peroxidation was calculated according to Formula (7).

2.9. Statistical Analysis

Each test was performed in triplicate, and the results are presented as the mean $\pm$ standard error of the mean (SEM). Statistical analysis was conducted using Student's *t*-test with SPSS software (version 25), considering a *p*-value of <0.05 ($p < 0.05$) as statistically significant.

3. Results

3.1. Phytochemical Analysis

The present study revealed moderate quantities of polyphenols, flavonoids, and condensed tannins in root extract, measured at 21.19 ± 0.37 mg GAE/g, 0.72 ± 0.013 mg QE/g, and 27.28 ± 1.04 mg TAE/g dry extract, respectively. These concentrations were determined using linear regression equations derived from calibration curves of gallic acid, quercetin, and tannic acid, respectively.

3.2. RP-HPLC Profile

The RP-HPLC profile of *C. caeruleus* L. root aqueous extract is summarized in Table 1. The results identified 22 chemical compounds, demonstrating a wide range of active components, which may contribute to the extract's potential therapeutic effects. As shown in Table 1, the identified biochemical molecules can be grouped into various categories, including phenolic acids and flavonoids (such as flavonols, flavanones, and flavones). Many of these compounds have been reported in the literature to exhibit antioxidant and/or anti-inflammatory activities when tested individually, as referenced in the table.

Table 1. Molecules identified by RP-HPLC in *C. caeruleus* L root aqueous extract and their classification.

Class	Components	References for Classification	Retention Time (min)	Area (%)	Biological Activity/References	
Phenolic acids	2,3-dimethyl cinnamic acid	[37]	14.760	1.5895	ND	
	3,4,5-Trimethoxy benzoic acid	[37]	10.908	2.1882	ND	
	Dihydroxycinnamic acid	[37]	7.256	2.1875	ND	
	Ferulic acid	[37]	9.250	2.8991	Antioxidant [38] Anti-inflammatory [39]	
	Isovanillic acid	[40]	7.545	4.1639	Antioxidant [40]	
	m-Anisic acid	[41]	12.169	0.9167	ND	
	p-Coumaric acid	[42]	9.616	2.0014	Antioxidant [42] Anti-inflammatory [43]	
	Rosmarinic acid	[44]	10.196	3.0362	Antioxidant [44] Anti-inflammatory [45]	
	Tannic acid	[46]	3.538	2.4771	Antioxidant [47] Anti-inflammatory [47]	
	Flavonoids	Flavonols	Myricetin	[48]	11.255	1.2076
Quercetin			[48]	12.932	1.6805	Antioxidant [50] Anti-inflammatory [51]
Rutin			[52]	8.832	2.5702	Antioxidant [52] Anti-inflammatory [52]
Flavanones		Hesperidin	[53]	15.579	0.4340	Antioxidant [54] Anti-inflammatory [54]
		Luteolin	[55]	12.658	1.2398	Antioxidant [55] Anti-inflammatory [56]
Flavones		Orientin	[57]	8.260	4.9447	Antioxidant [57] Anti-inflammatory [56]
		Vitexin	[58]	7.024	2.9931	Antioxidant [58] Anti-inflammatory [58]
		Caffeine (alkaloids)	[59]	6.370	1.5039	Antioxidant [60]
Other		Coumarin	[61]	11.784	1.4114	Antioxidant [50] Anti-inflammatory [50]
		Hydroxy-quinone (quinone)	[61]	3.895	3.3714	ND
	P-hydroxybenzaldehyde	[62]	7.892	2.7577	ND	
	Resorcinol	[63]	5.173	1.4332	Antioxidant [63] Anti-inflammatory [64]	
	Vanillin	ND	8.415	4.5950	Antioxidant [65] Anti-inflammatory [66]	

ND (not defined).

3.3. Antioxidant Activity

3.3.1. Total Antioxidant Capacity (TAC)

The total antioxidant capacity (TAC) of *C. caeruleus* L. root aqueous extract, measured using the phosphomolybdate method, is presented in Figure 1. The IC₅₀ value, determined graphically, was calculated using the linear regression equation derived from the calibration curve. A low IC₅₀ value (expressed in µg/mL) indicates high antioxidant activity. In this

study, the reducing potential of the standard ($471.34 \pm 1.41 \mu\text{g/mL}$) was significantly higher than that of the roots ($5405.1 \pm 4.42 \mu\text{g/mL}$) ($p < 0.05$). A dose-dependent antioxidant activity was noted.

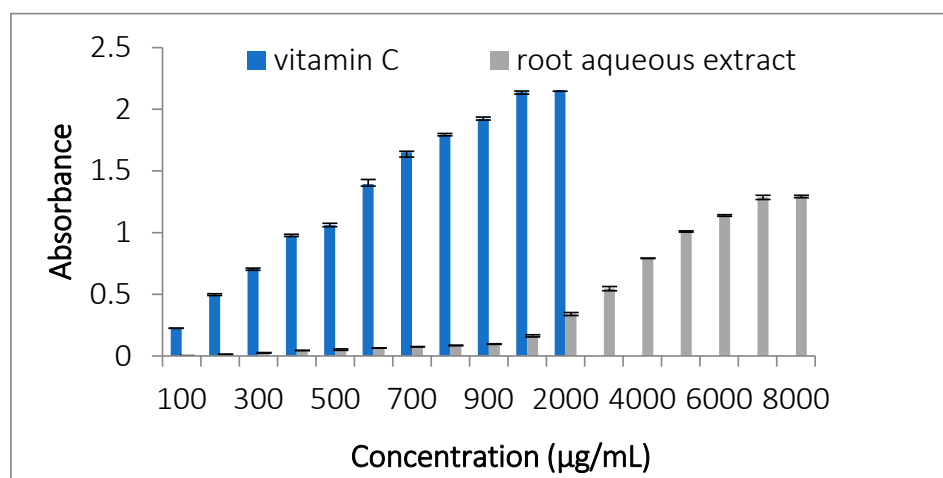


Figure 1. Total antioxidant capacity of vitamin C and *C. caeruleus* L. root aqueous extract.

3.3.2. Ferric Reducing Antioxidant Power (FRAP)

To assess the antioxidant potential, the iron-reducing capacity of vitamin C and root extract was evaluated. Figure 2 displays the dose–response curves for both samples, with the IC_{50} value representing the concentration needed to reduce half of the iron in the assay. The IC_{50} values obtained were $50.47 \pm 0.34 \mu\text{g/mL}$ for vitamin C and $1132.35 \pm 4.97 \mu\text{g/mL}$ for the root extract. Vitamin C showed significantly higher antioxidant activity than the root extract, as evidenced by its lower IC_{50} value ($p < 0.05$).

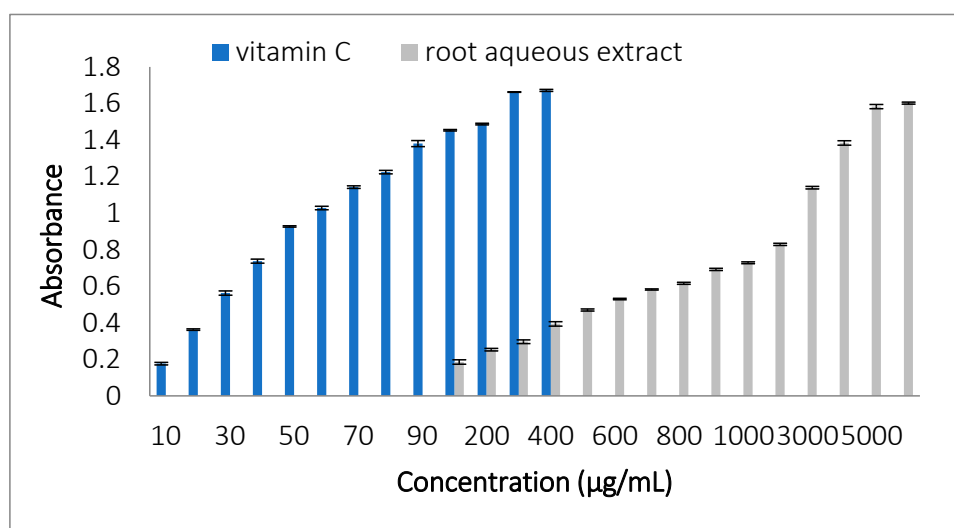


Figure 2. Ferric reducing power of vitamin C and *C. caeruleus* L. root aqueous extract.

3.3.3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Test

The DPPH scavenging activity was evaluated, and the results are presented in Figure 3. The IC_{50} values obtained were $6.29 \pm 0.04 \mu\text{g/mL}$ for vitamin C and $34.43 \pm 4.83 \mu\text{g/mL}$ for the root extract. Figure 3 shows the dose–response curves for both samples. Vitamin C exhibited significantly higher scavenging activity compared to the root extract ($p < 0.05$).

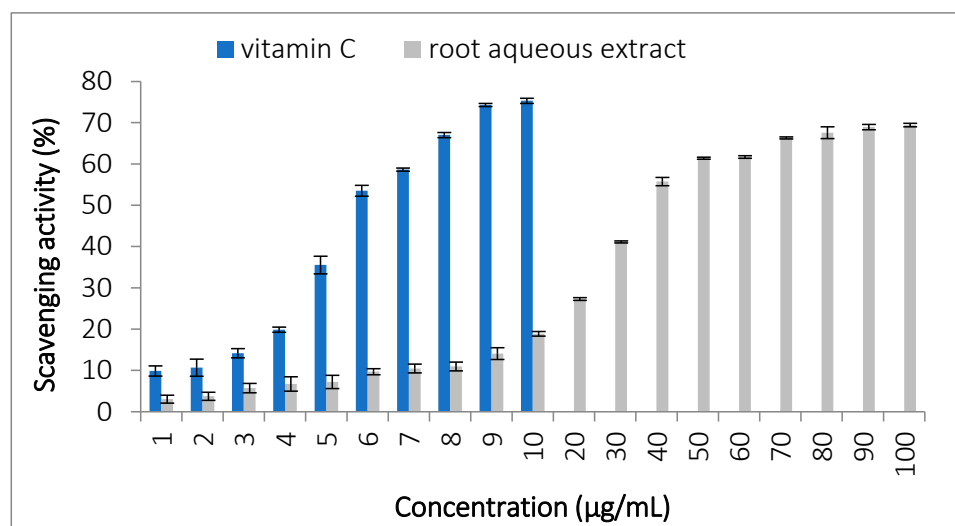


Figure 3. DPPH scavenging effect of vitamin C and *C. caeruleus* L. root aqueous extract.

3.3.4. Ferrous Ion Chelating Activity

This test demonstrates the extract's ability to chelate ferrous ions and inhibit the formation of the ferrozine-Fe²⁺ complex. The chelating activity of the root extract and EDTA is shown in Figure 4, with IC₅₀ values of 2462.76 ± 1.38 µg/mL for the extract and 57.97 ± 1.29 µg/mL for EDTA. EDTA exhibited significantly higher chelating activity compared to the extract ($p < 0.05$).

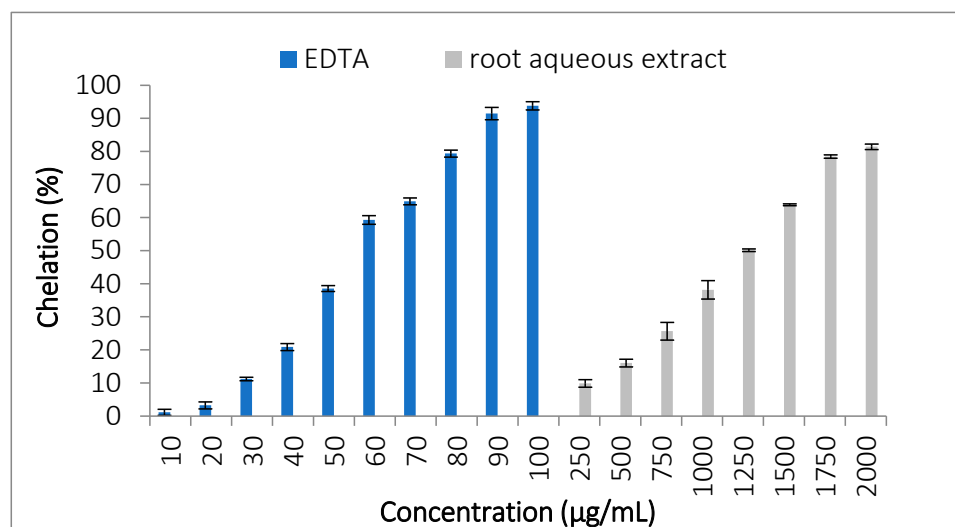


Figure 4. Chelating activity of EDTA and *C. caeruleus* L. root aqueous extract.

3.3.5. Antioxidant Activity in the Linoleic Acid System: Thiobarbituric Acid (TBA) Method

Linoleic acid peroxidation inhibition by the extract was assessed using the thiobarbituric acid method. The results, presented in Figure 5, show the percentage of protection against lipid peroxidation. BHT (1 mg/mL) provided the highest protection (77.36 ± 1.88%), followed by the root extract (22.32 ± 3.31%) ($p < 0.05$).

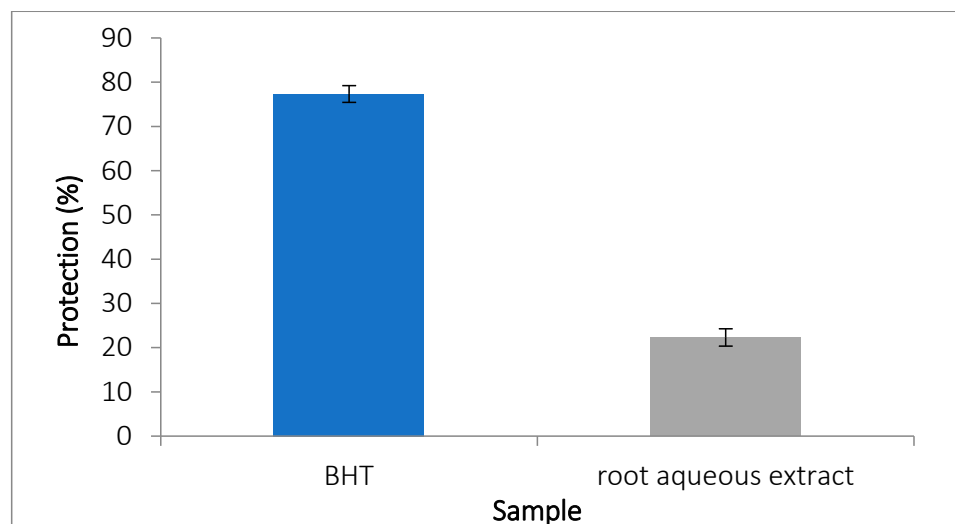


Figure 5. Inhibition of lipid peroxidation of BHT and *C. caeruleus* L. root aqueous extract.

3.3.6. Hydroxyl Radical Scavenging Assay

C. caeruleus L. root extract showed a higher hydroxyl radical ($\text{OH}^\bullet$) scavenging capacity compared to vitamin C, as demonstrated in Figure 6. The IC_{50} value for root extract was $512.81 \pm 9.46 \mu\text{g/mL}$, indicating its ability to neutralize hydroxyl radicals effectively. In contrast, vitamin C exhibited a higher IC_{50} value of $715.92 \pm 1.06 \mu\text{g/mL}$ ($p < 0.05$), showing weaker scavenging activity than the root extract. This study highlights, for the first time, the potential of *C. caeruleus* L. root extract to scavenge hydroxyl radicals, suggesting its significant antioxidant properties.

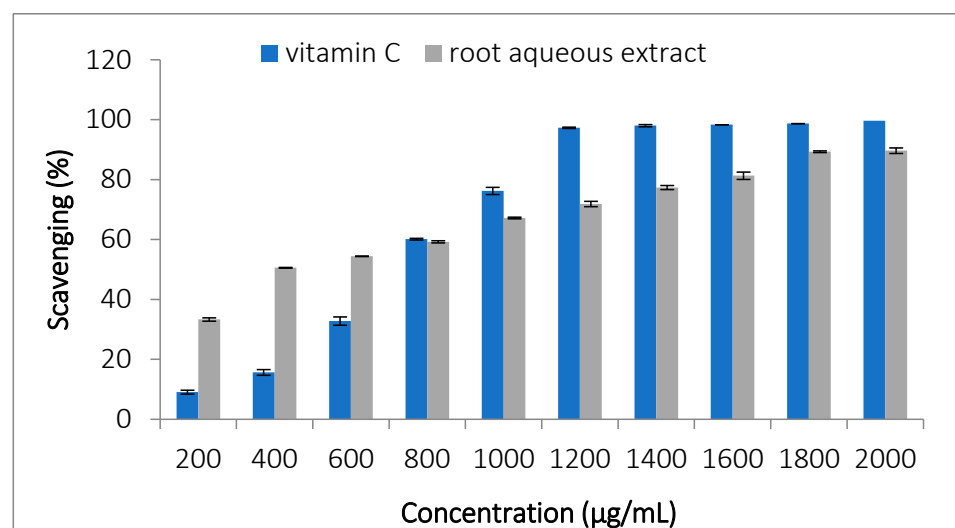


Figure 6. Hydroxyl radical scavenging effect of vitamin C and *C. caeruleus* L. root aqueous extract.

3.3.7. Hydrogen Peroxide (H_2O_2) Decomposition

This test assesses the ability of the extract to decompose H_2O_2 compared with a standard (vitamin C), as shown in Figure 7. A significant difference is recorded between the standard and the extract. Vitamin C exhibited the highest activity (IC_{50} of $37.84 \pm 5.98 \mu\text{g/mL}$), followed by roots (IC_{50} of $263.93 \pm 7.87 \mu\text{g/mL}$) ($p < 0.05$). This is the first study to document the ability of *C. caeruleus* L. root extract to decompose hydrogen peroxide.

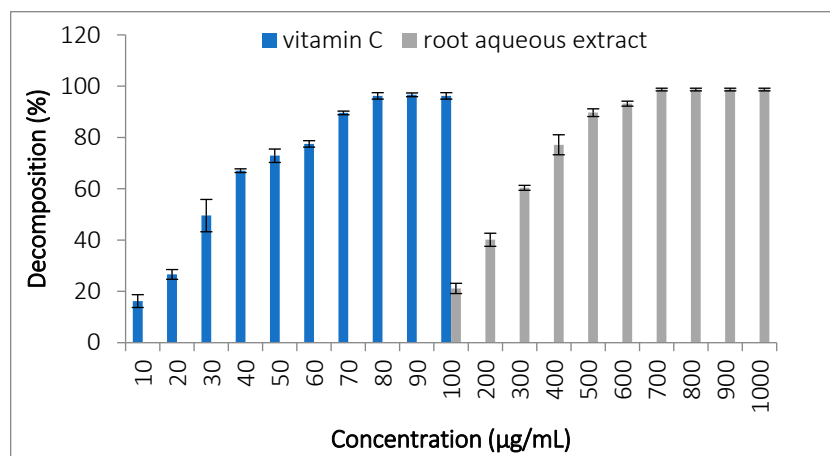


Figure 7. Hydrogen peroxide decomposition activity of vitamin C and *C. caeruleus* L. root aqueous extract.

3.4. Analysis of the Extract's Toxicity

To properly evaluate the anti-inflammatory activity of an extract, it is essential to assess its safety profile to rule out cytotoxicity, avoid interference with anti-inflammatory assays, and confirm its suitability for pharmacological applications [21]. As shown in Figure 8, the results demonstrate that the *C. caeruleus* L. root extract, at concentrations ranging from 1 to 8 mg/mL, does not induce hemolysis in erythrocytes, with hemolysis percentages between $0.045 \pm 0.12\%$ and $0.59 \pm 0.09\%$. In contrast, saponin caused significant hemolysis, with values ranging from $51.3 \pm 0.37\%$ to $98.88 \pm 0.18\%$ ($p < 0.05$).

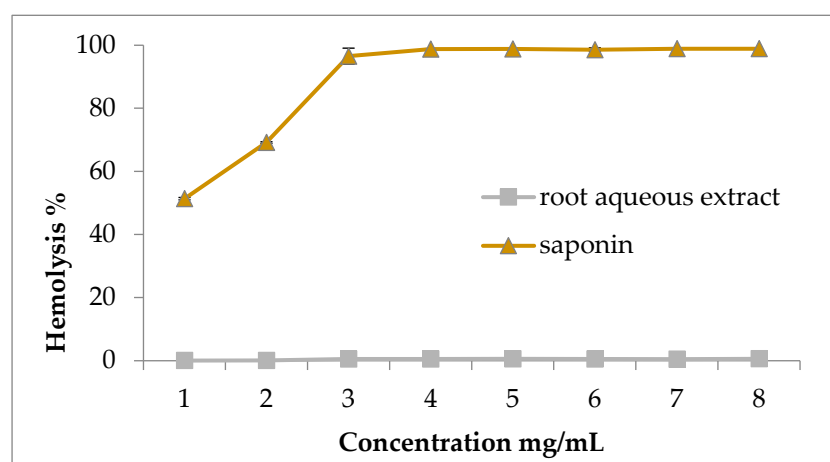


Figure 8. Hemolytic effect of *C. caeruleus* L. root aqueous extract.

3.5. Anti-Inflammatory Activity

The anti-inflammatory activity of *C. caeruleus* L. was evaluated through in vitro methods, as summarized in Table 2. This table shows the maximum activity of the extract, expressed as a percentage, compared to the standard in different assays. To our knowledge, this is the first in vitro analysis of the extract's anti-inflammatory effects, demonstrating its potential in preventing inflammation-related cellular damage and protecting against protein denaturation.

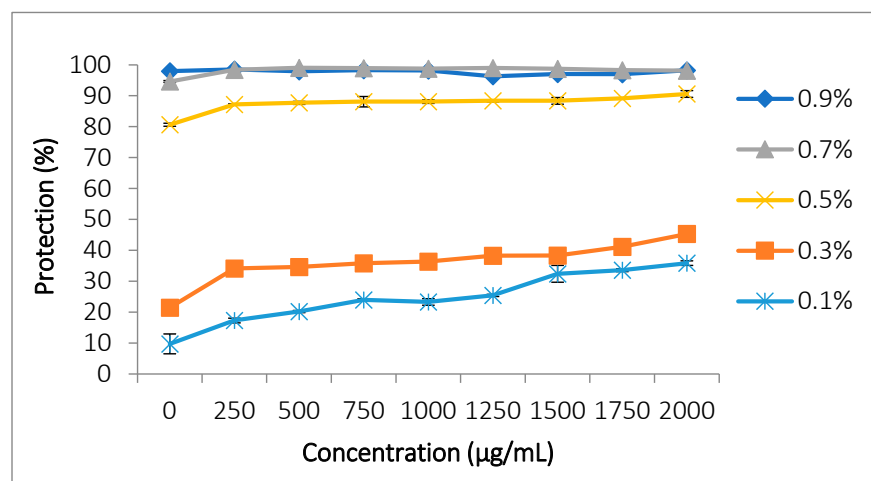
Table 2. Results of *C. caeruleus* L. root aqueous extract's anti-inflammatory activity.

Tests		Concentration ($\mu\text{g/mL}$)	Maximal Protection (%)	
			Extract	Standard
Hypotonicity-induced hemolysis at different concentrations of NaCl	0.7%	2000	98.13 ± 0.15^a	98.2 ± 0.13^a
	0.5%	2000	90.59 ± 1.07^a	98.2 ± 0.13^b
	0.3%	2000	45.3 ± 0.89^a	98.2 ± 0.13^b
	0.1%	2000	35.8 ± 0.75^a	98.2 ± 0.13^b
Heat-induced hemolysis		3000	70 ± 1.27^a	78.53 ± 0.51^b
HClO-induced hemolysis		2000	89 ± 0.87^a	99 ± 0.5^b
Albumin denaturation		2000	81.05 ± 2.2^a	85.28 ± 0.11^a
TBARS		4000	69.25 ± 0.89^a	77.2 ± 1.02^b

Values represent mean $\pm$ SEM. Significant differences are indicated by different superscript letters within the same row ($p < 0.05$). HClO: hypochlorous acid. TBARS: thiobarbituric acid reactive substance.

3.5.1. Protection Against Hemolysis Induced by Hypotonic Stress

Research on the stabilization of erythrocyte membranes aims to elucidate the anti-inflammatory mechanism of action. The results demonstrate that the extract effectively prevents membrane lysis induced by hypotonic stress (Table 2 and Figure 9). For the four hypotonic solutions tested (0.7%, 0.5%, 0.3%, and 0.1%), at a concentration of 2000 $\mu\text{g/mL}$, the extract demonstrated the following percentages of protection: $98.13 \pm 0.15\%$, $90.59 \pm 1.07\%$, $45.3 \pm 0.89\%$, and $35.8 \pm 0.75\%$, respectively. These values are compared with the protection percentage of the extract in the physiological medium of red blood cells (0.9% NaCl), which was $98.2 \pm 0.13\%$ at the same concentration. No significant differences were observed when comparing this value to that obtained for the 0.7% NaCl concentration in the presence of the extract. However, significant differences were noted with the other concentrations (0.5%, 0.3%, and 0.1%) compared to 0.9% NaCl ($p < 0.05$).

**Figure 9.** *C. caeruleus* L. root aqueous extract's protection against hypotonicity-induced hemolysis.

3.5.2. Protection Against Heat-Induced Hemolysis

In this study, the extract demonstrated significant erythrocyte protection, although this was significantly lower than that observed with aspirin at the maximum concentration of 3000 $\mu\text{g/mL}$ ($p < 0.05$). Protection percentages reported for the extract ranged from 19.2 ± 0.2 to $70 \pm 1.27\%$ at concentrations from 100 to 3000 $\mu\text{g/mL}$. Aspirin protection percentages ranged from 40.77 ± 0.59 to $78.53 \pm 0.51\%$ over the same concentration range (Table 2, Figure 10).

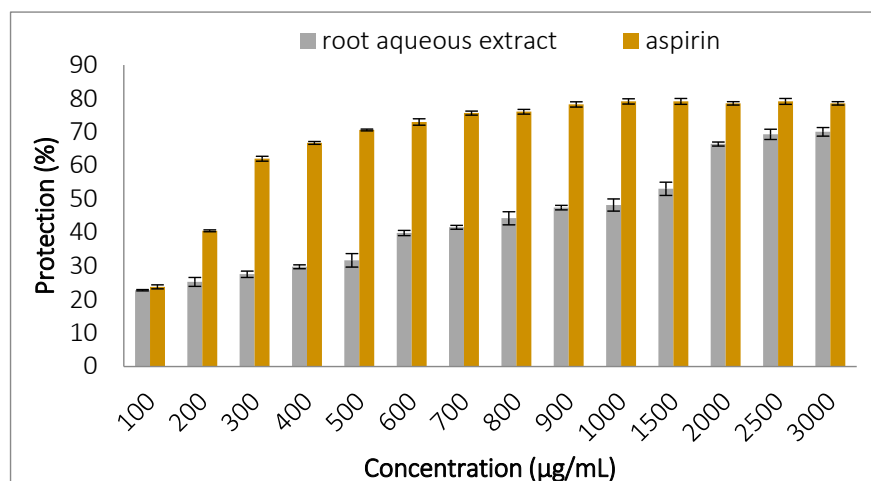


Figure 10. *C. caeruleus* L. root aqueous extract protection against heat-induced hemolysis.

3.5.3. Protection Against Oxidative Stress-Induced Hemolysis

To evaluate the anti-inflammatory potential of the extract, oxidative stress was induced in erythrocytes using hypochlorous acid (HClO). The results revealed a notable protective effect, with protection percentages ranging from $8.66 \pm 0.86\%$ to $89 \pm 0.87\%$, similar to those observed with aspirin ($5.47 \pm 2.16\%$ to $99 \pm 0.5\%$) across concentrations from 100 to 2000 µg/mL (Table 2, Figure 11). No difference was found between the extract and aspirin at the highest concentration of 2000 µg/mL ($p < 0.05$).

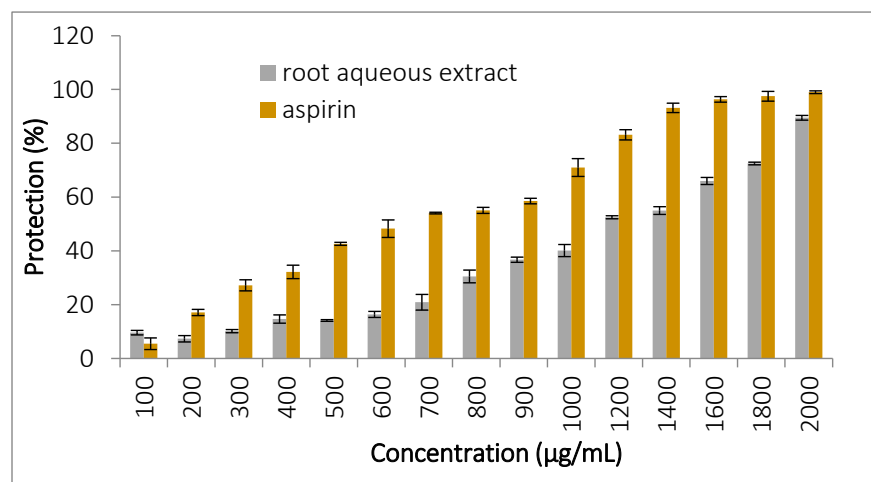


Figure 11. *C. caeruleus* L. root aqueous extract protection against hypochlorous acid-induced hemolysis.

3.5.4. Inhibition of Ovalbumin Denaturation

Protein denaturation plays a key role in several inflammatory conditions, and the capacity of *C. caeruleus* L. to inhibit denaturation suggests an anti-inflammatory potential. In this study, the root extract effectively inhibited heat-induced ovalbumin denaturation at concentrations ranging from 20 to 2000 µg/mL, showing protection percentages from $16.46 \pm 2.13\%$ to $80.86 \pm 2.23\%$. In comparison, aspirin demonstrated protection values between $17.86 \pm 0.3\%$ and $85.28 \pm 0.11\%$, showing no significant difference when compared to the extract at 2000 µg/mL (Figure 12).

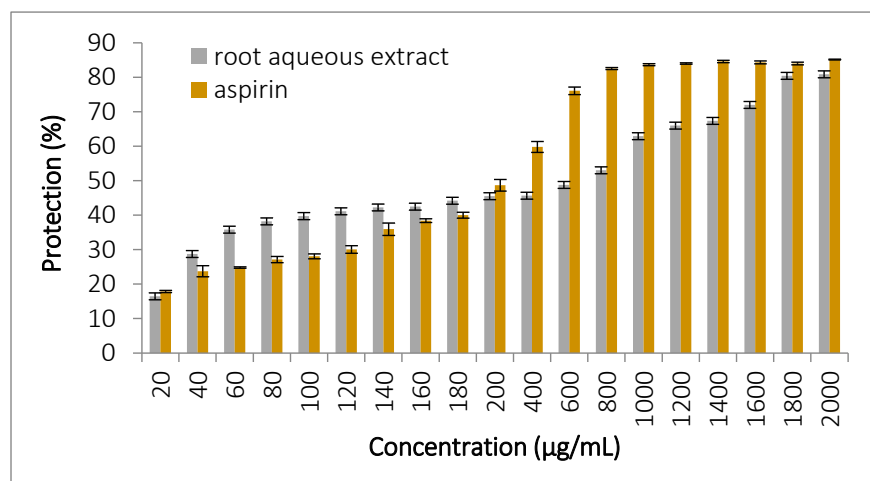


Figure 12. *C. caeruleus* L. root aqueous extract protection against ovalbumin denaturation.

3.5.5. Assessment of Lipid Peroxidation in Animal Tissues Using the TBARS Method

The results of lipid peroxidation assays on brain homogenate (TBARS) are presented in Table 2 and Figure 13. Significant activity was observed for the extract (23.69 ± 0.61 to $69.25 \pm 0.89\%$) compared with BHT (32.53 ± 1 to $77.2 \pm 1.02\%$) for concentrations ranging from 200 to 4000 µg/mL (Figure 13). At a concentration of 4000 µg/mL, a significant difference was observed between the extract and BHT ($p < 0.05$).

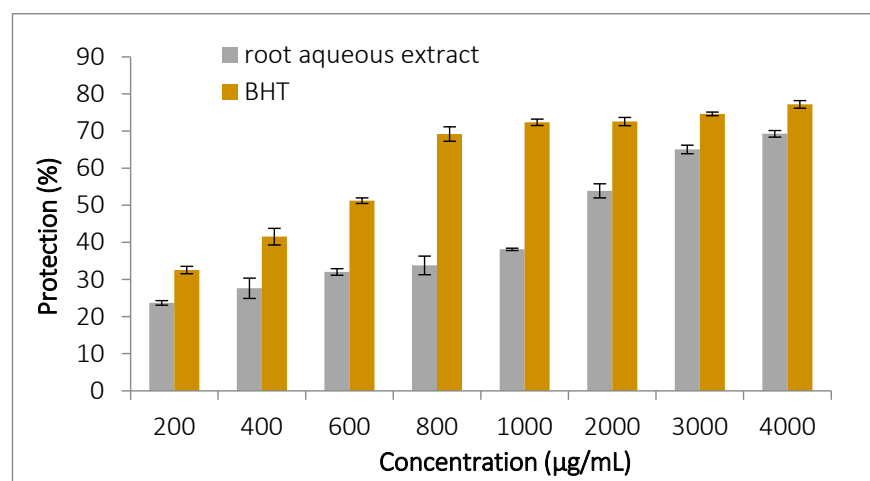


Figure 13. *C. caeruleus* L. root aqueous extract protection against lipid peroxidation in animal tissues.

4. Discussion

Polyphenols are a diverse group of bioactive compounds, with flavonoids, tannins, and phenolic acids being among the most widely studied [61]. These compounds are known for their significant antioxidant and anti-inflammatory activities, making them of particular interest in various medicinal applications. In this context, *C. caeruleus* L., a plant traditionally used in Algerian medicine, has garnered attention for its potential beneficial properties.

When comparing our results, which revealed moderate quantities of polyphenols in the *C. caeruleus* L. root aqueous extract (21.19 ± 0.37 mg GAE/g dry extract), Baghiani et al. [67] and Ouda et al. [68] reported lower polyphenol contents in *C. caeruleus* L. methanolic root extract, with values of 12.966 ± 0.727 and 13.08 ± 0.22 mg GAE/g extract, respectively. Both of these studies utilized methodologies similar to ours. Interestingly, while their studies indicated higher flavonoid contents, recorded at 2.231 ± 0.146 and 5.02 ± 0.55 mg

QE/g extract, respectively, our results for flavonoids content were significantly lower, at 0.72 ± 0.013 mg QE/g dry extract. These discrepancies may be attributed to the polarity of the solvents during extract preparation [69]. Solvent selection plays a crucial role in determining the effectiveness of polyphenol and flavonoid extraction: polar solvents extract hydrophilic compounds, while non-polar solvents extract lipophilic substances. Variations in solvent polarity can thus lead to differing amounts of these compounds from the same plant material [70]. In this regard, Moussa et al. [71] stated that polyphenols in *C. caeruleus* L. tend to be hydrophilic, and that adding water to methanol during extraction increases their quantity. This could explain the lower polyphenol content observed in the methanolic root extracts (13.08 ± 0.22 mg GAE/g extract) obtained by Baghiani et al. [67] and Ouda et al. [68]. Conversely, the higher flavonoid levels observed in these studies could be due to the chemical properties of flavonoids, which are more soluble in methanol, as noted by Ali-Rachedi et al. [69], which may explain the higher quantities reported in these two studies.

The assessment of plant extracts' antioxidant activity is a major area of research, with in vitro tests being the most frequently employed to estimate extract efficacy and facilitate rapid screening [72]. Indeed, low in vitro activity may reflect limited in vivo efficacy [73]. Although no single test can comprehensively summarize the total antioxidant activity of a plant due to its chemical complexity [67], various methods based on redox reactions have been developed. These methods involve electron or proton donation, as well as metal ion chelation [74]. This study employed various assays to assess the antioxidant capacity of *C. caeruleus* L. root aqueous extract.

The antioxidant activity of *C. caeruleus* L. is primarily attributed to its reducing capacity, a key mechanism in neutralizing free radicals. This capacity, which serves as an indicator of the extract's antioxidant potential, is further supported by the results from the TAC and FRAP assays, which are essential for evaluating the reducing properties of antioxidant agents [75,76]. The observed activity in these assays is linked to the extract's ability to reduce molybdate Mo(VI) ions to molybdenum Mo(V), or to convert iron into its ferrous form (Fe^{2+}), through the electron transfer capacity of the polyphenols present in the extract [77–79]. The bioactive compounds in the extract, including tannic acid, quercetin [78], luteolin [55], and myricetin [80], are likely to play a central role in these reduction processes.

Our extract is also capable of neutralizing free radicals, as demonstrated by the DPPH and $\text{OH}^\bullet$ assays. In line with previous studies by Baghiani et al. [67] and Moussa et al. [71], who reported DPPH radical scavenging activity in *C. caeruleus* L. methanolic root extracts (187.38 ± 2.36 $\mu\text{g/mL}$ and 1606 ± 0.05 $\mu\text{g/mL}$, respectively), our results confirm the significant antioxidant activity of the root extract. This variation in activity could be attributed to differences in extraction methods and solvent nature, both well-known factors influencing the type and quantity of polyphenolic compounds extracted [81]. Additionally, as noted by Habibou et al. [79], geographical variations may also contribute to differences in polyphenol content and antioxidant capacity. In this study, the observed antioxidant activity was further corroborated by the extract's ability to donate hydrogen atoms and/or electrons, which led to the disappearance of the characteristic purple color of DPPH, and the formation of the reduced radical (DPPH-H) [82]. The presence of bioactive compounds might explain the observed antioxidant effect. For example, caffeic acid [83], luteolin [55], hesperidin [54], rutin [52], quercetin, myricetin [80], and p-coumaric acid [76] are known for their capacity to stabilize the DPPH radical.

Furthermore, the $\text{OH}^\bullet$ radical, one of the most reactive species responsible for the degradation of biological biomolecules, particularly lipids, was also effectively scavenged by the *C. caeruleus* L. root extract. This inhibitory effect against $\text{OH}^\bullet$ radicals could be

attributed to the extract's ability to neutralize the radical directly or to inhibit the Fenton reaction, which generates $\text{OH}^\bullet$ radicals, through hydrogen peroxide decomposition and/or metal ion chelation, particularly iron. Studies have shown that the ability of extracts to neutralize free radicals is closely related to their chemical composition. For example, Parhiz et al. [84], Nile et al. [85], and Vieira et al. [60] demonstrated, respectively, that compounds such as hesperidin, quercetin, and caffeine are effective in scavenging $\text{OH}^\bullet$ radicals. According to Papuc et al. [74], the phenolic hydroxyl group of flavonoids, particularly their structural position, plays a crucial role in scavenging $\text{OH}^\bullet$ radicals. The superior scavenging activity of our extract compared to vitamin C may be attributed to a synergistic effect among its bioactive compounds, enhancing the collective radical scavenging capacity and surpassing the individual effect of vitamin C.

As previously mentioned, our extract may inhibit $\text{OH}^\bullet$ radicals through ferrous ion chelation, and the results from the chelation assay with *C. caeruleus* L. further support this hypothesis. Ouda et al. [68] also demonstrated the ability of *C. caeruleus* L. root extract to chelate ferrous ions. The significant activity observed in this study can be related to the presence of polyphenols in the extract, which are known for their strong metal ion binding properties. Among these compounds, flavonoids are particularly important due to their hydroxyl and carbonyl groups, which enable them to effectively bind metal ions [75]. Notably, quercetin stands out as a potent chelator due to its hydroxyl functional groups, including 3'-OH and 4'-OH from the B-ring and 3-OH and 4-oxo from the C-ring, as well as 4-oxo and 5-OH [86].

As previously suggested, our extract has exhibited antioxidant potential by decomposing hydrogen peroxide, a molecule with indirect oxidizing properties that can be converted into water through electron and proton transfer by polyphenols [74]. Hydrogen peroxide plays a role in the degradation of biological molecules by generating $\text{OH}^\bullet$ radicals, which underscores the importance of evaluating H_2O_2 decomposition activity [87]. In this study, *C. caeruleus* L. root aqueous extract demonstrated significant antioxidant activity by effectively decomposing hydrogen peroxide. This activity is attributed to the presence of polyphenols, such as tannic acid, which facilitate the conversion of hydrogen peroxide into water through electron and proton transfer [74,88].

Finally, root extract demonstrated a strong inhibitory effect on lipid peroxidation in vitro, as shown by the TBA test. These findings are consistent with those of Baghiani et al. [67], who reported the inhibitory activity of *C. caeruleus* L. extract against linoleic acid oxidation, measured by the β -carotene method. Lipid peroxidation is a key model for assessing antioxidant potential, involving free radicals and metal ions that lead to the formation of malondialdehyde (MDA) [61,89]. In this study, the extract reduced the formation of peroxidation products, likely due to polyphenols such as quercetin, rutin, and tannic acid, which may act by chelating Fe^{2+} ions [37,74] or scavenging peroxy ($\text{LOO}^\bullet$) and alkoxy ($\text{LO}^\bullet$) radicals through their conjugated cyclic structure and hydroxyl groups [9,50].

Polyphenols are renowned for their wide range of biological activities, which are largely attributed to the presence of their aromatic rings, hydroxyl groups, and specific configurations within the phenyl ring [90]. These compounds possess the ability to easily transfer protons and electrons during oxidative stress, a feature enhanced by the resonance stabilization provided by the aromatic ring [67]. Numerous studies have highlighted the relationship between structure and activity, demonstrating that the antioxidant properties of polyphenols are intricately linked to their chemical structures [50,52]. For example, *C. caeruleus* L. root extract contains tannic acid, which, with its hydrophobic core and hydrophilic outer shell, interacts effectively in various environments, neutralizing free radicals through its abundant hydroxyl groups [88]. Similarly, the ortho-dihydroxy configuration of rosmarinic acid has been shown to enhance its antioxidant activity [44].

Additionally, the presence of flavonoids such as quercetin, myricetin, hesperidin, and rutin likely contributes to the potent antioxidant activity of the extract. According to Borges-Bubols et al. [50] and Apak et al. [78], quercetin's 3,4-dihydroxy structure in the phenolic B ring aids in metal ion chelation and its 2,3 double bonds in the C ring facilitate electron delocalization, while myricetin, with its three OH groups on the B ring, proves effective in lipid systems. Furthermore, the strong antioxidant properties of hesperidin [54] and rutin [52] can be attributed to the presence of a 3'-OH group. Likewise, luteolin's 3',4'-dihydroxy structure on the phenolic B ring likely plays a key role in its antioxidant effects [55].

Lysozymes, which are produced by neutrophils during inflammation, contribute to the perpetuation of inflammation and tissue damage. Limiting their release by stabilizing lysosomal membranes is crucial to preventing such harm. Similarly to lysosomal membranes, erythrocyte membranes can be used to assess the extract's capacity to prevent lysozyme release [91]. Human erythrocytes serve as an important model for evaluating the anti-inflammatory potential of extracts. The release of hemoglobin following lysis allows for the measurement of hemolytic power spectrophotometrically, reflecting membrane integrity [92]. While little is known about the anti-inflammatory effects of phytochemicals, extracts may prevent erythrocyte hemolysis by stabilizing membrane integrity [35] and mitigating oxidative damage [39]. Investigating the extract's ability to stabilize erythrocyte membranes during lysis induced by osmotic stress [91], heat [93], or oxidative stress with HClO [33] provides a measure of its in vitro anti-inflammatory activity.

The presence of a hypotonic solution surrounding erythrocytes leads to an excessive accumulation of fluid within these cells, resulting in membrane rupture [94]. The activity of *C. caeruleus* L. in preventing this hemolysis thus highlights its ability to maintain membrane integrity, likely due to the presence of phytochemicals including flavonoids, alkaloids, and tannins [95]. While the exact mechanism of this protection remains unclear, AlSaffar et al. [96] suggested that the deformability and lysis of erythrocytes might be related to their calcium content. Indeed, intracellular calcium plays a crucial role in cellular function, but its concentration must be kept low to preserve cell integrity. An excessive accumulation of intracellular Ca^{2+} leads to irreversible changes in the membrane, causing the damage and destruction of erythrocytes. Similarly, it has been demonstrated that free radicals alter the structure of phospholipids by increasing intracellular calcium levels [97]. In this context, it can be suggested that *C. caeruleus* L. extract may protect erythrocytes by modulating calcium influx into erythrocytes and maintaining calcium concentration at low levels.

Thermal stress is a primary factor contributing to inflammation and cellular damage, leading to morphological changes and membrane lysis [98]. This hyperthermia induces protein unfolding, including membrane proteins, which leads to their aggregation and irreversible damage if not effectively managed [99]. In this context, several studies report that certain plant extracts can inhibit heat-induced protein denaturation [28]. Consequently, it may be inferred that *C. caeruleus* L. extract may protect cells against thermal stress by inhibiting the denaturation of membrane proteins, which, consequently, would explain their ability to prevent erythrocyte hemolysis. Various studies have highlighted that phytochemicals, like flavonoids, tannins, and alkaloids present in this extract, could be responsible for this capacity to stabilize membranes [100]. Consequently, these results could support the traditional use of this plant in treating skin burns, as shown by ethnobotanical investigations [101,102].

Hypochlorous acid (HClO) is a powerful natural oxidant generated by neutrophils and monocytes during inflammation which is responsible for tissue damage. While the exact mechanism of action of this oxidant remains unclear, research has shown that the primary site of action is the cell membrane, inducing cell lysis [33]. The protective activity of *C.*

caeruleus L. root aqueous extract appears to be closely linked to its chemical composition. Previous research, such as that conducted by Suwalsky et al. [103], demonstrated that polyphenols present in the extracts are capable of diffusing through and being distributed within the cell membranes, inhibiting the diffusion of free radicals. This mechanism contributes to membrane protection and the extract's anti-inflammatory effect. Furthermore, An et al. [56] also revealed that quercetin, vitexin, orientin, and luteolin protect red blood cells against H₂O₂-induced toxicity. These findings support the observations made by Suwalsky et al. [33], who, through electron microscopy analysis, showed that resveratrol neutralizes the oxidative effects of HClO and prevents modifications in human erythrocytes' forms. According to their conclusions, the insertion of resveratrol molecules into the lipid bilayer of cell membranes could disrupt the insertion of HClO, thus reducing its deleterious effects. They suggested that this protection results from the hydrogen atoms on its three hydroxyl groups, which can neutralize reactive oxidants and prevent them from attacking the cell membrane before they can penetrate.

The inflammatory response can also arise from protein denaturation [93]. Heat, a physiological response to inflammation, can act as a denaturant, causing conformational changes in proteins that disrupt their structure and biological functionality [104]. Non-steroidal anti-inflammatory drugs (NSAIDs) counter this effect by stabilizing proteins through specific binding interactions, preventing conformational changes that lead to denaturation and preserving their function under thermal stress [105]. In light of this, studying heat-induced protein denaturation or lipid peroxidation provides a useful model for evaluating the anti-inflammatory activity of extracts [93]. *C. caeruleus* L.'s capacity to inhibit ovalbumin denaturation, for instance, is likely due to the presence of phenolic compounds that interact with proteins, stabilizing them by complexing with ovalbumin. Similar findings have been reported for other plant extracts, such as *Rosa canina*, which inhibits bovine serum albumin (BSA) denaturation by binding to aliphatic regions around lysine residues [106].

In inflammation, the overproduction of free radicals can lead to cell damage and lipid peroxidation, which subsequently destroy membranes and exacerbate the inflammatory response [35]. Cell membranes, especially those of neurons, contain a large amount of polyunsaturated fatty acids [107]. According to numerous pharmacological studies, polyunsaturated fatty acids, being the molecules most sensitive to peroxidation, affect the biochemical characteristics of membranes, such as fluidity and permeability [68]. The TBARS assay measures the products of this peroxidation, thus providing insights into the degree of oxidative damage within membranes. Various cell types have been used as sources of the fatty acids—liver, spleen [35], red blood cells [108], and the brain—which were utilized in our study [36]. The measurement of malondialdehyde, a product of lipid peroxidation, in animal tissues is a method for assessing the extent of this reaction [36]. In this study, the extract's ability to prevent lipid peroxidation in brain homogenate can be attributed to its chemical composition. Louerrad et al. [94] demonstrated that flavonoids can inhibit lipid peroxidation of erythrocyte membranes by chelating iron, which prevents the formation of free radicals and, consequently, protects polyunsaturated fatty acids from damage. For example, quercetin is effective against H₂O₂-induced lipid peroxidation in erythrocyte membranes [51]. Other research has revealed that ferulic acid prevents peroxidation through its role in neutralizing free radicals, targeting hydroxyl and peroxy radicals [39]. In this context, further studies suggested that the observed activity may result from the capacity of polyphenols to insert into membranes and restrict their fluidity, thereby limiting the diffusion of free radicals that could induce lipid peroxidation [103].

Finally, this study further supports the safety profile of *Carthamus caeruleus*, which exhibits significant antioxidant and anti-inflammatory activities, as described in previous

sections. A prior *in vivo* toxicity assessment conducted on Wistar rats demonstrated no signs of toxicity when treated with root aqueous extract at doses of 300, 500, and 1000 mg/kg body weight over a 24 h observation period [68]. These findings are consistent with those of Dahmani et al. [109], who also investigated the toxicity of *C. caeruleus* L. root methanolic extract.

5. Conclusions

C. caeruleus L. root aqueous extract demonstrates significant antioxidant and anti-inflammatory activities, primarily attributed to its rich phytochemical composition. Its antioxidant effects are linked to its ability to reduce and chelate metal ions, especially iron, which are involved in generating harmful radicals, like hydroxyl and pyroxy. Additionally, the extract directly neutralizes free radicals, such as $\text{OH}^\bullet$ and DPPH, or decomposes hydrogen peroxide. Furthermore, the anti-inflammatory effects of the extract can be attributed not only to its antioxidant potential, but also to the ability of its chemical compounds to integrate into membranes, stabilizing their integrity and providing protection against protein denaturation and membrane lipid peroxidation. In light of these results, this study provides novel perspectives on the antioxidant and anti-inflammatory potential of *C. caeruleus* L., reinforcing its traditional use and efficacy against burns. However, further studies are recommended to identify new bioactive molecules and evaluate the beneficial potential of this plant *in vivo*.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pr13030878/s1>, Figure S1: Gallic Acid Calibration Curve; Figure S2: Quercetin Calibration Curve; Figure S3: Tannic Acid Calibration Curve; Figure S4: Chromatogram of *C. caeruleus* L. root aqueous extract at 230.4 nm; Figure S5: Chromatogram of *C. caeruleus* L. root aqueous extract at 255.4 nm; Figure S6: Chromatogram of *C. caeruleus* L. root aqueous extract at 280.4 nm; Figure S7: Chromatogram of *C. caeruleus* L. root aqueous extract at 300.4 nm; Figure S8: Chromatogram of *C. caeruleus* L. root aqueous extract at 355.4 nm; Figure S9: Spectrum of the mixture of reference standards.

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Abbreviations

A: absorbance; ANOVA, analysis of variance; BHT, butyl hydroxy toluene; DAD, diode array detector; DPPH, 2,2-diphenyl-1-picrylhydrazil; EDTA, ethylenediamine tetraacetic acid; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; H_2O_2 , hydrogen peroxide; HClO : hypochlorous acid; HPLC, high-performance liquid chromatography; IC50, inhibition concentration 50%; $\text{OH}^\bullet$, hydroxyl radical; QE, quercetin equivalents; RT, retention time; SEM, standard error of mean; TAC, total antioxidant capacity; TAE, tannic acid equivalents; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substance.

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