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DOI: 10.2478/auoc-2025-0002

Ovidius University Annals of Chemistry Volume 36, Number 1, pp. 13 - 19, 2025

Phenolic profile, antioxidant capacity, and *in vivo* sub-acute toxicity evaluation of *Calligonum comosum* L. aerial part

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Abstract. This study provides a comprehensive analysis of the phenolic constituents and antioxidant properties of *C. comosum*, complemented by an evaluation of its acute toxicity in vivo. Employing spectrophotometric techniques, we quantified the total polyphenol content (TPC) and total flavonoid content (TFC) using gallic acid and quercetin standards, respectively. High-performance liquid chromatography (HPLC) was utilized to identify and quantify individual phenolic compounds. Antioxidant efficacy was measured through assays assessing total antioxidant capacity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, and reducing power (RP). The analysis revealed a substantial polyphenol content of 185.073 mg GAE/g DW and a flavonoid content of 21.75 mg QE/g DW. HPLC detected eight distinct phenolic compounds, with quercetin and rutin emerging as the most predominant. The aqueous extract of *C. comosum* exhibited pronounced antioxidant activity, with significant inhibition across the tested assays. Acute toxicity studies on Wistar rats indicated a favorable safety profile, showing no mortality or significant behavioral changes at doses up to 2000 mg/kg. In conclusion, *C. comosum* demonstrates a rich reservoir of secondary metabolites with substantial antioxidant potential, affirming its potential as a candidate for therapeutic applications. However, additional in vivo studies are essential to fully elucidate its therapeutic efficacy and safety profile.

***Keywords*:** *Calligonum comosum* L; HPLC analysis; anti-oxidant capacity; sub-acute toxicity.

1. Introduction

The *Calligonum* genus, a prominent member of the Polygonaceae family, comprises approximately 80 species that are widely distributed across diverse regions, including Europe, North Africa, West Asia, and Central Asia [1]. Among these, *Calligonum comosum* L'Her, commonly known as “arta”, stands out for its ecological and medicinal significance. Thriving in the sandy soils of North African deserts, particularly in Algeria, *C. comosum* has adapted remarkably well to harsh environmental conditions [2, 3]. This resilient shrub, with its woody branches, typically reaches heights of 4 to 10 feet and exhibits a unique growth pattern, spreading horizontally through suckers that emerge from adventitious roots [4, 5]. Its exceptional ability to withstand extreme environments, including prolonged summer droughts, positions *C. comosum* as an ideal candidate for ecological restoration projects, particularly in degraded landscapes, arid regions, and agro-forestry systems [6-8].

In traditional medicine, arta is revered for its extensive therapeutic properties, which are attributed to its rich array of bioactive compounds. The aerial parts of *C. comosum* have been extensively studied and found to possess anti-osteoporotic, anti-ulcer, anti-inflammatory, anti-diabetic, and antioxidant activities, showcasing its diverse pharmacological potential [9-12]. Additionally, the plant exhibits notable antibacterial properties, as highlighted by Ahmed et al. [2]. The increasing concerns over the side effects and high production costs associated with synthetic antioxidants have fueled a growing interest in natural alternatives. These natural antioxidants are not only safer but often more efficacious [13]. Extensive research has revealed that *C. comosum* is a rich source of beneficial phytochemicals, including flavonoids and phenolic compounds. For instance, Okla et al.[14] identified 16 ester and non-ester acids among 22 compounds present in the seed extract of *C. comosum*. Furthermore, comprehensive chemical analysis of the aerial parts from Emiratean *C. comosum* bushes has uncovered a diverse spectrum of compounds, such as polyphenols, flavonoids, tannins, carbohydrates, alkaloids, proteins, steroids, terpenoids, saponins, and phlobatannins, underscoring the plant’s potential for therapeutic applications.

Despite the growing interest in medicinal plants, research on *C. comosum* remains scarce, particularly regarding its phytochemical composition. This study seeks to fill this knowledge gap by performing an in-depth phytochemical investigation of the aerial parts of *C. comosum*. The primary goal is to meticulously analyze its phenolic profile, employing high-performance liquid chromatography (HPLC) to identify and quantify key phenolic compounds. In addition to

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characterizing the phenolic content, the study evaluates the antioxidant activity of the extract through various *in vitro* assays, including total antioxidant capacity, DPPH radical scavenging, and reducing power assays. To ensure a comprehensive assessment of the plant's safety and potential therapeutic benefits, the study also includes an acute toxicity evaluation in Wistar rats. This holistic approach aims to elucidate the bioactive properties of *C. comosum*, providing a scientific foundation for its potential applications in health and medicine.

2. Experimental

2.1. Materials and reagents

The study utilized high-purity chemicals, including sodium chloride (NaCl), monobasic potassium phosphate (KH_2PO_4), trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), aluminum trichloride ($AlCl_3$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, gallic acid, and ferric chloride ($FeCl_3$). These were sourced from Sigma-Aldrich (USA).

2.2. Preparation of plant material

In March 2023, during the flowering period of *Calligonum comosum*, aerial parts of the plant were collected from the El Ghenami region in Southeast Algeria (Province of Ouargla). The plant material was authenticated by Professor Atef CHOUIKH from the Faculty of Natural Science and Life, El Oued University. The collected aerial parts were thoroughly rinsed under running water to eliminate dust and other impurities, then dried, powdered, and stored for subsequent analyses.

2.3. Preparation of aqueous extract

To prepare the aqueous extract, 10 grams of the powdered *C. comosum* aerial parts were soaked in 100 mL of distilled water at room temperature, shielded from light, for 24 hours. The mixture was then filtered using filter paper to remove any residues. The filtrate was carefully dried at 40 °C and stored at 4 °C for future experiments, following the protocol of Murugan and Parimelazhagan [15].

2.4. Phytochemical screening

The aqueous extract was subjected to phytochemical screening using established methods to identify the presence of phenolic compounds, tannins (catechol and gallic acid), alkaloids, steroids, saponins, flavonoids, and triterpenoids [16].

2.5. Quantification of phytochemicals compounds

2.5.1. Estimation of total phenolics. The total phenolic content was quantified using the Folin-Ciocalteu reagent method. Specifically, 1 mL of 10% Folin-Ciocalteu reagent was combined with 0.2 mL of the *C. comosum* aqueous extract. After 4 min incubation, 800 µL of saturated sodium carbonate solution (75 g/L) was added. The mixture was then incubated for 2 hours at room temperature, and absorbance was recorded at 765 nm. The total phenolic content was quantified in milligrams equivalent to gallic acid per gram of extract using the linear calibration equation based on gallic acid as the standard: $y = 0.0096 \cdot x + 0.052$ ($R^2 = 0.994$) [17].

2.5.2. Estimation of total flavonoids

The total flavonoid content was assessed using the aluminum chloride ($AlCl_3$) colorimetric method, following a standardized procedure. A 1 mL aliquot of 2 % $AlCl_3$ solution prepared in distilled water was mixed with 1 mL of the sample extract, with quercetin serving as the standard. After 30 min of reaction at room temperature, the absorbance was measured at 430 nm. Results were determined using a linear calibration equation with quercetin as the standard: $y = 0.0104 \cdot x + 0.0819$ ($R^2 = 0.9925$) [18].

2.6. Qualitative analysis by HPLC

High-performance liquid chromatography (HPLC) was employed to analyze the phenolic compounds in the *C. comosum* aqueous extract. The analysis was conducted using a Shimadzu LC20 AL system equipped with a UV-Vis detector (SPD 20A) and a Shim-pack VP-ODS C18 analytical column (4.6 x 250 mm, 5 µm). The mobile phase consisted of a gradient mixture of acetonitrile and 0.1% acetic acid. The flow rate was set to 1 mL/min, and detection was performed at a wavelength of 268 nm. Injection volumes for both samples and standards were 20 µL. To identify and confirm the retention time (RT) of the compounds, we used nine standards of known compounds, including caffeic acid (RT (min): 16.27), *p*-coumaric acid (RT (min): 23.81), gallic acid (RT (min): 5.29), vanillic acid (RT (min): 15.53), chlorogenic acid (RT (min): 13.39), naringin (RT (min): 34.78), rutin (RT (min): 28.37), quercetin (RT (min): 45.04), and vanillin (RT (min): 21.46). These standards were run under the same HPLC conditions as the sample extracts to establish baseline retention times.

2.7. Antioxidant activity

2.7.1. DPPH free-radical scavenging activity (DPPH). To evaluate the antioxidant activity of the *C. comosum* extract, different concentrations of the aqueous extract were tested using the DPPH radical scavenging assay. Specifically, concentrations of 5, 25, and 50 µg/mL were prepared, representing the amounts of the extract used to assess its ability to neutralize DPPH radicals. The antioxidant activity was determined by mixing 1 mL of the extract with 1 mL of DPPH solution, followed by incubation at room temperature in the dark for 30 min. The absorbance was measured at 517 nm, with ascorbic acid serving as the reference standard [19].

2.7.2. Reducing Power Assay (RP). The reducing power of the extract was determined using the method described by Oyaizu [20]. The reaction mixture consisted of the extract at various concentrations, phosphate buffer (2.5 mL, 0.2 M, pH 6.6), and potassium ferricyanide (2.5 mL, 1%). After incubation at 50 °C for 20 min, trichloroacetic acid (2.5 mL, 10%) was added, followed by centrifugation at 3000 rpm for 10 min. The supernatant was mixed with distilled water and 0.1% $FeCl_3$ solution, and absorbance was measured at 700 nm [21].

2.8. Sub-acute toxicity study

A sub-acute toxicity study of the *C. comosum* aqueous extract was conducted according to OECD guidelines 425 [22]. Four groups of male albino rats (n = 3 per group) were administered single doses of the extract at 100, 1000, and 2000 mg/kg, respectively, via intragastric gavage. A control group received normal water. Observations for toxicity signs, changes in body weight, side effects, and mortality were recorded over a 14-day period.

2.9. Statistical analysis

Experiments were conducted in triplicate, and results were analyzed using Microsoft Excel. Data are presented as mean ± standard deviation (n = 3). IC_{50} and EC_{50} values were calculated using GraphPad Prism 7.

3. Results and discussion

3.1. Phytochemical screening

Phytochemical analysis confirmed the presence of various bioactive compounds, including tannins, flavonoids, saponins, alkaloids, and terpenoids, in the aqueous extract of *C. comosum* (Table 1). The phytochemicals present in *C. comosum* are known to be biologically active, exhibiting a range of properties such as antioxidant, antimicrobial, antifungal, and anticancer activities. This comprehensive evaluation will provide valuable insights into the plant's health-promoting properties and its potential use in various therapeutic applications [23-25].

Table 1. Phytochemical screening of *C. comosum* aqueous extract.

Phytochemical compounds		***C. comosum* (aqueous extract)**
Polyphenols		(+)
Alkaloids	Mayer	(-)
	Wagner	(+)
Tannins	*Catechi*n	(+)
	Gallic	(+)
Flavonoids		(+)
Saponins		(+)
Steroids		(+)
Terpenoids		(+)

(−) Absence of phytochemicals compounds.
(+) Presence of phytochemicals compounds

According to the preliminary results of the phytochemical screening (see Table 1), the *C. comosum* extract has a number of beneficial phytochemicals, including tannins, phenols, alkaloids, terpenoids, saponins, and flavonoids. These chemical components, which are frequently present in higher plants, play protective roles and help the plant acquire medicinal qualities. These results align with a phytochemical analysis conducted by Alzahrani et al. [4].

3.2. Qualitative analysis by HPLC

The HPLC analysis of *C. comosum* aqueous extract revealed the presence of eight phenolic compounds, emphasizing its rich phytochemical profile. Quercetin and rutin, identified as the most abundant phenolic compounds (6499.419 µg/g and 4618.419 µg/g, respectively), are well-documented for their diverse pharmacological activities. Quercetin is a potent antioxidant known for its role in neutralizing reactive oxygen species (ROS), reducing inflammation, and protecting against cardiovascular diseases [23-25]. Similarly, rutin is recognized for its vascular-strengthening properties, anti-inflammatory effects, and potential in managing oxidative stress-related conditions. Moderate levels of chlorogenic acid (677.156 µg/g) and gallic acid (529.050 µg/g) further enhance the therapeutic potential of the extract. Chlorogenic acid is widely studied for its antioxidant and anti-inflammatory effects, as well as its potential to regulate glucose metabolism, which may have implications in diabetes management. Gallic acid, another phenolic compound, is known for its antimicrobial, anticancer, and hepatoprotective properties [26-29]. The detection of vanillin (78.914 µg/g) and *p*-coumaric acid (12.958 µg/g) in trace amounts underscores the complexity of the phenolic composition. Vanillin, beyond its flavoring applications, exhibits antioxidant and anti-inflammatory activities, while *p*-coumaric acid contributes to antimicrobial and UV-protective effects, albeit in smaller concentrations [30].

These findings align with prior studies on the phenolic composition of *C. comosum*, supporting its use as a natural source of bioactive compounds. The high abundance of quercetin and rutin positions *C. comosum* as a promising candidate for developing antioxidant-rich therapeutics, while the diversity of other phenolics contributes to a broad spectrum of pharmacological benefits. This comprehensive profile reinforces the traditional medicinal applications of *C. comosum* and highlights its potential in combating oxidative stress and associated health conditions [31, 32].

Table 2. Concentrations and retention times of phenolic compounds detected in the aqueous extract of *Calligonum comosum* L'Hér.

Phenolic compound	**Retention time (min)**	**Equation**	**Concentration (µg/g extract)**
Chlorogenic acid	13.392	y = 21665x	677.156
Caffeic acid	16.277	y = 84066x	201.624
p-Coumaric acid	23.817	y = 49495x	12.958
Gallic acid	5.29	y = 54681x	529.050
Vanillic acid	15.53	y = 65077x	159.189
Naringin	34.788	y = 19379x	ND
Rutin	28.37	y = 28144x	4618.419
Quercetin	45.047	y = 45378x	6499.760
Vanillin	21.46	y = 58930x	78.914

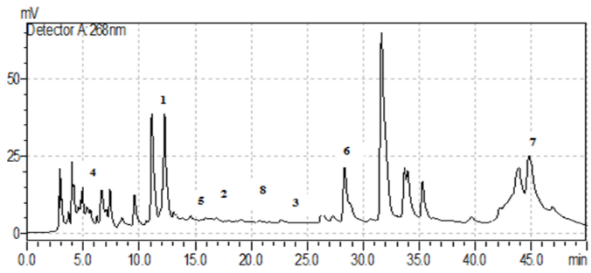

Figure 1. HPLC chromatograms of the *C. comosum* extract: **1** - Chlorogenic acid; **2** - Caffeic acid; **3** - *p*-coumaric acid; **4** - Gallic acid; **5** - Vanillic acid; **6** - Rutin; **7** - Quercetin; **8** - Vanillin.

3.3. Quantification of phytochemical compounds

Quantification of the phytochemical compounds in the *C. comosum* aqueous extract was carried out using calibration curve methodologies for total phenolics and flavonoids.

The total phenolic content was determined using the Folin-Ciocalteu method, with the phenolic compounds in the extract quantified based on a calibration curve derived from gallic acid standards. The phenolic content of the extract is expressed as milligrams of gallic acid equivalent per gram of dry extract (mg GA eq/g dry extract).

Similarly, the total flavonoid content was assessed using the aluminum chloride colorimetric method. The flavonoid content was quantified using a calibration curve based on quercetin standards, with results expressed as milligrams of quercetin equivalent per gram of dry extract (mg Q eq/g dry extract). This finding underscores the potential therapeutic benefits of these phenolic constituents, which are known to offer a range of pharmacological advantages. The identification of these secondary metabolites provides additional validation of the traditional use of *C. comosum* in treating various ailments [26, 33]. Our study underscores the significant levels of total phenols and flavonoids in *C. comosum*. Given the established antioxidant properties and diverse pharmacological effects of phenolic compounds, the high phenolic content in this plant suggests a robust antioxidant capacity [28]. This enhanced antioxidant potential highlights *C. comosum*'s potential as a valuable natural resource for therapeutic applications [31, 32].

Table 3. Quantitative evaluation of the aqueous *C. comosum* extract's total phenolic and flavonoid composition.

Compounds	*C. comosum*
Total phenolic compound (mg GAE/g extract)	185.073±4.013
Flavonoids (mg Q eq/g dry wt)	21.75±2.86

3.4. Antioxidant activity

3.4.1. DPPH radical scavenging activity

The antioxidant activity of the *C. comosum* extract was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, which measures the ability of the extract to neutralize free radicals. The DPPH radical scavenging activity of the extract was evaluated at various concentrations, specifically 5, 25, and 50 µg/mL. At a concentration of 5 µg/mL, the *C. comosum* extract demonstrated a scavenging activity of 57.34%, which increased to 60.08% at 25 µg/mL, and further enhanced to 66.55% at 50 µg/mL. These results indicate a dose-dependent increase in antioxidant activity.

Table 4. Shows the percentage of *C. comosum* and vitamin C that scavenges free radicals (DPPH).

Concentration (µg/mL)	% inhibition	
	C. comosum	Vitamin C
5	57	73.073
25	60.068	81.22
50	66.55	81.56

3.4.2. Reducing power. The reducing power of the *C. comosum* extract was assessed to determine its ability to act as an electron donor, which is indicative of its antioxidant potential. This activity was measured using the reducing power assay, where the ability of the extract to reduce Fe^{3+} to Fe^{2+} was quantified. The IC_{50} value, representing the concentration required to achieve 50% of the maximum reducing power, was found to be 141 ± 0.01 µg/mL for *C. comosum* extract. This indicates that the extract possesses a potent reducing ability.

The DPPH and reducing power assays are two different in vitro assays that were used to thoroughly evaluate the antioxidant capabilities of *C. comosum*. An essential criterion for evaluating the antioxidant capacity of plant extracts is the removal of reducing power and DPPH free radicals. The findings show that the *C. comosum* extract has strong free radical scavenging properties. These results concur with Cheruth et al.'s [5]. They found that the most effective radical scavenger was the fruit's ethanolic extract. Additionally, existing literature indicates that extracts derived from the leaves, bark, and cones of *C. comosum* exhibit a potent ability to neutralize DPPH free radicals, which correlates with their phytochemical composition. The findings reported by Alzahrani [4] emphasizing a significant presence of total phenolic and flavonoid content in extracts from the leaves and bark of the specified plants, align with our observations. These results are also consistent with the study conducted by Jahromi et al. [34] on the antioxidant properties of *C. comosum* species extracts. Thus, the primary contribution to the antioxidant activity of the selected plant is attributed to its phenolic and flavonoid constituents [29, 31, 32, 35, 36].

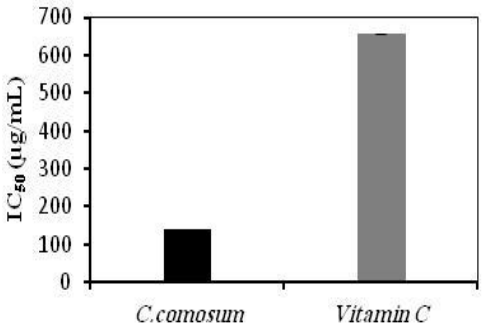

Figure 2. Antioxidant efficacy of *C.comosum* using reducing power assay.

3.5. Sub-acute toxicity study

The sub-acute toxicity of *C. comosum* aqueous extract was assessed in compliance with OECD 425 guidelines, with a focus on its physiological and behavioral impacts over 24 hours, 72 hours, and up to two weeks. Throughout the study, no significant alterations in physiological parameters were observed at any time point. Behavioral and physical activity levels remained stable across all test groups during the observation periods, with no recorded fatalities among the test subjects. Table 5 provides a detailed summary of the key observations and measurements, reinforcing the extract's excellent safety profile within the tested dosage range. These findings demonstrate that *C. comosum* extract, at the administered concentrations, exhibits no acute toxicity or notable adverse effects on the health of the rats. This underscores its potential as a safe and effective therapeutic agent.

Table 5. Sub-acute toxicity test of aqueous extract of *C. comosum* on physiological parameters of Wister albino rats.

	0 h		**3 h**		**24 h**		**7 days**		**14 days**	
Parameters	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Dead rats	0	0	0	0	0	0	0	0	0	0
Movement	N	N	N	N	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N	N	N

4. Conclusions

The aqueous extract of *C. comosum* is distinguished by its substantial content of phenolic compounds and flavonoids, which contribute significantly to its impressive antioxidant activity. These results underscore the potential of *C. comosum* as a valuable source of natural antioxidants with applications in nutraceuticals and functional foods. The study confirms a favorable safety profile of the extract, evidenced by the absence of acute toxicity at the administered concentrations. The *in vitro* antioxidant assays robustly validate the biological activity of *C. comosum* extract, supporting its efficacy in combating oxidative stress. Despite these promising findings, a comprehensive understanding of the underlying mechanisms driving these antioxidant effects necessitates further in vivo investigations. Such studies will be critical in fully elucidating the therapeutic potential of *C. comosum* and advancing its application in preventive and therapeutic health strategies.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Received: 05.07.2024
Received in revised form: 07.02.2025
Accepted: 10.02.2025