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Antioxidant potential and phenolic composition of *Cistanche tinctoria*: A comparative study of crude, flavonoid, and tannin extracts

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Abstract

The antioxidant capacity and phenolic composition of *Cistanche tinctoria* (Orobanchaceae) were evaluated in the crude, flavonoid, and tannin extracts prepared from air-dried flowers using methanol, ethyl acetate, and acetone, respectively. The highest extraction yield (11.3 %) was obtained with the crude extract. The elevated total phenolic (168 ± 24 mg GAE/g) and flavonoid (27 ± 3 mg QE/g) contents in the crude extract were revealed. The tannin extract exhibited the highest antioxidant activity (IC_{50} 8 ± 2 μg/mL), but, in terms of antioxidant and antiradical properties, all extracts were significantly less effective than ascorbic acid. The flavonoid extract demonstrated the greatest hemolysis inhibition (23 %). The highest absorbance (0.432) was observed for the tannin extract at a concentration of 0.1 mg/mL. Based on the high-performance liquid chromatography analysis, chlorogenic acid and naringenin were identified as the major phenolic compounds in the crude extract. The results validate the health benefits of phenolic compounds in *C. tinctoria* and highlight further research priorities for its applied and medicinal use.

Keywords: *Cistanche tinctoria*, antioxidant activity, phenolics, flavonoids, tannins, DPPH, hemolysis, reducing power, high-performance liquid chromatography

Institutional Review Board Statement. This study was conducted in accordance with the Declaration of Helsinki (2000).

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Антиоксидантная способность и состав фенольных соединений *Cistanche tinctoria*: сравнительное исследование неочищенного, флавоноидного и дубильного экстрактов

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Аннотация

Изучены антиоксидантная способность и состав фенольных соединений *Cistanche tinctoria* (Orobanchaceae) на основе анализа неочищенного, флавоноидного и дубильного экстрактов, полученных из воздушно-высушенных цветков с помощью метанола, этилацетата и ацетона соответственно. Наибольший выход экстракта (11.3 %) достигнут при использовании метанола. Показано высокое содержание фенольных (168 ± 24 мг-экв. галловой кислоты/г) и флавоноидных (27 ± 3 мг-экв. кверцетина/г) соединений в неочищенном экстракте. Наибольшую антиоксидантную активность проявил дубильный экстракт (IC_{50} 8 ± 2 мкг/мл). При этом антиоксидантные и антирадикальные свойства всех экстрактов оказались менее выражены по сравнению с аскорбиновой кислотой. Наилучшую способность к защите мембран эритроцитов от перекисного гемолиза продемонстрировал флавоноидный экстракт (23 %). Максимальное значение оптической плотности (0.432) зафиксировано для дубильного экстракта в концентрации 0.1 мг/мл. Методом высокоэффективной жидкостной хроматографии установлено, что основными фенольными соединениями неочищенного экстракта являются хлорогеновая кислота и нарингенин. Полученные результаты свидетельствуют о выраженном терапевтическом потенциале фенольных соединений в составе *C. tinctoria* и указывают на перспективность дальнейших исследований их прикладного и лекарственного применения.

Ключевые слова: *Cistanche tinctoria*, антиоксидантная активность, фенольные соединения, флавоноиды, таннины, ДФПГ, гемолиз, восстановительная способность, высокоэффективная жидкостная хроматография

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Introduction

Natural antioxidants have attracted much attention in recent years due to their potential health benefits for preventing and treating diseases induced by oxidative stress [1].

Oxidative stress, driven by an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify these harmful compounds, is implicated in the pathogenesis of many chronic diseases, including cardiovascular dysfunction, cancer, and neurodegenerative disorders [2]. Natural oxidants from plants effectively neutralize ROS, thereby mitigating or averting oxidative damage [3]. Phenolic compounds, such as flavonoids and tannins, are potent antioxidants, which is attributed to their ability to donate hydrogen atoms or electrons and stabilize free radicals [4].

Among the wide variety of medicinal plants known for their antioxidant properties, *Cistanche tinctoria* (Orobanchaceae) is especially rich in bioactive compounds, particularly phenolics and flavonoids. This desert parasitic plant is native to North Africa, the Arabian Peninsula, and Asia [5]. It attaches itself to the roots of its primary host plants (*Tamarix gallica*, *Calligonum comosum*, and *Pulicaria*) and derives nutrients from them for growth.

C. tinctoria has been traditionally utilized not only for its aesthetic and decorative value but also for its medicinal effects [6] to treat and relieve many ailments such as abdominal pains, diarrhea, dystonia, bruises, gynecological diseases, delayed lactogenesis, and diabetes [5].

This study aims to explore the antioxidant capacity of *C. tinctoria*, identify key phenolic compounds in its extracts that reduce oxidative stress, and validate its potential as a source of natural antioxidants.

1. Material and Methods

1.1. Laboratory equipment used in sample preparation and analysis. A Buchi R-200 rotary evaporator was used for solvent removal during the extraction process. Filtering was performed with standard filter paper. Absorbance of the samples in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power assays was measured with a Shimadzu UV-Vis spectrophotometer (Japan). In the hemolysis and reducing power assays, a centrifuge was employed to separate the particles suspended in the prepared extracts. High-performance liquid chromatography (HPLC) analysis was carried out using a Shimadzu LC 20 AL system (Japan) equipped with a Hamilton 25 μL universal injector, a Shim-pack VP-ODS C18 analytical column (4.6 mm × 250 mm, 5 μm), and a UV-VIS detector (SPD 20A, Shimadzu, Japan) to determine and quantify phenolic compounds.

1.2. Plant material. *C. tinctoria* flowers were collected in the full flowering stage (Fig. 1) on March 29, 2018 from the Taleb Larbi region in the El-Oued state of Algeria (33°42'27.8" N, 07°18'57.1" E), approximately 60 km along the Tebessa road.

The collected plant material was air-dried in the dark at room temperature for 3 to 4 weeks. Once dried, it was ground into a fine powder using a mechanical grinder. The obtained powder was stored at room temperature in airtight containers shielded from bright light until experimentation.

1.3. Preparation of methanolic extract. To prepare a methanolic extract of *C. tinctoria*, 10 g of the dried plant material were macerated in 150 mL of methanol in the dark at room temperature for 24 h and then filtered through filter paper. The solvent was evaporated to dryness under reduced pressure in a rotary evaporator at 50 °C to obtain the crude extract, which was stored away from light and protected from moisture intake [7].

Fig. 1. *C. tinctoria* plant in full flowering stage, Taleb Larbi region (El-Oued state, Algeria)

1.4. Extraction of flavonoids. To extract flavonoids, the same amount (10 g) of the dried plant material was macerated in 150 mL of methanol in the dark at room temperature for 24 h. After the filtration through filter paper, the solvent was evaporated under reduced pressure in a rotary evaporator at 50 °C. Then, 150 mL of warm distilled water and 150 mL of ethyl acetate were added to the methanolic extract, and the mixture was placed in a separatory funnel. The ethyl acetate phase was collected, evaporated at 50 °C, and used as the flavonoid extract [8].

1.5. Extraction of tannins. Tannins were extracted by macerating 30 g of the *C. tinctoria* powder in 60 mL of distilled water and 140 mL of acetone in the dark at room temperature for 72 h. The resulting solution was filtered through filter paper. To remove acetone, the solvent was evaporated under reduced pressure in a rotary evaporator at 50 °C. Then, to remove lipid-soluble substances, 150 mL of dichloromethane was added to the remaining solution. The mixture was allowed to stand in a separatory funnel for about 2 h. The ethyl acetate phase was collected and evaporated to dryness at 50 °C to obtain the tannin extract [9].

1.6. Determination of total phenolic contents. The total phenolic contents (TPC) of the crude extract were determined using the Folin–Ciocalteu method with modifications [10]. A mixture containing 0.2 mL of the extract, 1 mL of the Folin–Ciocalteu reagent (diluted 1 : 10 with water), and 0.8 mL of sodium carbonate solution (7.5 %) was incubated for 30 min. The absorbance was measured by a spectrophotometer at a wavelength of 765 nm. Gallic acid was used as a standard, and the TPC content was expressed as mg of gallic acid equivalents (GAE) per gram of the extract.

1.7. Determination of total flavonoid contents. The total flavonoid contents were measured using the method adapted from Chouikh et al. [11]. For this purpose, 1 mL of the sample solution was mixed with 1 mL of aluminum trichloride in methanol (2 %) and incubated at room

temperature for 10–15 min. The absorbance was measured at a wavelength of 430 nm. Quercetin was used as a standard, and the flavonoid content was expressed as mg of quercetin equivalents (QE) per gram of the extract.

1.8. DPPH radical scavenging assay. The DPPH radical scavenging activity was estimated by the method of Brand-Williams [12] with modifications. Exactly 1 mL of each extract at different concentrations was added to 1 mL of DPPH solution (0.1 mM in methanol). Following the incubation at room temperature for 10–15 min, the absorbance was measured at a wavelength of 517 nm. Ascorbic acid, a strong antioxidant compound, was used as a standard.

1.9. Hemolysis assay. The hemolysis assay was conducted following the protocol by Chouikh et al. [13]. A volume of 40 μL of human erythrocytes was mixed with 2 mL of the plant extract and incubated at 37 °C for 5 min. Then, 40 μL each of H_2O_2 (30 mM), $FeCl_3$ (80 mM), and ascorbic acid solution (50 mM) were added. After 1 h of incubation at 37 °C, the mixture was centrifuged at 700 rpm for 10 min, and the absorbance of the supernatant was read at a wavelength of 540 nm.

1.10. Ferric reducing antioxidant power assay. The ferric reducing antioxidant power (FRAP) assay was performed according to Mesbahi et al. [14]. A mixture of 0.5 mL of the plant extract, 1.25 mL of phosphate buffer (0.2 M, pH 6.6), and 1.25 mL of potassium ferricyanide (1 %) was incubated at 50 °C for 20 min. The reaction was stopped by adding 1.25 mL of trichloroacetic acid (10 %), followed by centrifugation at 3000 rpm for 10 min. After the centrifugation, 1 mL of the supernatant was mixed with 0.25 mL of $FeCl_3$ (0.1 %) and 1.25 mL of distilled water. The absorbance was read at a wavelength of 700 nm. Ascorbic acid was used as a positive control.

1.11. High-performance liquid chromatography. The high-performance liquid chromatography **(**HPLC) analysis was carried out as described by Ben Ali et al. [15]. For this procedure, 20 μL of the crude extract was injected into the HPLC system. The mobile phase solvent was forced through the column using a high-pressure pump to separate the compounds based on their polarity. To identify the compounds, present in the sample, chromatograms were generated by a detector connected to the column and a computer.

2. Results and Discussion

2.1. Determination of extraction yield. Extraction yield is a key metric, which quantifies the proportion of bioactive components recovered from raw plant material.

In this study, notable differences were revealed in the yields of the crude, flavonoid (ethyl acetate phase), and tannin extracts of *C. tinctoria*. The crude extract had the highest yield (11.3 %). The flavonoid extract ranked second (1.3 %). The lowest yield (0.5 %) was obtained for the tannin extract.

The above differences may be related to a variety of factors, among which is the type and polarity of the solvent, with ethyl acetate being less polar compared to methanol and thus yielding lower extraction efficiency [16]. Other factors significantly affecting extraction yield values are sampling conditions, drying and storage methods, extraction techniques such as maceration, solvent-to-plant material ratio, and extraction duration [17]. The quality of extracted compounds also depends on environmental stresses altering plant physiology [18]. Of particular importance is the age of the plant, with older perennial plants characterized by lower yields. In many cases, the quality and quantity of extracted compounds are determined by climate and the plant's growth environment [19].

2.2. Total phenolics and flavonoids and their antioxidant effects. Phenolics and flavonoids are the two secondary metabolites commonly found in *C. tinctoria* and directly contributing to its antioxidant capacity.

In the crude *C. tinctoria* extract, the TPC determined using the Folin–Ciocalteu method was 168 ± 24 mg GAE/g DM. The total flavonoid content was 27 ± 3 mg QE/g DM. These levels are considered high.

Generally, elevated levels of phenolics and flavonoids may be related to high solubility of these compounds in methanol, which is known to inhibit polyphenol oxidase and evaporates quicker compared to water [20]. They are also influenced by various environmental factors, including season, sampling dates, soil composition, climate, temperature, light, humidity, and water stress [21], as well as the plant's age and growth stage. Pre-extraction drying and selection of an appropriate extraction method are crucial as well for the recovering of bioactive compounds like phenolics and flavonoids [22]. With few studies focusing on phenolics and flavonoids contained in *C. tinctoria* flowers, our findings fill an important gap existing in the literature.

2.3. Antioxidant activity evaluated by DPPH assay. The antioxidant activity of *C. tinctoria* constituents was determined by calculating IC_{50} from the DPPH assay with ascorbic acid as a standard. IC_{50} was evaluated by plotting the extract concentrations versus DPPH inhibition (Fig. 2). The inhibitor with lower IC_{50} was considered more potent under identical testing conditions, as suggested by Johari and Khong [23]. Fig. 2 shows that the tannin extract had the lowest IC_{50} value (8 ± 2 μg/mL), indicating that it exhibited the most pronounced antiradical activity among the tested extracts. The crude and flavonoid extracts with the higher IC_{50} values (23 ± 6 and 20 ± 6 μg/mL, respectively) were less effective in DPPH scavenging. Compared to the plant extracts, ascorbic acid used as a reference antioxidant exhibited even higher scavenging activity with IC_{50} of 5 ± 1 μg/mL.

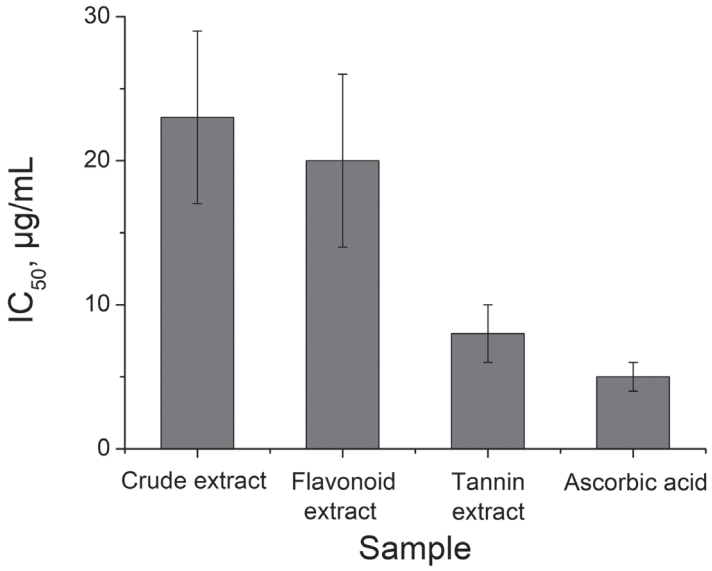

Fig. 2. IC_{50} values (μg/mL) of the *C. tinctoria* extracts and ascorbic acid in DPPH assay

Therefore, higher concentrations of all plant extracts were needed in order to achieve the same effect as with ascorbic acid. However, in the study by Mahtout et al. [24], the ethyl acetate extract demonstrated higher DPPH scavenging activity than BHT and ascorbic acid. In our study, the differences observed in the DPPH scavenging activity between the plant extracts may be attributed to the structure, type, and concentration of phenolic compounds [25]. In [26, 27], the antioxidant strength of plant extracts varied depending on the content of polyphenols, which are strong ROS scavengers due to the presence of hydroxyl groups on the aromatic ring. The flavonoid content is of no less significance. The study by Zheng et al. [28] reports that the position of hydroxyl groups

and their hydrogen-donating ability, along with the presence of a double bond between C2 and C3 atoms, play a crucial role in the inhibition of radicals [29]. Ascorbic acid, a well-established antioxidant and radical scavenger [30], was used as a positive standard due to concerns about the direct determination of the DPPH radical scavenging activity from the calibration curve [31].

2.4. Hemolysis evaluation. The hemolysis assay is a simple and robust screening test to evaluate the antioxidant activity of compounds by using erythrocytes as a model to study the interactions between oxidants and antioxidants. The membranes of erythrocytes are rich in unsaturated fatty acids, which are highly sensitive to free radicals. These membranes are also responsible for oxygen transport to hemoglobin molecules [13]. Lipid oxidation in erythrocyte membranes induces oxidative stress by affecting membrane fluidity and receptor functions, leading to erythrocyte degeneration [32].

Fig. 3 illustrates the percentage of hemolysis (break down of red blood cells) in the presence of the plant extracts and ascorbic acid at a concentration of 1 mg/mL. The lowest value was observed for ascorbic acid (17 %). The crude, flavonoid, and tannin extracts had higher values (34, 23, and 44 %, respectively).

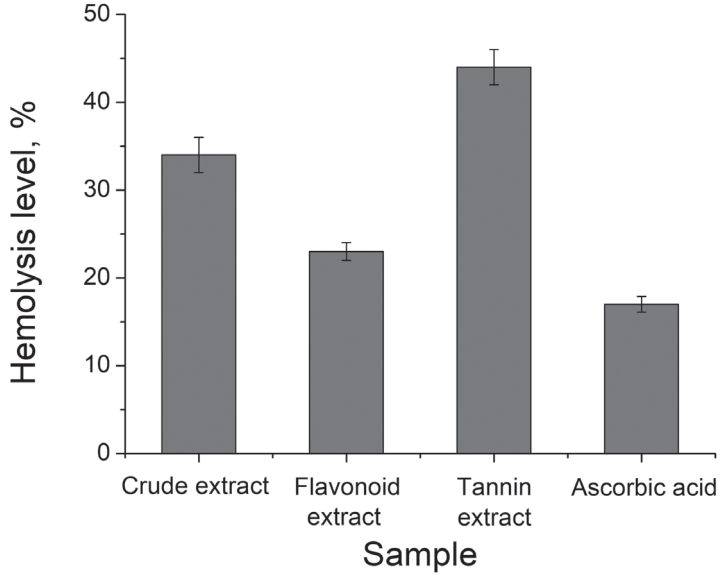

Fig. 3. Hemolysis levels with the *C. tinctoria* extracts and ascorbic acid, 1 mg/mL

In our study, the ability of erythrocytes to resist free radicals in the extracts was monitored using spectrophotometry. The antioxidant activity of the extracts varied considerably, with significant differences from ascorbic acid, which aligns with the results of the DPPH assay. Nevertheless, the extracts still displayed notable antioxidant potential due to their phenolic compounds, which protect biofilms from oxidation by free radicals and thus act as antioxidant agents terminating the chain reaction associated with these reactive metabolites [33].

2.5. Evaluation of reducing power. Reducing power is associated with the ability of a compound to reduce Fe^{3+} to Fe^{2+} and serves as an indicator of its antioxidant activity. During the FRAP test, the yellow color of the solution changes to various shades of green and blue, depending on the reducing power. At higher concentrations of a reducing agent, the amount of Fe^{2+} increases, which is evident from greater absorbance of the sample. Reducing agents act as antioxidants by breaking the free radical chain through hydrogen atom donation and reacting with certain peroxide precursors, preventing peroxide formation [34].

As seen from Fig. 4, at a concentration of 0.1 mg/mL, the tannin extract demonstrated the highest ferric reducing ability compared to all other tested extracts.

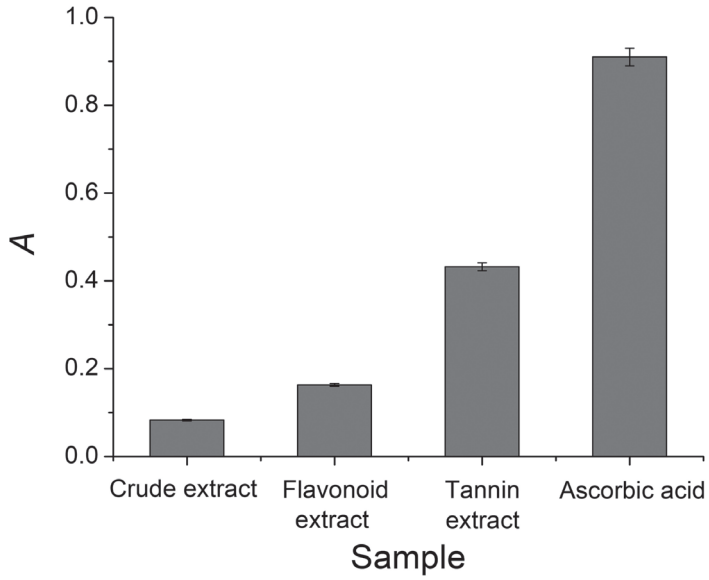

Fig. 4. Reducing power of the *C. tinctoria* extracts and ascorbic acid, 0.1 mg /mL

Previous studies have shown a direct correlation between the antioxidant capacity and reducing power of certain plant extracts [35]. In this study, the reducing power of all extracts increased with concentration, closely correlating with their antioxidant capacity. Thus, the antioxidant properties of the extracts were enhanced by reducing agents [36]. The FRAP assay was also consistent with the DPPH assay, suggesting a correlation between the reducing power and the DPPH scavenging activity due to similar underlying mechanisms [37].

2.6. High-performance liquid chromatography. To analyze and separate phenolic compounds present in the extracts, a HPLC analysis was performed (Fig. 5, Table). Based on the chromatogram of the methanolic (crude) extract (Fig. 5), two phenolic compounds can be identified (chlorogenic acid and naringin). Their concentrations differed significantly (1.152 and 27.46 μg/mg Ext for chlorogenic acid and naringin, respectively).

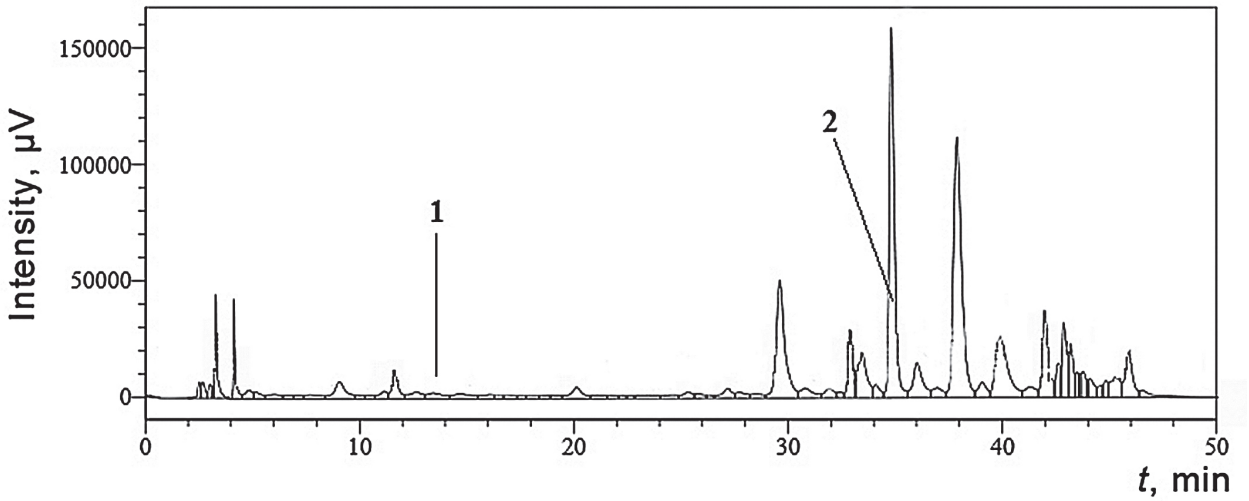

Fig. 5. Chromatogram of the methanolic extract of *C. tinctoria*. 1 – chlorogenic acid, 2 – naringin

Table. Phenolic compounds in the methanolic extract of *C. tinctoria* and their concentrations

Compound	Retention time, min	Concentration, µg /mg Ext
Gallic acid	5.275	—
Chlorogenic acid	13.437	1.152
Vanillic acid	15.562	—
Caffeic acid	16.225	—
Vanillin	21.462	—
p-Coumaric acid	23.911	—
Rutin	28.868	—
Naringin	34.092	27.46
Quercetin	45.018	—

Chlorogenic acid, also known as 5-*O*-caffeoylquinic acid (5-CQA), is an ester of cinnamic acids, such as caffeic and quinic acids [38]. It exhibits various bioactive properties, including antibacterial, antioxidant, and anticarcinogenic [39], as well as donates hydrogen atoms to reduce free radicals and inhibit oxidation reactions [40]. Naringin, a predominant flavanone glycoside (flavonoid) that occurs naturally in many plants, has been reported to possess antioxidant and antimicrobial activities [41].

Conclusions

The antioxidant capacity and phenolic composition of *C. tinctoria* extracts (crude, flavonoid, and tannin) were examined. All extracts exhibited antioxidant activity in the DPPH, hemolysis, and FRAP assays, with the tannin extract being the most effective. However, they were still less potent than ascorbic acid used as a reference compound. The HPLC analysis revealed the presence of two antioxidant phenolic compounds, chlorogenic acid and naringin, in the crude extract. The antioxidant efficacy of the extracts varied significantly, which may be attributed to the differences in their phenolic contents and the influence of environmental factors on the plant's phytochemical characteristics. The findings provide valuable insights into the potential of *C. tinctoria* as a natural source of antioxidants, as well as into the health benefits its offers. The mechanisms underlying the antioxidant capacity and medicinal value of *C. tinctoria* set an agenda for future research.

Conflicts of Interest. The authors declare no conflicts of interest.

Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов.

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