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Pyrrolizidine Alkaloids Content and Antioxidant Activity of Two *Heliotropium* Species from Basrah - Southern Iraq

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ABSTRACT

Background: *Heliotropium* species (Boraginaceae) have long been used in traditional medicine, yet their antioxidant properties remain underexplored. This study investigates and compares the antioxidant activity of two species—*H. curassavicum* (a newly recorded species in Iraq) and *H. crispum*—collected from Basrah, Iraq. **Methodology**: Whole plants of *H. curassavicum* and *H. crispum* were collected, shade-dried, and ground into coarse powder. Methanolic maceration was performed over 5 days. The crude extracts were analyzed for PA content using High-Performance Liquid Chromatography (HPLC). Antioxidant activity of the extracts and isolated heliotrine was evaluated using *in vitro* assays: Total Antioxidant Capacity (TAC), DPPH radical scavenging assay, and Ferric Reducing Antioxidant Power (FRAP). Ascorbic acid served as the standard for comparison. Two-fold serial dilutions (50–800 µg/ml) were used in the antioxidant assays. **Results**: The TAC of *H. crispum* (0.413 mg AAE/g) was higher than that of *H. curassavicum* (0.191 mg AAE/g). Heliotrine exhibited strong antioxidant activity in DPPH (29.36%–62.92%) with an IC_{50} of 151.40 µg/ml and good reducing capacity in FRAP assays (0.36–3.67 FRP) across concentrations of 50–800 µg/ml, comparable to ascorbic acid (31.52%–77.24%, IC_{50} = 137.17 µg/ml). Statistical analysis revealed no significant differences between heliotrine and ascorbic acid across all concentrations tested ($p < 0.05$).

Conclusion: Both *H. crispum* and *H. curassavicum* demonstrated notable antioxidant activity, with *H. crispum* exhibiting higher TAC. Heliotrine displayed strong scavenging and reducing power, comparable to ascorbic acid, supporting the medicinal potential of these species and encouraging further pharmacological exploration.

Keywords: DPPH assay, *Heliotropium curassavicum*, HPLC, Pyrrolizidine alkaloids (PAs), Total antioxidants capacity (TAC).

INTRODUCTION

Throughout the history of civilization, plants have consistently served as a crucial source of survival, shelter, transportation, and medicine. Ancient humans utilized specific plants for analgesic effects and applied plant leaves to wounds to promote healing. It was an era when natural remedies were the exclusive method for addressing ailments and injuries. Yet, the discovery of novel drugs has consistently provided a difficulty for scientists in identifying promising lead options [1]. Drug discovery is a process that advances from the screening of natural products to the identification of new isolates, demanding expertise and experience, and is a costly endeavor. Nonetheless, recent high-throughput techniques have transformed the

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screening of natural products and the identification of newly developed drugs in a time-efficient and cost-effective manner [2]. Approximately 40% to 50% of pharmaceutical medications utilized globally are sourced from nature, primarily due to the harmful effects associated with synthetic chemical medications in many cases [3].

The Boraginaceae family is a prevalent angiosperm family, including 110 genera and 1,600 species, primarily located in temperate zones, especially within the Mediterranean region [4]. The genus *Heliotropium*, belonging to the family Boraginaceae, is a complex and extensive group within this family. Approximately 250-300 species within this genus have been documented worldwide in temperate and tropical regions. Al-Rawi (1964) classified a total of 15 species under the genus *Heliotropium*, and Rechinger (1964) identified 8 species. In the history of traditional medicine, species of the genus *Heliotropium* have gained significant pharmacological interest [5]. *Heliotropium curassavicum* (salt heliotrope) is a newly recorded species in Iraq, recorded by Yusra Al-Rudainy [6]. This species is utilized in certain nations to deal with several human ailments, including ulcers, edema, wounds, localized infections, erysipelas, gonorrhea, cancer, diabetes, constipation, rheumatism, and arteriosclerosis [7] [8].

Pyrrolizidine alkaloids (PAs), flavonoids, terpenoids, phenols, and naphthoquinones are diverse bioactive compounds isolated and classified from *Heliotropium* species [9]. Over 660 PAs have been identified in more than 6000 plant species, including nearly 200 PAs isolated from *Heliotropium* [10]. The biochemically active constituents of *Heliotropium* species demonstrate antibacterial, antifungal, antitumor, antiviral, antioxidant, wound-healing, antiplatelet, anti-inflammatory, cardiotonic, contraceptive, and prostaglandin effects [10] [11]. The current study investigated the antioxidant properties of the methanol crude extract of *H. crispum* and *H. curassavicum* and isolated heliotrine, which may be associated with the plant's potential medicinal benefits as indicated in traditional medicine.

METHODOLOGY

Plant Materials

The entire plant of *H. curassavicum* and *H. crispum* was gathered at full flowering from Basrah province, specifically along the Airport Road and Al-Seiba, in April and November 2023, respectively. The plants were authenticated by Dr. Ula Almosawi, an expert in plant taxonomy at the College of Pharmacy, University of Basrah. All samples were rinsed with water to eliminate sand and dust particles, thereafter air-dried in the shade, and pulverized into minute fragments using a mechanical grinder. Plants were collected and subsequently utilized to investigate their medicinal properties and conduct an initial assessment of their phytochemical contents.

Extraction Methods

Each plant species, in a quantity of 100 grams, was separately macerated in 1 liter of 70% methanol for 5 days with stirring. Filtration was subsequently performed utilizing Whatman No. 1 filter paper. The filtrate of the crude extract of methanol was additionally concentrated with a rotary evaporator at 40°C and 60 rpm, and then the extract was dried at ambient temperature to produce a dry extract.

Pyrrolizidine Alkaloids Characterization by HPLC

The investigation conducted to identify and quantify PA content in two species of *Heliotropium* employed an HPLC system (Shimadzu) at the Government's Ministry of Science and Technology in Baghdad.

In Vitro Free Radical Scavenging Activity (RSA)

Determination of Total antioxidant capacity

The total antioxidant activity of both plant extracts was assessed using the phosphomolybdenum method as outlined by Prieto *et al.* [12]. 1.0 ml of the extract was added to 1.0 ml of the standard reagent

solution, which consists of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The tubes were then covered and maintained in a thermal block at 95°C for 90 minutes. Following cooling to ambient temperature, the absorbance was assessed at 695 nm against a reagent blank. Ascorbic acid served as a positive control, and the results were quantified as milligrams of ascorbic acid equivalence (AAE) per gram of the extract (mg AAE/g extract).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The DPPH radical scavenging assay is a frequently utilized method for assessing the free radical scavenging capacity of naturally occurring substances; it is proportional to the disappearance of DPPH•.

The application of a UV spectrometer to monitor DPPH• has emerged as the predominant method owing to its simplicity and precision. DPPH• exhibits an intense absorption peak at 517 nm (purple). The shade transitions from purple to yellow, accompanied by the creation of DPPH, following the absorption of hydrogen from an antioxidant. The resulting process is stoichiometric concerning the quantity of hydrogen atoms consumed. As a result, the antioxidant impact can be readily assessed by monitoring the reduction in the absorption of UV at 517 nm [13].

The free radical scavenging activity for heliotrine was assessed in vitro utilizing the DPPH radical, according to Shimada *et al.*, with minor modifications: 1.0 ml of heliotrine at several concentrations (12.5, 25, 50, 100, and 200) µg/ml was combined with 1.0 ml of 0.8 mM DPPH solution. The entire mixture was agitated thoroughly and left to settle for 30 minutes, and then discoloration of the solution was evaluated to determine the antioxidant activity of heliotrine. The sample absorbance was read at 517 nm against a reagent blank. Ascorbic acid was used as a positive control, and the radical scavenging activity of heliotrine was expressed as mg of ascorbic acid equivalents (mg AAE/g extract).

Reducing Power FRAP Assay

The FRAP (ferric reducing antioxidant power) assay has been used to determine the reductive activity of heliotrine. Experimental techniques were conducted as previously outlined [14].

Different concentrations of heliotrine (µg/ml) were combined with 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [K3Fe(CN)6] solutions. The resultant mixture was thoroughly agitated and thereafter underwent incubation at 50°C for 20 minutes utilizing a vortex shaker. Upon completion of the incubation, 2.5 ml of 10% trichloroacetic acid was incorporated into the mixture and centrifuged for 10 minutes at 3,000 rpm. 2.5 ml of supernatant was combined with 2.5 ml of deionized water and 0.5 ml of 0.1% ferric chloride. The colored solution was detected at 700 nm against the blank. Ascorbic acid served as a positive control, and the reducing power of heliotrine was calculated as mg to that of Ascorbic Acid Equivalents AAE (mg AAE/g heliotrine).

RESULTS

Extraction

Methanolic maceration as the extraction method yielded 2.425 mg of crude extract from *H. crispum* and 2.656 mg from *H. curassavicum*.

High-Performance Liquid Chromatography (HPLC) Analysis

HPLC was used to detect and quantify PAs, employing ≥98% pure heliotrine as a standard. Analyte identities were confirmed by matching retention times (Rt) with the standard. (Figure 1) displays the characteristic peaks of heliotrine in both *H. crispum* and *H. curassavicum* extracts, (Table 1) summarizes Rt, area, and height values, offering qualitative and quantitative insight into heliotrine content in the two species. Isolation of heliotrine

(Figure 2) by HPLC demonstrates a well-isolated and symmetrical peak. The raised peak area and height confirm a significant presence of this PA in the analyzed samples.

The quantitative assessment demonstrated disparities in the quantities of heliotrine between the two species, where heliotrine showed high concentration: 61 and 54 mg/g in *H. curassavicum* and *H. crispum*, respectively (Table 2).

A calibration curve was established for the standard solution across the given concentration range (3, 5, 10, and 20 ppm for heliotrine) and injection volume of 100 µl to quantify the concentrations of heliotrine. Figure 3 displays the calibration curve, equation, and correlation factor (R^2) obtained for the PA standard.

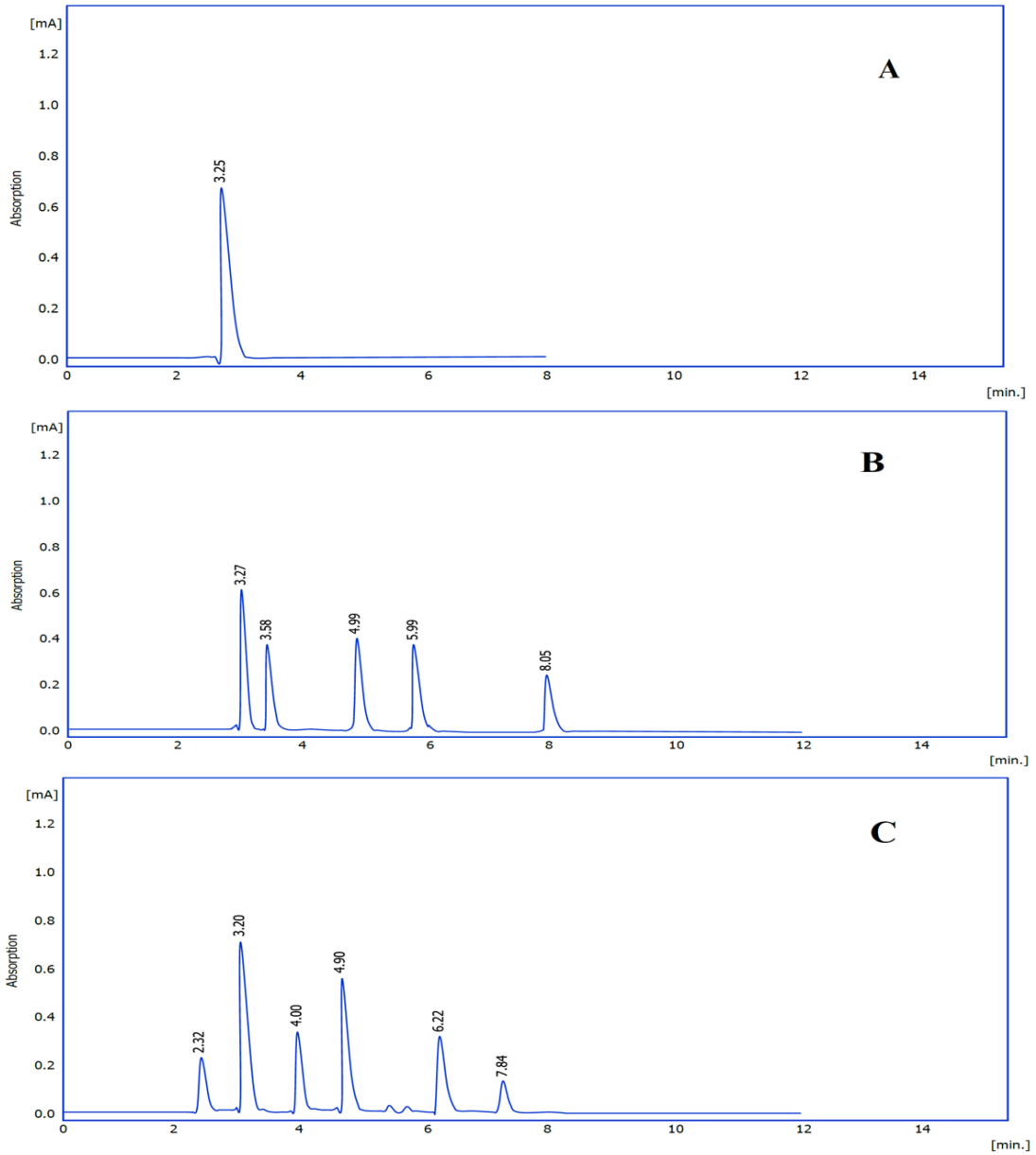
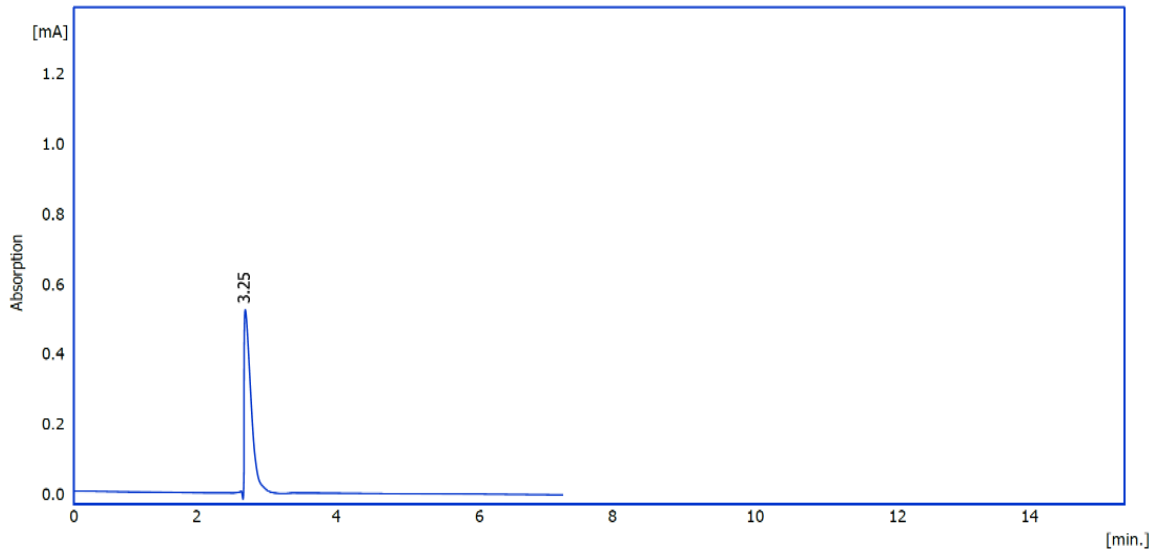


Figure 1. HPLC for **(A)** Heliotrine standard, **(B)** *H. crispum* extract **(C)** *H. curassavicum* extract

Table 1. HPLC detection for Heliotrine in methanol extract of *H. crispum* and *H. curassavicum*

Heliotrine st./ *H.* species	**Reten. Time [min]**	**Area [mAU.s]**	**Height [mAU]**	**Area [%]**	**Height [%]**
Heliotrine st.	3.25	3215.90	688.95	100	100
H. crispum	3.27	875654.08	600.12	25.00	25.00
H. curassavicum	3.20	985160.24	784.15	27.00	27.00



Result chromatography Table (Uncal - F:\ heliotrine isolation)

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	3.25	845.90	580.44	100.00	100.00	0.20	
	Total	845.90	580.44	100.00	100.00		

Figure 2. HPLC for isolated heliotrine.

Table 2. The concentration of heliotrine in *H. crispum* and *H. curassavicum.*

Heliotropium	**Heliotrine conc.**
H. crispum	54 mg/g
H. curassavicum	61 mg/g

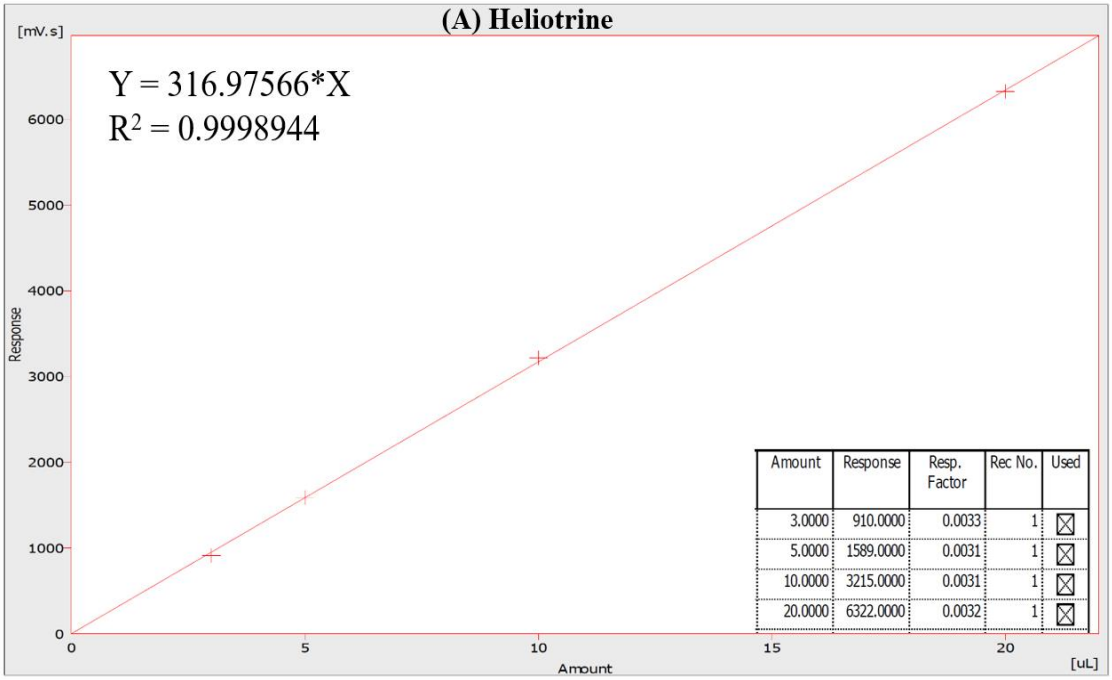


Figure 3. Calibration curve for heliotrine standard

In Vitro Free Radical Scavenging Activity (RSA)

Determination of Total antioxidant capacity

The result presented that the total antioxidant activity was higher in the crude extract of *H. crispum* (0.413 mg of AAE/g) than in *H. curassavicum* (0.191 mg of AAE/g). In our study, we want to discover the free radical scavenging activity of pyrrolizidine alkaloids, so we chose heliotrine for further investigation by DPPH and FRAP assays in order to contrast outcomes and link the antioxidant potential of the crude extract with heliotrine.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

Heliotrine exhibited antioxidant activity at all tested concentrations, with DPPH radical scavenging percentages of (30.01%, 43.48%, 54.78%, 66.66%, and 79.65%) at concentrations of (50, 100, 200, 400, and 800) µg/ml, respectively. These values are comparable to those of the standard ascorbic acid, which demonstrated 31.98%, 45.06%, 57.13%, 68.36%, and 79.55% at the same concentrations (Figure 4). The IC_{50} value of heliotrine was determined to be 151.40 µg/ml, slightly higher than that of ascorbic acid (137.17 µg/ml), indicating a strong radical scavenging capacity (Table 3). Upon addition to the DPPH solution, heliotrine donates an electron or hydrogen atom, converting DPPH• to the non-radical DPPH-H, resulting in a measurable color change. Statistical analysis showed no significant differences between heliotrine and ascorbic acid at all tested concentrations (p >0.05).

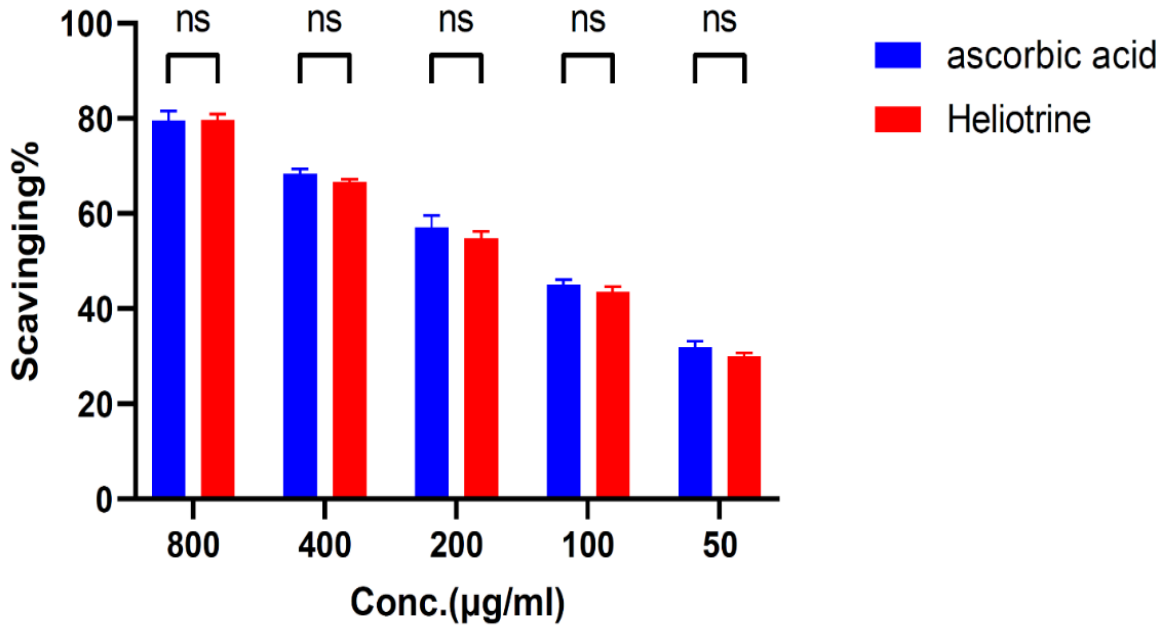


Figure 4. Scavenging activity of heliotrine vs. ascorbic acid by DPPH assay.

Table 3. IC_{50} for heliotrine vs. ascorbic acid by DPPH assay.

Compound	**IC_{50} µg/ml**
Ascorbic acid	137.37
Heliotrine	151.40

Reducing Power FRAP Assay

The reducing power of heliotrine was evaluated using the potassium ferric cyanide (FRAP) assay. At 700 nm, the absorbance increased with concentration, indicating a dose-dependent reducing ability. Heliotrine displayed ferric reducing power values of (0.36, 0.52, 0.72, 1.94, and 3.67) at concentrations of (50, 100, 200, 400, and 800) µg/ml, respectively. In comparison, ascorbic acid, used as a reference standard, demonstrated slightly higher values of (0.46, 0.65, 0.89, 2.13, and 3.91) at the same concentrations (Figure 5). Statistical analysis using GraphPad Prism indicated no significant difference between the activities of heliotrine and ascorbic acid across all tested concentrations (p >0.05).

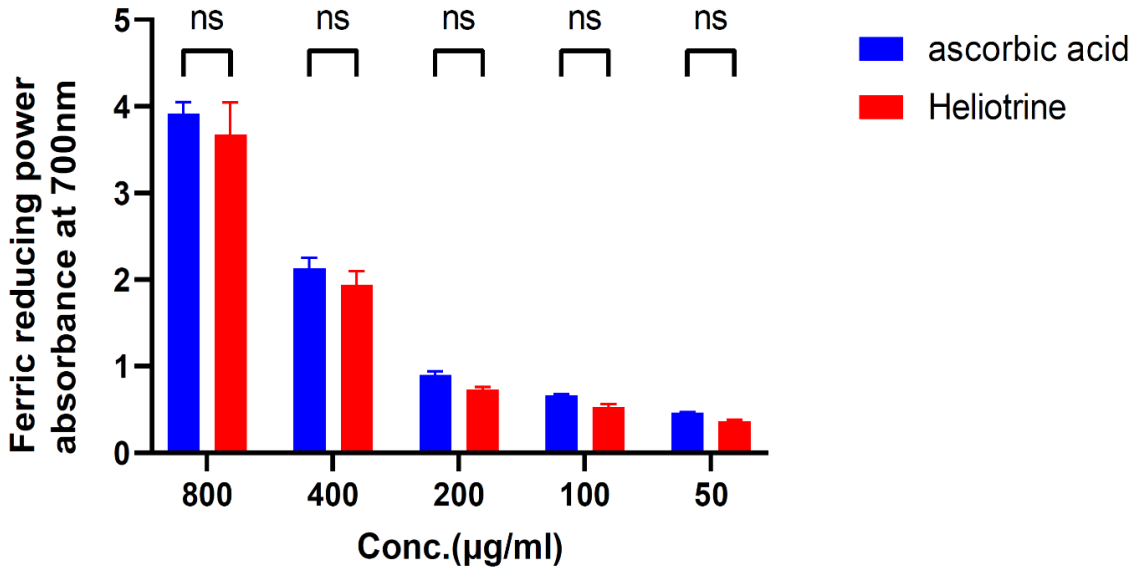


Figure 5. Reducing power of heliotrine vs. ascorbic acid by FRAP assay.

DISCUSSION

Extraction

The choice of extraction conditions and solvent significantly influences both the yield and quality of the targeted PAs. Given that PAs, and their N-oxide forms, have limited solubility in non-polar solvents and are better extracted with polar ones, 70% methanol was selected as the extraction solvent. Since elevated temperatures have been shown to reduce PA yield [15]Maceration at room temperature was employed. This method, carried out over five days with continuous agitation, enhances the extraction of PA tertiary bases, aligning with the findings reported by Tomasz Mroczek [16].

High-Performance Liquid Chromatography (HPLC) Analysis

HPLC provides greater efficacy in separating and detecting PAs. The use of an authenticated PA standard ensures accurate and reliable results; therefore, we employed an authentic standard of heliotrine with a purity of ≥98%. HPLC is highly effective for the separation, identification, and quantification of pyrrolizidine alkaloids (PAs) and their N-oxides, offering several advantages such as high-resolution separation, broad compound detection, and simple sample preparation [15]. Quantitative analysis revealed higher heliotrine levels of 61 mg/g in *H. curassavicum*, while 54 mg/g in *H. crispum*. The linearity of the calibration curve confirms the reliability of the quantification method for heliotrine across the tested concentration range. The observed variation in heliotrine levels between *H. curassavicum* and *H. crispum* suggests species-specific differences in alkaloid biosynthesis. The higher concentration in *H. curassavicum* may indicate a more active or efficient biosynthetic pathway for heliotrine, which could influence its pharmacological properties and potential applications.

The first documentation of *H. curassavicum* in Iraq marks a significant botanical discovery, underscoring the potential for unrecognized plant diversity within the region. Identifying and recording new plant species is vital to enriching the nation's floral inventory and advancing botanical research [6]. Heliotrine was identified as the major PA in both *Heliotropium* species examined, consistent with its prevalence in 11 out of 20 *Heliotropium* species previously studied. It has also been reported at the

highest concentrations in the inflorescences of *H. lasiocarpium* and *H. suaveolens*, supporting the observations by AL-Shekhany *et al.* [17]. This suggests that heliotrine may be the primary alkaloid produced by these plants. Its isolation using HPLC (Figure 2) revealed a sharp, symmetrical peak, with the large peak area and height indicating a high concentration of this PA in the analyzed samples.

Determination of Total Antioxidant Capacity

The difference in TAC (0.413 mg of AAE/g in *H. crispum*, and 0.191 mg of AAE/g in *H. curassavicum*) could be attributed to the variation of active phytochemicals of each species, which may interact synergistically, such as flavonoids and phenolics enhancing each other's radical scavenging effects. Studies have reported quercetin, kaempferol, anthocyanin, and coumarin in the crude extract of *H. crispum* that could result in substantial antioxidant activity [18]. Research by Amir Sh. *et. al.* revealed that the *H. crispum* extract in polar solvents had greater antioxidant capacity compared to non-polar extracts, a finding with which we concur [19]. The reported findings of the methanolic extract of *H. crispum* studied by Arshad *et. al.* by phosphomolybdenum assay, using trolox equivalent instead of ascorbic acid, support our results [20].

The antioxidant activity works through different mechanisms, such as donating hydrogen atoms or electrons, neutralizing reactive oxygen species (ROS) [21]Metal chelating activity in biological systems [22], enzyme modulation by enhancing the antioxidant enzymes (such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT)), and inhibition of lipid peroxidation to stabilize cellular membranes [23].

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The DPPH assay results confirm heliotrine's notable antioxidant potential, supporting its role as a natural phytoconstituent with free radical scavenging ability. The IC_{50} value specifies the concentration of a compound required for inhibiting 50% of DPPH radicals, with lower values signifying enhanced antioxidant activity. The comparable IC_{50} values between heliotrine and ascorbic acid suggest heliotrine's effectiveness in mitigating oxidative stress. This antioxidant activity is attributed to heliotrine's chemical nature as a tertiary amine; the electrophilic iminium ion may play a significant role in its redox behavior and metabolism. Antioxidants typically function by donating hydrogen atoms to neutralize reactive oxygen species such as hydroxyl, peroxyl, and superoxide radicals. By doing so, they help inhibit lipid peroxidation, stabilize cell membranes, and prevent oxidative cellular damage [24]. These findings are in line with the study by AL-Shekhany *et al.* [17], who reported strong antioxidant activity in *H. lasiocarpium* and *H. suaveolens* collected throughout the Kurdistan region of northern Iraq. Similarly, *H. ellipticum* (Singh *et al.* [25] and *H. indicum* have also demonstrated significant antioxidant effects [26], reinforcing the therapeutic potential of *Heliotropium* species.

Reducing Power FRAP Assay

The FRAP assay results confirm that heliotrine possesses significant electron-donating capacity, enabling it to reduce Fe^{3+} (ferricyanide complex) to Fe^{2+} (ferrous complex). This reduction capability reflects its potential to terminate free radical chain reactions, thereby contributing to its antioxidant profile [26]. The increase in absorbance at 700 nm with increasing concentration supports the dose-dependent nature of its reducing power. While heliotrine's activity was slightly lower than that of ascorbic acid, the lack of significant difference suggests a comparable antioxidant strength. These findings, consistent with the DPPH results, highlight heliotrine's role as a natural antioxidant agent capable of participating in redox reactions and mitigating oxidative damage.

CONCLUSION

This work underlines the considerable antioxidant potential of *Heliotropium* species, especially *H. crispum*, which exhibited a superior total antioxidant capacity relative to *H. curassavicum*. Both species

demonstrated favorable outcomes in free radical scavenging experiments. Heliotrine demonstrated significant antioxidant activity, equivalent to ascorbic acid, as indicated by DPPH and FRAP experiments. These findings support the conventional medical application of *Heliotropium* and highlight the potential of heliotrine as a natural antioxidant agent.

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